Vikas Srivastava · Shakti Mehrotra
Sonal Mishra *Editors*

Hairy Roots

An Effective Tool of Plant Biotechnology

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This book is dedicated to
Late Dr. Arun Kumar Kukreja
(Ex-Chief Scientist, CSIR-Central Institute of Medicinal and Aromatic Plants, India)

Preface

The escalating industrial magnitude of plant-based chemicals has resulted great interest in the evolution of methods to meet their desired production. These phytochemicals are widely used in cosmetics, flavor and fragrance, dyes and pigments, food additives, and insecticide/pesticide industries as a whole or as an important ingredient in various formulations. Out of various conventional and unconventional strategies that have been applied for the production of these phytochemicals, the development of *Agrobacterium rhizogenes*-mediated hairy root cultures (HRCs) is considered as the most practically feasible approach. The HRCs are usually stable in their biosynthetic potential and, thus, offer a sustainable production system for desired metabolites. Additionally, several proof-of-principle experiments have also revealed the practical feasibility of HRCs in plant-based remediation of environment pollutants, biotransformation of important compounds, and production of therapeutic proteins. It is pertinent to mention here that perspectives in the upscaling of hairy root cultures also offer ceaseless opportunities in various objectives. Nevertheless, an easy to establish and maintain, economic, renewable and above all, the sustainability of HRCs justify the attention of the global scientific community.

At this juncture, where hairy root biotechnology is recognized as most sought-after and very dynamic research area, it is relevant to get judicious update in recent advances along with hitherto biotechnological progress of the subject. Thus, considering HRCs as a multifaceted biological tool for various applications, the present book entitled *Hairy Roots: An Effective Tool of Plant Biotechnology* has been designed. The editorial team members (**Vikas Srivastava; Shakti Mehrotra; Sonal Mishra**) have been working on various aspects of hairy root research since long time and published many articles in journals/books of international repute. The present book provides the details of conceptual as well as pragmatic information of HRCs-based research along with relevant case studies. Furthermore, an attempt has also been made to investigate the loopholes in existing methodologies and challenges and to find out possible solutions through scientific discussions from various eminent research groups working on hairy root biotechnology. The book has been

framed on the basis of three major areas and thus presents three broad parts as (i) Hairy Roots and Secondary Metabolism, (ii) Progressive Applications, and (iii) Novel Approaches and Future Prospects.

The first part (**Hairy Roots and Secondary Metabolism**) comprised of seven chapters that deals with comprehensive discussion about hitherto gradual progression of hairy root research from a simple biotechnological tool to mimic the natural phenomenon of bacterial gene transfer and occurrence of disease syndrome to a most preferred and dynamic technology for secondary metabolite production and other value-added applications. This part deals with the discovery of nature's own genetic engineer *A. rhizogenes*, its journey since then, and its successful exploitation in various fields of biotechnology and related prospects. Further discussion includes the regeneration of pRi-transformed plants, various types of HRCs-mediated secondary metabolite production, and impact of various extrinsic factors over HRCs-mediated secondary metabolite production. This part also provides an inclusive account on biotechnological interventions in HRCs of tropane and terpene alkaloid-bearing plants. The part culminates with the description of the design and development of bioreactors to achieve maximum productivity in plant cell and hairy root cultivations.

The second part (**Progressive Applications**) comprised of five chapters that provide an inclusive account on further advancement of HRCs research. This part offers vivid account on the capacity of HRCs for the biotransformation of a variety of substrates for value addition and its utility as a potential system for the production of imperative biopharmaceuticals. Further the competence of HRCs for the remediation of the environment and its utilization to study signaling pathways during nodule formation has also been incorporated. Lastly, this part also highlights the methodologies developed to generate composite plants and the applications of co-transformed hairy roots for studying gene function.

The third part (**Novel Approaches and Future Prospects**) comprised of three chapters and includes current attention on HRCs research. Here, the exploration of transcriptome sequencing in HRCs of medicinal plants and in silico perspective of HRCs growth monitoring and modeling have been presented. Finally, the utility of CRISPR/Cas9-mediated editing will offer new directions for HRCs metabolic engineering. This edited book is an attempt to ensure the research and teaching community, about the major progress in HRCs-based interventions in plant biology and applications thereof. Besides, the emerging thrust that still needs time to grow will also be considered to project the prospect trajectory of HRCs research. The book will surely provide endless opportunities in the ongoing and future research in this fascinating area.

Samba, India — Vikas Srivastava
Lucknow, India — Shakti Mehrotra
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Part I
Hairy Roots and Secondary Metabolism

Chapter 1
Progress and Prospects of Hairy Root Research

Suvi T. Häkkinen and Kirsi-Marja Oksman-Caldentey

Abstract Nature's own genetic engineer *Agrobacterium rhizogenes* was discovered more than 40 years ago, and an increasing number of publications on the use of hairy roots in biotechnology have been published since – with more than 85% of all the publications during the past 15 years. Hairy roots have been successfully exploited in various fields in biotechnology, including secondary metabolite research, recombinant protein production, and bioremediation, to mention a few. In the following chapter, we will deal with the current state of the art of hairy root research starting from evolutionary facets of hairy root generation and host-bacteria association to a range of applications where hairy roots are efficiently exploited.

Keywords Hairy root · History · Applications · Secondary metabolites · Bioreactor

1.1 Hairy Roots: Where It All Started from?

Already as early as in the late 1950s, Dr. Armin Braun from The Rockefeller University first demonstrated that tumor cells in plants are transformed, i.e., they can be freed from *Agrobacteria* – a gram-negative soil bacteria – and grown in vitro without the supplemental auxin and cytokinin required by normal plant cells in vitro (Braun 1958). Later, metabolites called octopine and nopaline were discovered from tumor cells (Petit et al. 1970). Indirect genetic evidence that *Agrobacterium* might carry a virus or plasmid with tumor-inducing genes emerged from two kinds of experiments (Hamilton and Fall 1971; Kerr 1971). It was discovered that tumor-inducing trait is recuperated in bacteria after the loss of virulence, indicating that the trait would be plasmid- or virus-borne. Simultaneously, existence of an extrachromosomal element was indicated via experiments showing transfer of virulence by

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Kerr and co-workers (1971). The exciting journey which eventually led to gene transfer of plants by *Agrobacterium* had an important step when *Ti* mega-plasmid was discovered in Ghent (Zaenen et al. 1974). Virulence of the *Agrobacterium* was located in *vir*-region (Stachel et al. 1985). The genetic engineering of plant cells was finally simultaneously accomplished by four independent research groups (Herrera-Estrella et al. 1983; Bevan et al. 1983; Fraley et al. 1983; Murai et al. 1983), which eventually made history for molecular biology and plant sciences.

The term "hairy root" dates back to 1900 when it was first associated with diseased fruit crops. Back then, hairy root syndrome, affecting mainly dicotyledonous plants, caused substantial losses in vineyards, orchards, and vegetable nurseries (Georgiev et al. 2012). Investigations revealed that causative agents for this disease were phytopathogenic *Agrobacterium rhizogenes* strains carrying an *Ri* plasmid (root-inducing plasmid) (reviewed by Sinkar et al. 1987), which displayed high resemblance to *Ti* plasmid carried by *A. tumefaciens*. While the latter causes the formation of crown gall tumor tissues in infected plants, *A. rhizogenes* induces the hairy root disease (Chilton et al. 1982). All strains of *A. rhizogenes* are known to produce opines of agrocinopine group and all or a few opines of the agropine group. The strains which produce all the agropine-type opines (agropine, mannopine, agropinic acid, and mannopinic acid) are known as the agropine-type strains, whereas the strains which produce all agropine-type opines excluding agropine are known as the mannopine-type strains (Petit et al. 1983) (Willmitzer et al. 1983). *Ri* plasmid of the mannopine strain 8196 contains only one T-DNA (Hansen et al. 1991), while two T-DNA regions have been identified in agropine *Ri* plasmids, which are separated by a 15 kb nontransferred region. The right T-DNA contains the regions similar to *Ti* plasmid, including *tms1* and *tms2*, which are responsible for the auxin biosynthesis (Inzé et al. 1984). The left T-DNA, however, does not possess close resemblance to any sequences with *Ti* plasmids (Huffman et al. 1984). Interestingly, while *virE1* and *virE2* genes are important for T-DNA transfer in *Ti* plasmids, they are not found in *Ri* plasmids (Moriguchi et al. 2001). Hairy roots induced by agropine strains frequently contain only the TL-DNA (Jouanin et al. 1987). However, in some cases, the information carried on the TL-DNA is not sufficient, and the presence of the TR-DNA greatly extends the host range of the infection. Sequence analysis has identified 18 open reading frames (ORFs) on the TL-DNA of pRiA4, and 8 of those *loci* were shown to affect the root formation, denoted as *rolA*, *rolB*, *rolC*, and *rolD* (Slightom et al. 1986). While mutants induced to *rolA*, *rolC*, and *rolD* resulted in attenuated growth or altered phenotype, mutants in *rolB* were totally avirulent confirming the very crucial role of this gene in hairy root formation (Spena et al. 1987). Furthermore, when *rolB* is introduced into the host plant genome as a single gene, it is capable of hairy root induction (Altamura 2004). Diverse and also synergistic effects of individual *rol* genes were shown by Palazón and co-workers (Palazón et al. 1997) who reported differential effects of these genes in tobacco hairy root growth and alkaloid production. Hairy roots easily regenerate into whole plants and transmit their *Ri* T-DNA into next progeny

(Oksman-Caldentey et al. 1991). Such plants display a significantly altered phenotype (reviewed in Nilsson and Olsson 1997).

Initially it was thought that monocotyledonous plants are insensitive to *Agrobacterium*-mediated gene transfer. Various molecular mechanisms for transformation resistance in monocotyledonous plants were suggested, including production of antimicrobial compounds (Sahi et al. 1990), a lack of *vir* gene inducers (Usami et al. 1987), inefficient T-DNA integration (Narasimhulu et al. 1996), and programmed cell death induced by *Agrobacterium* (Hansen 2000). A significant breakthrough occurred in 1993–1994, when highly regenerable explants of rice, immature embryos, or calli derived from mature seeds were inoculated with disarmed *Agrobacterium* harboring plant selectable marker genes resulting in fertile transgenic rice plants (Chan et al. 1993; Hiei et al. 1994). Transformation frequencies of monocotyledonous plants were improved by applying different selection markers (Negrotto et al. 2000), by modification of medium components, by optimization of co-culture and resting time periods, and by addition of *Agrobacterium* growth-inhibiting agent or bacteriocide such as silver nitrate (Zhao et al. 2001; Zhang et al. 2003). Spurred on by the success of Hiei and colleagues, there was significant interest in transforming other agronomically important crop species, such as barley and wheat. By the use of "super-virulent" *A. tumefaciens* strains and/or acetosyringone, a phenolic compound inducing expression of *vir* genes on the *Ti* plasmid, transformation via *A. tumefaciens* has become a major method also in monocots. Various factors have been identified of being important for successful transformation of monocotyledonous plants, as reviewed by Cheng et al. (2004). These include plant genotype, explant, *Agrobacterium* strain, pretreatment, and chosen selectable marker. However, there are also very few examples of successful hairy root transformation of monocotyledonous plants. Of monocotyledonous plants, onion and asparagus have been reported to be susceptible to *A. rhizogenes* transformation (Dommisse et al. 1990; Christey 1997). Maize hairy roots were recently generated offering platform for studying host-parasite interactions (Runo et al. 2012). Problems associated with difficulties of *Agrobacterium* (*tumefaciens*) transformation in monocots are reviewed by Sood and co-workers (Sood et al. 2011).

1.2 Characteristics of Hairy Roots

A. rhizogenes infects wounded plant cells because of the production of phenolic compounds that attract *A. rhizogenes*. Bacteria move to the wound site by chemotaxis. Subsequent infection at wound site followed by integration of *Agrobacterium*-derived T-DNA into the plant genome results in development of hairy root disease. Hairy root disease is characterized by high growth rate, a high degree of lateral branching, profusion of root hairs, lack of geotropism, and the tissue maintaining a highly differentiated and functional root organ (Tepfer 1984; Sevón and Oksman-Caldentey 2002). Hairy roots offer an attractive alternative for the production of a

range of high-value secondary compounds for various biotechnologically important reasons. Hairy roots are able to accumulate, e.g., the same alkaloids as the parent plant, even in higher quantities than the intact plants or undifferentiated cell cultures (Sevón and Oksman-Caldentey 2002; Ramachandra Rao and Ravishankar 2002; Akhgari et al. 2015).They gain biomass rather rapidly and have simple cultivation medium requirements, being able to grow without phytohormones. They also show high genetic stability as well as more stable metabolic production than that of undifferentiated cell cultures (Peebles et al. 2009; Häkkinen et al. 2016). This has largely been related to chromosomic stability displayed by the hairy roots (Weber et al. 2008, 2010; Dehghan et al. 2012). The chromosomic number and karyotype of hairy roots are typically the same as in the parent plant. In addition, the ability of hairy roots to grow without additional auxins increases the stability, since when exposed to growth regulators, even organized tissues modify their chromosomic numbers and display somaclonal variation (Baíza and co-workers, Baíza et al. 1999). *Catharanthus roseus* hairy roots displayed genetic and metabolic stability during a 5-year study (Peebles et al. 2009). Similarly Maldonado-Mendoza and co-workers (Maldonado-Mendoza et al. 1993) analyzed the tropane alkaloid production of hairy roots of *Datura stramonium* during 5 years and reported growth rates and alkaloid contents to be stable. In our recent study, hairy roots of *Hyoscyamus muticus* showed genetic and metabolic stability during continuous subculturing in the laboratory during 16-year follow-up (Häkkinen et al. 2016). Hitherto, this is the longest time period reported for continuous subculturing of hairy roots. Very similar results were reported by Sun and co-workers (Sun et al. 2017), with *C. roseus* hairy roots expressing anthranilate synthase. The stability was proven after 11 years of continuous subculturing. On the other hand, also contradictory findings related to high stability of hairy roots have been reported. Hairy roots of *Daucus carota* showed unstable phenotype and unstable transgene expression during a 2-year follow-up (Guivarc'h et al. 1999). Also, unstable production of tropane alkaloids in hairy roots of *Scopolia japonica* was reported, although the follow-up was rather short, 2 months (Mano et al. 1986), and usually adaptation to culture conditions requires time. Taken together, hairy roots have shown a great potential for viable industrial applications due to their high genetic and metabolic stability which surpasses that of undifferentiated cultures (Figs. 1.1 and 1.2).

1.3 Applications of Hairy Root Platform

The main applications of hairy root cultures include the biotransformation, production of high-value plant metabolites, phytoremediation, and production of artificial seeds (Georgiev et al. 2012; Guillon et al. 2006). Some of these examples are discussed further below. A number of studies related to biochemical research especially around plant secondary metabolism have been performed exploiting hairy roots. Alkaloids are compounds which are typically highly bioactive and are

Fig. 1.1 Hairy roots of *Catharanthus roseus* emerging from the wound site

Fig. 1.2 Hairy roots of *Hyoscyamus muticus* cultivated on solid and liquid medium

produced approximately in 20% of all plant species. For their interesting applications, the biosynthesis research related to alkaloids has been active, with hairy roots having an important role as research tools. Such examples are given plenty, as comprehensively listed in review by Giri and Narasu (2000). In the following section, examples of application of hairy root platform in the field of alkaloid research are described in more detail.

Tropane alkaloids are a class of alkaloids many of which are pharmaceutically interesting for their anticholinergic activities. The biosynthetic pathway of tropane alkaloids starts from amino acids arginine and ornithine and on the other hand from phenylalanine. The pathway leading to active pharmaceuticals hyoscyamine and scopolamine is rather well described. Perhaps the most significant finding related to tropane alkaloid research was reported by Hashimoto an co-workers, with isolation and characterization of an enzyme hyoscyamine-6β-hydroxylase (H6H) which converts hyoscyamine into scopolamine in a two-step process (Hashimoto and Yamada 1986) (Matsuda et al. 1991). The gene encoding for H6H has since been overexpressed in various *Solanaceae* plant species (Hashimoto et al. 1993; Parr et al. 1990; Palazón et al. 2003b; Jouhikainen et al. 1999) together with other pathway genes resulting in high accumulation of hyoscyamine and/or scopolamine

(Kang et al. 2011; Rocha et al. 2002). A remarkable yield of scopolamine (411 mg/L) was achieved in hairy root cultures of *Hyoscyamus niger*, by simultaneous overexpression of genes encoding for putrescine methyltransferase and H6H (Zhang et al. 2004). In addition to tropane alkaloid-producing species, overexpression of *h6h* was shown to catalyze the conversion of exogenously applied hyoscyamine into scopolamine in hairy root systems (Häkkinen et al. 2005; Rocha et al. 2002) and even in microbes (Kai et al. 2011; Cardillo et al. 2012). As other notable examples, Robins and co-workers (Robins et al. 1990; Hagan et al. 1999) investigated the tropane alkaloid pathway and revealed the flux regulation and littorine rearrangement pattern in *Datura* hairy roots.

Tobacco alkaloids such as nicotine, nornicotine, and anabasine are synthesized in plant roots where they are transported to plant leaves for storage and for their biological function. These tobacco alkaloids accumulate also in hairy roots, in much higher amounts than in undifferentiated cells (Hamill et al. 1986; Häkkinen et al. 2004). Similar to tropane alkaloids, also tobacco alkaloid pathway is well described except for final steps leading to nicotine and also to other nicotinic acid-derived alkaloids. Tobacco BY-2 cell culture is a widely used plant cell culture for various aspects of plant biochemistry and especially cell cycle research, due to its very high multiplication rate and easy genetic transformation (Nagata et al. 1992). Tobacco BY-2 produces alkaloids after elicitation (Goossens et al. 2003). However, it was unclear why BY-2 cell accumulates anatabine and only small amounts of nicotine after methyl jasmonate elicitation, before Shoji and Hashimoto showed the reason for this to lie in the transcriptional regulation of methyl putrescine oxidase (MPO) (Shoji and Hashimoto 2008). As BY-2 culture does not spontaneously produce alkaloids and as a result constitutive overproduction of alkaloids via genetic engineering might be detrimental to this culture, hairy roots offer an attractive alternative for tobacco pathway engineering (Häkkinen et al. 2007; Lackman et al. 2011). Recently, the biosynthetic pathway of anabasine was further revealed by using hairy root platform with ^{15}N-labelled lysine (Bunsupa et al. 2014). It was interesting to note that no significant labelling was detected in nicotine, anatabine, nor anatalline, indicating that anabasine could be synthesized via nicotinic acid-independent route.

Terpenoids are another group of important secondary compounds with a largest diversity of compound structures and are well known for their many applications in the pharmaceutical, fragrance, and cosmetics industries. Hairy root platform has mainly been exploited with *Catharanthus roseus* for pathway engineering leading to bioactive terpenoid indole alkaloids (TIAs) such as vincristine and vinblastine (Peebles et al. 2011; Hughes et al. 2004). Several TIA pathway genes have been overexpressed in hairy roots including anthranilate synthase holoenzyme (Chung et al. 2007), tryptophan decarboxylase (Hughes et al. 2004), and deacetylvindoline 4-*O*-acetyltransferase (Magnotta et al. 2007). TIA pathway genes have also been expressed in heterologous hosts. As an example, geraniol synthase gene was successfully expressed in tobacco hairy roots resulting in accumulation of geraniol and its glycosides (Vasilev et al. 2014). Engineered hairy roots were also cultivated in larger scale yielding mg amounts of geraniol. Hairy roots of *Cinchona officinalis*

expressing genes encoding for tryptophan decarboxylase and strictosidine synthase yielded high amounts of both tryptamine and strictosidine, as well as quinine and quinidine (Geerlings et al. 1999). However, many examples show that overexpression of a single gene in a specific pathway does not lead to higher accumulation of the desired metabolite, and feedback inhibition is often an encountered problem in metabolic engineering events (Palazón et al. 2008). One problem associated with homologous gene expression or expression of even heterologous genes with high sequence homology with the native genes is co-suppression. In addition, secondary metabolism in plant systems is commonly highly compartmentalized between different cellular organs, and sometimes tissue-specific expression is required, and thus the expression may not be achieved in hairy root systems. Transcription factors (TFs) are promising metabolic engineering targets due to their ability to regulate multiple biosynthetic pathway genes (Memelink and Gantet 2007). The transcription factors regulating TIA biosynthesis include the activators ORCA2, ORCA3, BIS1, BPF1, MYC1, MYC2, and WRKY1 and the repressors JAZ, ZCT1, ZCT2, ZCT3, GBF1, and GBF2 (Zhou and Memelink 2016; Rizvi et al. 2016).

1.4 Advantages and Challenges of Hairy Root Culture Systems

As a plant-based production platform, hairy roots offer several advantages over microbial- or mammalian-based systems (Häkkinen and Ritala 2010). Plant cells exhibit a potential to produce a number of small molecular weight compounds, which some are very difficult or impossible to make via chemical synthesis in an economic way. The risk of endotoxins or oncogenes in the product is nonexisting, while in microbial and mammalian systems, these risk factors should always be considered. Other advantages include the high product homogeneity and easy separation of cells and culture medium for product purification purposes. To date there are some examples of successful production of plant-based natural compound using microbial hosts (Paddon et al. 2013; Galanie et al. 2015), although sometimes the yields have remained rather low. Common problems encountered when trying to transfer the plant-based biochemical pathway to microbes are the availability of precursors; expression and activity of enzymes in prokaryotes, e.g., difficulties associated with expression of cytochrome P450s; and lack of *S*-adenosyl methionine, required in many methylation steps in plant pathways (Khosla and Keasling 2003). A notable study reported by Galanie et al. (2015) showed that the complete biosynthetic pathway of opioids could be reconstructed in yeast; however the final yields remained very low, less than 1 μg/L. While artemisinic acid, a precursor of important antimalarial compound, was successfully produced in yeast after several years of extensive research efforts with very high titers (25 g/L), the final step in the process requires a chemical conversion to reach artemisinin (Paddon et al. 2013).

When it comes to plant-based natural products, cell and tissue cultures, such as hairy roots, offer a viable option for large-scale production due to limitations posed by isolating the compounds from whole plants. Cell and tissue cultures can be cultivated in controlled and contained environment, enabling the optimization efforts for high productivities with possibility to apply GMP (good manufacturing practice). In addition, in whole plants, many plant-derived compounds accumulate in certain plant organs or in specific developmental stage making the yield optimization and production process demanding. Cell culturing enables the use of synthetic growth media, and usually the variation in yields or product quality is low (Häkkinen and Ritala 2010). When it comes to hairy roots, a specific advantage is displayed by their ability to grow relatively fast without growth hormones, reducing the costs deriving from culture medium (Georgiev et al. 2007; Häkkinen et al. 2018). Hairy roots, as other cell culture systems, offer also advantage via reduced costs deriving from product isolation and purification, since unlike whole plants, cell cultures do not possess by-products such as waxes, chlorophyll, oils, or fibers, which often are complicating these processes. However, the choice of the production host and platform should always be made by evaluating the properties of the final product against the total production costs by techno-economic feasibility assessment. It was estimated that the production of a natural product with cell and tissue culture-based host becomes economic when the price of the final product exceeds $500–1000/kg (Sajc et al. 2000). Therefore naturally this system is beneficial for high-value, complex molecules. Nielsen and Keasling estimated that engineering of microbial strains that overproduce a target compound to economically relevant levels takes 6–8 years and over US$50 million, which means much higher numbers for more complex plant cells (Nielsen and Keasling 2016).

Biotransformation has also shown to be viable option for applications with hairy root systems (Banerjee et al. 2012). Perhaps the most often hairy root-catalyzed reaction has been glycosylation, including the reactions leading to digitoxigenin glycosides (Kawaguchi et al. 1990), glycyrrhetinic acid glycosides (Asada et al. 1993), dehydroabietic acid, and phenolic acid glycosides (Fons et al. 1999; Häkkinen et al. 2012). When it comes to high-value commercial compounds, recently we showed that natural raspberry ketone, which is estimated to be the most expensive natural flavor compound after vanillin, was successfully produced in tobacco hairy roots by bioconversion strategy (Häkkinen et al. 2015). Diversity of examples shows that hairy root cultures are entering into a new era of applied research in generating pharmaceutical lead compounds by accomplishing chemical transformations aided through these unique biological systems.

1.5 Bioreactor Design for Hairy Roots

Hairy root morphology sets criteria for bioreactors suitable for cultivation of hairy roots. Tightly packed hairy roots, which are also generally considered as rather shear sensitive, typically form clumps in bioreactors causing mass transfer limitations,

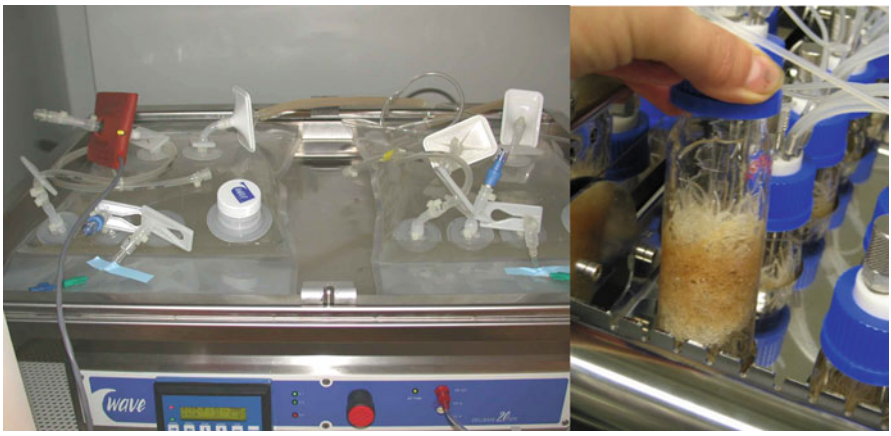

Fig. 1.3 Bioreactors for hairy root cultivation. Wave bioreactor (left) and Medicel Explorer Cultivation Unit (right)

including oxygen and nutrient availability (Georgiev et al. 2007; Eibl and Eibl 2008). Efficient ways to overcome these constraints have been shown by the use of various immobilization techniques, e.g., meshes, cages, or polyurethane foam. Thus, bioreactors with diverse configurations have been used for cultivating hairy roots, including mechanically driven reactors (e.g., stirred tank, wave, and rotating drum reactors), pneumatically driven reactors (e.g., bubble column and airlift reactors), and bed reactors (e.g., trickle bed and mist reactors) (Liu et al. 2009; Georgiev et al. 2010). Disposable bioreactors have demonstrated as promising tools for hairy root cultivations (Lehmann et al. 2014). Major advantages with the use of disposable bioreactors are the minimal cleaning and sterilization and reduced costs via reduced cleaning needs, capital investments, and maintenance (Eibl et al. 2011). Hairy roots are successfully cultivated in bioreactors with wave-induced mixing and aeration (Fig. 1.3). The performance and ginsenoside production of *Panax ginseng* hairy roots in wave bioreactors showed that both factors were significantly improved in wave cultivation compared to shake flask cultivations (Palazón et al. 2003a). Large-scale wave systems with capacities up to 600 L are now commercially available (source: Wave Biotech AG®, Tagelswangen, Switzerland). The most cited and largest hybrid bioreactor (bubble column-spray reactor) to grow hairy roots (*Datura stramonium*) so far is the 500 L Wilson Bioreactor (Wilson 1997).

1.6 Predicting the Future

Since the discovery three decades ago, hairy roots have been a tool for studying the molecular mechanism of a number of basic phenomena in plant behavior, biochemistry, and physiology. Nowadays hairy roots can be induced from practically any

plants; one of the important focuses in hairy root research should be the conservation of biodiversity and production of useful, rare, and exotic compounds from, e.g., endangered plant species. Plant kingdom has an enormous, still largely underutilized potential for the discovery of natural compounds (Newman and Cragg 2016), which may be exploited for human use. Especially for many medicinal plants, the biochemical pathways leading to interesting compounds are still much unknown, and hairy roots offer an excellent platform for pathway discovery.

The main challenge in hairy root biotechnology is still the relatively low yields of production leading to high costs for the desired product. When it comes to large-scale production of natural compounds, bioreactor technology plays a crucial role. Although hairy root cultivation technology has been studied intensively (see reviews by Mehrotra et al. 2015 and Banerjee et al. 2017), there are no flagship cases existing in hairy root-produced commercial products. However, intensive research and development work of both bioreactor design and novel computational tools applying, e.g., modelling, neural networks, and artificial intelligence, will definitely improve the understanding of processes related to hairy root technology and will lead to improved yields (Gallego et al. 2011; Mehrotra et al. 2015; Sweetlove et al. 2017).

Undoubtedly, plant metabolic engineering involving the overproduction of specialized metabolites is a technology which has resulted in great success (Farré et al. 2014). Recently, Sweetlove and co-workers showed how even primary metabolism of plant systems can be successfully engineered using computational modelling (Sweetlove et al. 2017). Another development that will clearly revolutionize plant metabolic engineering is CRISPR-Cas9-mediated genome editing. This technique is being rapidly adopted by the plant community as a robust and simple way to create targeted mutations, and it has also resulted in successful cases with application of hairy roots (Cai et al. 2015; Michno et al. 2015).

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Chapter 2
A Critical Review on Biotechnological Interventions for Production and Yield Enhancement of Secondary Metabolites in Hairy Root Cultures

Mihir Halder, Dipasree Roychowdhury, and Sumita Jha

Abstract In the past three decades, differentiated hairy root culture-related researches gained a great attention due to the equal or greater bio-production capacity of low amount, high-value secondary metabolites as compared to their parent plants with several advantages over undifferentiated cell suspension cultures in plants. This was mainly because hairy roots are capable of auxin-independent rapid growth and are genetically and biochemically stable, with high productivity and suitability for adaptation to large-scale systems. Nowadays, hairy root cultures of various plant species offer a novel promising opportunity and great prospects for in vitro mass production of economically important bioactive metabolites. At present, the productivity of desired compounds by hairy root cultures is generally too low to fulfill the demands of pharmaceutical industry owing to various biological and technological limitations. Screening and selection for high-yielding root lines and optimization of the culture media and the culture conditions like type of nutrient medium, salt strength, source of carbon and concentration, source of nitrogen and the ratio of NH_4^+/NO_3^-, concentration of phosphate, inoculum density, hydrogen ion concentration, temperature, and light intensity and quality have been taken as yield enhancement strategies among others, to produce desired secondary metabolites using hairy root cultures. Feasibility of commercial application of hairy root culture in bioreactors requires several optimization steps. This review highlights some of the recent progress and outlines future prospects for metabolite production and yield enhancement approaches in hairy root cultures for producing bioactive substances.

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2.1 Introduction

Hairy root culture of different plant species has been established by utilizing different strains of *Agrobacterium rhizogenes* (Roychowdhury et al. 2013; Benyammi et al. 2016; Thiruvengadam et al. 2016; Srivastava et al. 2017b; Bathoju et al. 2017), a Gram-negative soil bacterium, which transfers its T-DNA (transfer DNA) of root-inducing plasmid into the host genome during infection (Tepfer 2017; Gelvin 2017; Vaghari et al. 2017). In the past three decades, differentiated hairy root culture-related researches gained a great attention due to its rapid growth in phytohormone-free basal culture medium, genetic and biochemical stability in long-term culture, and the comparable or greater bio-production capacity for secondary metabolites (SMs) as compared to their parent plants with several advantages over undifferentiated cell suspension cultures (Parr 2017; Roychowdhury et al. 2017; Vaghari et al. 2017). Hairy root cultures are well known for high productivity and suitability for adaptation to large-scale bioreactor system (Patra and Srivastava 2015, 2017; Thakore et al. 2017). Additionally, de novo synthesis of secondary metabolites and biotransformation can also be achieved through the hairy root cultures (Bakkali et al. 1997; Sangwan et al. 2008; Banerjee et al. 2012; Srivastava et al. 2017a).

Although SMs derived from field-grown plants have long been a rich source of a wide variety of medicinal drugs that provide chemical scaffolds in the pharmaceutical industry, numerous challenges are encountered such as availability of the plant materials, low abundance, phytogeographical and seasonal variations, tissue-/organ-specific metabolite production, variability in impurities, difficulties in purification process, and economic costs involved in the selection and implementation of appropriate high-throughput screening bioassays (Almagro et al. 2013; Nandagopal et al. 2017). Due to chirality and structural complexity of the targeted SM, chemical synthesis is usually not always possible and if possible, it is economically not viable due to high production cost (Almagro et al. 2013). Thus, hairy root culture emerges as an excellent, convenient, and efficient organ-based tissue culture system alternative to harvesting natural or in vitro grown plants to produce important bioactive metabolites in less time period (Murthy et al. 2014; Srivastava et al. 2017b). Additionally, large-scale production of different SMs is possible through scale-up from laboratory shake culture to hairy root culture in bioreactors. Although large-scale production of SM in bioreactors is quite difficult due to its complexity as it requires investigations on several optimization steps, production of desired SM by hairy root-based technologies in bioreactors has been achieved in few plant species through recent advancements in bioreactor design and construction (Patra and Srivastava 2014a, b, 2015, 2017; Pillai et al. 2015; Thakore et al. 2017).

Preliminary biotechnological approach for plant SM production includes production of hairy root culture of plant species to assess their growth and secondary metabolite accumulation efficacy compared to field-grown or in vitro grown plants or roots (Georgiev et al. 2010; Nourozi et al. 2014; Kumar et al. 2014). Georgiev et al. (2010) reported betalain extracts obtained from hairy root cultures of *Beta vulgaris* cv. Detroit Dark Red showed 20-fold higher total phenolic than extracts of mature intact plant roots. Similarly, production of ~4-fold higher rosmarinic acid (RA; 213.42 μg g^{-1} DW) compared to non-transformed (NT) roots (52.28 μg g^{-1} DW) has been achieved using hairy root cultures of *Agastache foeniculum* (Nourozi et al. 2014). Hairy root culture of *Helicteres isora* developed by transformation with *A. rhizogenes* strain ATCC-15834 using leaf explants showed ~8-fold higher diosgenin yield than the seeds of *H. isora* (Kumar et al. 2014).

At present, although several reports demonstrated that established hairy root culture produced comparable or enhanced amount of secondary metabolites compared to NT plants, in most of the species, the productivity of desired compounds by hairy root cultures is generally too low to fulfill the demands of pharmaceutical industry owing to various biological and technological limitations (Dehghan et al. 2012; Almagro et al. 2013; Murthy et al. 2014). Productivity of any SM in hairy root culture is controlled by two-stage events – biomass accumulation and metabolite content and yield. Biomass accumulation represents first stage of the ultimate yield of the desired secondary metabolite that is greatly influenced by the parameters that control the growth and multiplication of cultured cells/organs, whereas the second stage is represented by the content of the metabolite in cells/organ which is controlled by the parameters that influence biosynthesis of secondary metabolites (Murthy et al. 2014).

Hence, various biotechnological strategies have been developed to evaluate their effectiveness toward improvement of the growth and productivity of secondary metabolites utilizing hairy root culture of different plant species. This includes the selection of high-yielding rhizoclone(s); optimization of culture medium and culture conditions such as optimum level of salt, sugar, nitrogen, and phosphate and physical factors such as temperature, illumination, light quality, medium pH, agitation, aeration, and environmental gas (e.g., oxygen and carbon dioxide); replenishment of nutrient and precursor feeding (Srivastava and Srivastava 2014; Zhu et al. 2014; Chashmi et al. 2016); elicitation (Wang and Wu 2013; Belabbassi et al. 2016; Harfi et al. 2016; Hashemi and Naghavi 2016; Li et al. 2016; Piątczak et al. 2016); application of phytohormones in medium and metabolic engineering (Goklany et al. 2013; Zhao et al. 2013; Shi et al. 2016; Sun and Peebles 2016); and scale-up to bioreactors (Dehghan et al. 2012; Stiles and Liu 2013; Murthy et al. 2014). By following stage-specific strategies, it is possible to produce large amounts of biomass with an increase in the accumulation of secondary compounds (Murthy et al. 2014). This review highlights some of the recent (during 2010–2017) reports on selected biotechnological strategies adapted by different researches for optimum yield of desired secondary metabolites in hairy root cultures.

2.2 Screening and Selection for Fast-Growing and High-Yielding Root Lines

The genotype, constituent of the culture medium, and surrounding environment are the three important factors that control the growth, development, morphogenesis, and secondary metabolite production in vitro. Species- or genotype-specific secondary metabolite accumulation in plants (Almagro et al. 2013; Murthy et al. 2014) signified the necessity of proper selection of high-yielding species or genotype as source of explants for hairy root culture induction for production of target compound (s). As each hairy root line, initiated owing to individual transformation events, may differ from the other showing great variability in the copy number, position, and length of integrated T-DNA into the host plant genome that can be correlated with the variability in morphology, growth rates, and ability to accumulate desired phytochemical(s) in different hairy root lines (Murthy et al. 2014; Basu et al. 2015; Halder and Jha 2016). In perspective of SM productivity, generally root lines have been selected via analysis of the growth of root lines followed by quantification of desired product through UV-Vis (ultraviolet-visible) spectrophotometry, TLC (thin-layer chromatography), HPTLC (high-performance thin-layer chromatography), HPLC (high-performance liquid chromatography), GC-MS (gas chromatography-mass spectrometry), and NMR (nuclear magnetic resonance spectroscopy). Several reports (Basu et al. 2015; Gai et al. 2015a; Jiao et al. 2015; Benyammi et al. 2016; Halder and Jha 2016) clearly demonstrated the importance of screening and proper selection of root line(s) with high biomass and SM yielding capacity among the induced root lines.

Two cultivars of *Catharanthus roseus* transformed with *A. rhizogenes* strains AR15834 and A4 induced 1229 hairy root lines, among them three root lines were selected on the basis of vigorous growth for the production of ajmalicine and catharanthine, and other root lines were discarded as they failed to sustain vigorous growth (Benyammi et al. 2016). Eventually, one root line was selected as high-productive line in terms of biomass production (24.48-fold compare to control) and alkaloid accumulation (3.8 mg g^{-1} DW ajmalicine and 4.3 mg g^{-1} DW catharanthine) and utilized for further studies (Benyammi et al. 2016). Similarly, *A. rhizogenes*-mediated transformation of *Arachis hypogaea* followed by meticulous screening among 150 Ri-transformed root lines of three different *A. rhizogenes* strains, namely, LBA9402, A4, and R1000, helped to achieve 19-fold higher *trans*-resveratrol production compared to NT roots (Halder and Jha 2016).

The variability in growth [growth index (GI) on dry weight (DW) basis] and plumbagin content (4.81–6.69 mg g^{-1} DW) was observed among ten selected highly growing LBA9402-transformed hairy root lines of *Plumbago zeylanica* on solid modified MS (Murashige and Skoog 1962) medium after 4 weeks irrespective of the morphotype of roots (Basu et al. 2015). The identification of high plumbagin yielding transformed root lines such as PzIX33, PzIX11, PzIX15, and PzIX28 of *P. zeylanica* through the screening procedure has been reported by Basu et al. (2015).

Proper screening on the basis of growth parameters such as total root elongation, lateral root density, and biomass accumulation led to the selection of two root clones, AV1 and AV2, that accumulated substantial amount of essential oils than NT roots among the established 92 hairy root clones of *Artemisia vulgaris*, developed by *A. rhizogenes*-mediated genetic transformation with different strains of *A. rhizogenes* (Sujatha et al. 2013). The importance of proper screening and selection of hairy root line(s) among induced and/or established root lines also has been reported in *Astragalus membranaceus* for production of total astragalosides (TAG) (Jiao et al. 2015) and isoflavonoids (Jiao et al. 2014), in *Plumbago zeylanica* for production of plumbagin (Nayak et al. 2015), and in *Isatis tinctoria* for production of flavonoids and total alkaloids (Gai et al. 2015a, b).

Thus, meticulous screening and proper selection of appropriate hairy root line (s) with fast growth rate and high productivity have been reported to be very important factor for the future application of this biotechnological system as a promising platform for large-scale secondary metabolite production. Primary selection of stable high-yielding root line(s) might help us to provide the best potential root line that can be used for further yield enhancement through the application of other strategies like optimization of culture medium and culture condition, application of elicitors, precursor feeding, etc.

2.3 Optimization of Culture Medium for Growth and Secondary Metabolite Production

Several chemical and physical factors such as the type of basal medium, salt strength of the medium, types and levels of carbohydrates, nitrate, phosphate, and growth regulator have been known to affect accumulation of biomass and secondary metabolites in plant cell and organ cultures (Murthy et al. 2014). Thus, optimization of culture media is one of the key strategies for improvement of SM production in hairy roots as discussed below.

2.3.1 Influence of Nutrient Medium and Salt Strength

Several studies demonstrated that biomass accumulation and SM production in hairy root cultures can be improved by manipulation of nutrient medium (Murthy et al. 2014; Carlín et al. 2015; Hanafy et al. 2016). Strength of the macro- and micronutrients in the basal medium (full or half), physical status of the medium (solid or liquid), and individual salt strength of the medium can influence growth and productivity of the SM in the hairy roots depending on species or genotype (Carlín et al. 2015; Chung et al. 2016; Hanafy et al. 2016; Thiruvengadam et al. 2016; Bathoju et al. 2017; Perassolo et al. 2017).

In a number of plant species, MS medium had been reported to be the most suitable medium for optimum growth and target SM production. Hairy root of *Momordica dioica* cultured on MS medium (full-strength) supplemented with 3% sucrose showed optimum biomass accumulation compared to different media tested, namely, half-strength MS, full- and half-strength B5 (Gamborg et al. 1968), NN (Nitsch and Nitsch 1969), and LS (Linsmaier and Skoog 1965) medium (Thiruvengadam et al. 2016). The accumulation of biomass and phenolic compounds such as flavonols (2529.53 μg g^{-1}), hydroxycinnamic acid (1337.69 μg g^{-1}), and hydroxybenzoic acid (1704.61 μg g^{-1}) was found to be significantly higher in hairy roots cultured in full-strength MS medium as compared to roots of NT plant. In comparison to NT roots, significantly higher total phenolic and flavonoid contents were observed in such hairy roots (Thiruvengadam et al. 2016).

Among the different media formulations tested for species *Chlorophytum borivilianum*, viz., B5, NN, MS, LS, and White (White 1963), MS medium was found to be the most suitable for growth of the selected two transformed rhizoclones (2364a and 2364b) with significantly high biomass accumulation (~21.89–22.52 g) compared to NT roots (~4.52 g) (Bathoju et al. 2017). On MS medium, root line 2364b showed the highest accumulation of hecogenin (2.33-fold) and optimum content of stigmasterol (21-fold) compared to the controls (Bathoju et al. 2017).

The optimized growth and anthraquinone production (emodin 211.32 μg g^{-1} DW and physcion 353.23 μg g^{-1} DW) in hairy roots of *Polygonum multiflorum* were achieved in liquid MS basal medium compared with other culture media evaluated such as SH (Schenk and Hildebrandt 1972), B5, and N6 (Chu et al. 1975) media (Thiruvengadam et al. 2014). Although qualitatively 23 types of polyphenolic compounds were detected in both NT and hairy root cultures, hairy root of *P. multiflorum* showed higher amount of total phenolic content including higher amount of pyrogallol, hesperidin, naringenin, and formononetin, as well as significantly higher flavonoid content and antioxidant and antimicrobial activity in optimized condition compared to untransformed roots (Thiruvengadam et al. 2014). Similarly, optimum growth (i.e., up to 19.30-fold) and significantly higher accumulation of anthraquinone constituents (Rhein 2.495 μg g^{-1}; 2.55-fold than in wild-type plant) were obtained when hairy root of *P. multiflorum* culture in full-strength MS medium was compared to other half-strength MS, B_5, and White media (Huang et al. 2014a).

In MS medium, ATCC 15834-transformed root of *Arnica montana* (root clone T4) showed the optimum growth and biomass accumulation (7.6-fold higher than NT roots) with 4.72 cm average root length and 9.15 cm^{-1} lateral root density, followed by growth in B5 medium [with 3.0 g FW (fresh weight), 3.87 cm average root length, and 6.10 cm^{-1} lateral root density] and SH medium (with 2.21 g FW and 4.25 cm^{-1} lateral root density) after 40-day culture (Petrova et al. 2013). Recently, Chung et al. (2016) showed that hairy root culture of *Brassica rapa* ssp. *rapa* shows maximum biomass accumulation in full-strength MS medium, followed by half-strength MS medium and NN, B5, and LS media. The full-strength MS medium was similarly found to be most suitable for the hairy root growth and SM production in *Astragalus membranaceus* (Jiao et al. 2015) and *P. zeylanica* (Nayak et al. 2015).

The effect of different strengths of solid and liquid MS medium on growth and indole-alkaloid production was well studied in hairy root cultures of *C. roseus* (Hanafy et al. 2016). Growth of hairy roots increased ~46-fold and ~34-fold (FW basis) in full- and half-strength liquid MS medium, respectively, compared to wild type. Hanafy et al. (2016) showed 1474.3-fold higher accumulation of vincristine compared to NT roots (~0.3 ng mg^{-1} FW) in hairy roots of *C. roseus*. In addition, the hairy roots showed accumulation of catharanthine (maximum 0.7 ng mg^{-1} FW) which was not detected in the roots of NT grown in full- or half-strength liquid MS medium. Vincristine, catharanthine, and vinblastine were also secreted in the liquid culture medium of hairy root cultures (Hanafy et al. 2016).

On contrary, hairy root culture of *Fagopyrum tataricum* showed significantly higher, i.e., more than twofold, biomass accumulation and rutin production in half-strength MS liquid medium compared to full- and half-strength B5 and N6 medium (Huang et al. 2016). Similarly, when hairy root line W16A4-1 of *Vitis vinifera* subsp. *sylvestris* was cultured in full- and half-strength MS medium, better biomass production was obtained in half-strength MS medium (Hosseini et al. 2017). Saravanakumar et al. (2012) also demonstrated that half-strength MS liquid medium was found to be superior for the biomass accumulation (attained 0.46 g per flask after 30 days) and withaferin A accumulation (72.3 mg g^{-1} DW) in hairy roots in *W. somnifera* in comparison to full-strength MS or B5 medium and half-strength B5 medium. Out of the four media compositions, half-strength MS medium was reported to support better biomass production of hairy root culture of *A. vulgaris* (Sujatha et al. 2013).

Carlín et al. (2015) clearly demonstrated the need of media optimization at a species-specific manner, as growth responses differed among taxa. Twelve different basal culture media, viz., Anderson basal media (Anderson 1978, 1980), DCR (Douglas-fir cotyledon revised medium; Gupta and Durzan 1985), DKW (Driver and Kuniyuki 1984), B5, Heller (Heller 1953), K&M (Kao and Michayluk 1975), MS, N6, NN, Westvaco WV3 (Coke 1996), White, and WPM (Woody Plant Medium; Lloyd and McCown 1981), were tested for biomass production of hairy root culture of six cactus species, namely, *Escobaria chaffeyi*, *Ferocactus peninsulae*, *Mammillaria bocasana* subsp. *bocasana*, *Turbinicarpus lophophoroides*, *T. pseudopectinatus*, and *T. schmiedickeanus* subsp. *schwarzii.* Both the hairy roots of *E. chaffeyi* and *T. schmiedickeanus* subsp. *schwarzii* showed maximum threefold increase of biomass on DKW medium, whereas *M. bocasana* subsp. *bocasana* exhibited optimum twofold increase of biomass on WPM. However, best growth of hairy root culture of *F. peninsulae* and *T. lophophoroides* was achieved on N6 medium. *T. pseudopectinatus* showed optimum growth without callus induction in Heller medium, whereas callus induction was associated with root growth in all other basal medium which suggested that hairy root's morphology was also affected by the culture medium type (Carlín et al. 2015).

Both full- and half-strengths of four different basal media, viz., MS, B5, WP, and NN, were used for optimizing the yield potential of the selected hairy root clone of *Picrorhiza kurroa*, and full-strength B5 medium was found suitable for the maximum biomass yield (GI ~32.72 on the 40th day) which was 2.68 and 4.17 times

higher as compared to root growth in half-strength MS and WP media (Verma et al. 2015). Moreover, the optimum level iridoid glycoside production (1.1- and 1.3-fold higher kutkoside and picroside I) was observed in half-strength B5 medium instead of the full-strength B5 medium (Verma et al. 2015). Similarly, B5 liquid medium was found to be superior among various solid and liquid media tested as it resulted maximum root biomass (36-fold higher) in root culture of *Gentiana scabra* (Huang et al. 2014b).

Recently, the effect of medium on growth kinetics and anthraquinone (AQ) production of hairy root cultures of *Rubia tinctorum* has been well evaluated in half-strength B5 medium and WPM by Perassolo et al. (2017). Interestingly, WPM promoted 58.6% higher growth compared to half-strength B5 medium, whereas enhanced accumulation of intracellular AQ was observed in half-strength B5 medium after 42-day culture (Perassolo et al. 2017). Moreover, AQ release (maximum of ~10% of total AQs) to the half-strength B5 medium was observed, while extracellular AQs were almost undetectable in WPM (Perassolo et al. 2017).

Interestingly, diploid and tetraploid hairy root cultures of *H. muticus* in MS medium supplemented with 50 g L^{-1} sucrose produced 27.5- and 26.5-fold enhanced tropane accumulation than parental plants in spite of both diploid and tetraploid hairy root clones that had higher growth and biomass production in liquid B5 medium compared to liquid MS medium (Dehghan et al. 2012)

Hairy roots of *A. acutangulus* showed optimum growth and tropane alkaloid yield (9.5 mg L^{-1}) in N6 medium as compared to MS, B5, and White (Liu et al. 2013), whereas hairy roots of *Scutellaria baicalensis* grown in full-strength SH medium showed the highest growth and the higher levels of the flavones, baicalin, baicalein, and wogonin in half-strength B5 than in the other basal media used in this study (Kim et al. 2012).

Hairy root culture of *Persicaria minor* showed maximum 35-fold increase of GI and production of β-caryophyllene (major sesquiterpenes) in half-strength MS basal medium supplemented with Gamborg vitamins (1/2 MS-B5) after 8 weeks of culture which was significantly higher than the MS, MS-B5, and 1/2 MS media (Ashraf et al. 2015). These results showed the crucial role of the B5 vitamins for hairy root biomass production in *P. minor* (Ashraf et al. 2015). The first report of production of reserpine in quantifiable amounts from LBA 9402-transformed root lines of *Rauvolfia serpentina* was reported by Ray et al. (2014). The medium optimization and screening among hairy root lines resulted in selection of high reserpine-producing root line RsIX6 showing 5.65-fold increase in accumulation of reserpine on modified MS medium as compared with non-transformed roots of in vitro plantlets (Ray et al. 2014).

The numerous reports on different plant species discussed above emphasize on the critical role of the type of culture medium, physical status of the medium, and the salt strength of the medium for optimum biomass and SM yield in hairy roots and the importance of optimization of these parameters for each species.

2.3.2 *Influence of the Carbon Source and Concentration*

The nature of carbon source used in plant tissue culture medium and its concentration can influence the growth and the accumulation of secondary metabolites in hairy root cultures. Thus, several researchers examined the effects of different types of carbon sources and their different concentrations in optimized culture medium on the growth and/or accumulation of SM in hairy root cultures (Shinde et al. 2010; Praveen and Murthy 2012; Kochan et al. 2014; Verma et al. 2015; Chung et al. 2016; Weremczuk-Jeżyna et al. 2016). In plant tissue culture, usually a single simple sugar or a combination of simple sugars such as glucose, fructose, maltose, and sucrose is used as carbon source for unorganized cell cultures and organ cultures (Murthy et al. 2014). Sucrose is the most significant carbon source for plant tissue cultures, used as the chief energy source and an important constituent in secondary metabolite biosynthesis (Praveen and Murthy 2012; Nagella et al. 2013).

Liu et al. (2013) verified the effect of different sugars including sucrose, glucose, fructose, and galactose contained in a medium on biomass and alkaloid yield of *A. acutangulus* hairy root cultures and reported the best biomass and alkaloid yield was obtained in medium supplemented with 3% sucrose. In hairy root culture of *A. membranaceus*, the effect of different carbohydrates and different concentrations of sucrose was tested, and sucrose was found to be the ideal carbohydrate for biomass production and astragaloside (AG) accumulation (Jiao et al. 2015). Culture medium supplemented with ~3% sucrose favored optimum AG accumulation, but sucrose concentration ~4% was suitable for biomass production (Jiao et al. 2015).

Different concentrations (10–50 g L^{-1}) of sucrose, D-glucose, or D-fructose were used in full-strength MS media to evaluate their effect on biomass growth of transformed hairy roots of *P. zeylanica*. Medium containing sucrose at concentration of 20 g L^{-1} favors maximum biomass accumulation of both A4- and LBA9402-transformed hairy roots, whereas MS medium supplemented with D-glucose and D-fructose showed negative influence on the root biomass accumulation (Nayak et al. 2015).

For testing the effect of individual sugars, viz., glucose, fructose, sorbitol, mannitol, ribose, lactose, rhamnose, galactose, market grade sugar, and sucrose (used as control) as carbon source on yield potential of the hairy root clone 14-P of *P. kurroa*, each was added to the half-strength B5 medium at 4% concentration (Verma et al. 2015). The highest biomass yield and secondary metabolites (1.2-fold, 1.3-fold, and 1.4-fold higher yield of total glycoside, kutkoside, and picroside I, respectively) were obtained when market grade sugar was used as compared to other carbohydrates (Verma et al. 2015).

However, Vinterhalter et al. (2015) reported that the concentration than the type of carbohydrate played a significant role on hairy root growth of *G. dinarica*. Moreover, most of the root clones showed a positive correlation between the carbohydrate concentration up to 116.8 mM in the culture medium and phenolic

content. Higher sugar concentration (175.2 mM) showed negative impact on phenolic production (Vinterhalter et al. 2015). Similarly, xanthone content increased with sucrose concentration (up to 116.8 mM), whereas glucose- or fructose-supplemented medium showed the highest xanthone accumulation at 175.2 mM (Vinterhalter et al. 2015).

Of the various sucrose concentrations (10–120 g L^{-1}) assessed in *Panax quinquefolium* hairy roots cultivated in shake flasks and a nutrient sprinkle bioreactor, medium containing 3–5% sucrose favored root growth, whereas 20 g L^{-1} or more than 70 g L^{-1} sugar concentrations inhibited growth of hairy roots in shake flasks (Kochan et al. 2014). The highest amount of ginsenosides in hairy roots was observed in the medium with 30 g L^{-1} sucrose (Kochan et al. 2014). In bioreactor, similar correlate with saponin content and the sucrose concentration in the medium was also noticed. Lower sucrose concentrations (20 and 30 g L^{-1}) favored accumulation of protopanaxadiol derivatives, while higher sucrose concentrations (50 and 70 g L^{-1}) stimulated accumulation of Rg group saponins (Kochan et al. 2014).

Among the different sucrose concentrations (10–50 g L^{-1}) tested for optimization of hairy root culture of *F. tataricum*, selected hairy root line showed maximal biomass accumulation (45-fold) along with maximum rutin content (4.11-fold) in full-strength MS liquid medium supplemented with 30 g L^{-1} sucrose compared to control (Huang et al. 2016). Similarly, hairy root of *M. dioica* demonstrated the highest biomass accumulation in liquid MS medium supplemented with 3% sucrose, among the tested sucrose concentration, i.e., 1–4% (Thiruvengadam et al. 2016). Of the various concentrations of sucrose (10–40 g L^{-1}) tested for hairy root of *Papaver bracteatum*, MS medium containing 30 g L^{-1} sucrose showed most stimulating effect on growth rate and accumulation of biomass (Sharafi et al. 2013). Similar type of result was observed in hairy root cultures of *A. foeniculum* (Nourozi et al. 2014). Biomass production and phenolic accumulation in hairy roots of *Dracocephalum forrestii* were studied in respect to changes in initial concentration of sucrose (1–7%) using WPM (Weremczuk-Jeżyna et al. 2016). WPM supplemented with 3% sucrose showed optimal biomass accumulation and production of phenolic compounds.

Interestingly, in *B. rapa* ssp. *rapa*, liquid MS medium supplemented with 4% sucrose produced significantly higher hairy root biomass accumulation (FW 97.25 g L^{-1} and DW 10.11 g L^{-1}) in comparison with different concentrations (1–5%) of sucrose (Chung et al. 2016). Hairy root culture of *Psoralea corylifolia* cultured in MS medium containing 40 g L^{-1} sucrose produced the highest biomass 15.6 g L^{-1} on 28th day, whereas the growth of hairy roots was inhibited in lower sucrose concentrations (Shinde et al. 2010). Although maximum 2.08% DW daidzein and 0.37% DW genistein production in hairy roots was reported in MS medium supplemented with 40 g $L^{-1}$and 20 g L^{-1} sucrose, respectively, prolonged isoflavone production, even after 35 days of cultivation, was observed in MS medium with 50 g L^{-1} sucrose (Shinde et al. 2010). Similarly the highest biomass accumulation by hairy root culture of *A. vulgaris* was observed in medium supplemented with 40 g L^{-1} sucrose (Sujatha et al. 2013).

Similar type of study was performed by Praveen and Murthy (2012) using individual or different combinations of various types of carbohydrates such as

sucrose, glucose, fructose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1), and sucrose + glucose (1:1) in the hairy root cultures of *Withania somnifera*. Sucrose-containing MS medium showed optimum withanolide A production. Among the tested sucrose concentration (1–8%), optimum biomass accumulation and withanolide A production were obtained in MS medium supplemented with 3% and 4% sucrose, respectively (Praveen and Murthy 2012). Optimization of carbon source in half-strength MS liquid medium for hairy root growth and withaferin A and withanone production in *W. somnifera* was reported by Sivanandhan et al. (2012). Medium containing 2% sucrose was found best suited for hairy root biomass accumulation (1.41 g DW) after 40 days of culture (Sivanandhan et al. 2012). However, the maximum withaferin A (2.21 mg g^{-1} DW) and withanone (2.41 mg g^{-1} DW) accumulation was achieved in half-strength liquid MS medium with 4% sucrose on the 40th day of culture, followed by sucrose + glucose (4 + 1%) as compared to glucose, fructose, maltose, and other combinations tested (Sivanandhan et al. 2012).

Wawrosch et al. (2014) reported 10.9-fold and 7.6-fold increased leoligin (LG) and 5-methoxy-leoligin (MLG) production, respectively, in hairy root line K8A of *Leontopodium nivale* ssp. *alpinum* when cultured in liquid-modified MS medium supplemented with 6% sucrose compared to control.

Therefore, from the above reports, it is evident that effects of different carbon source and their different concentrations should be evaluated during formulation of the optimized culture medium as it can affect both the growth and/or accumulation of secondary metabolites in hairy root cultures. Enhancement of both biomass accumulation and SM yield can be manipulated by using these strategies.

2.3.3 *Influence of Nitrogen Source and the Ratio of NH_4^+/ NO_3^-*

Although both nitrate and ammonium serve as nitrogen sources in the most common plant tissue culture media such as MS, LS, SH, and B5, the source of nitrogen, the overall concentration of total nitrogen, and the ratio of ammonium (NH_4^+) to nitrate (NO_3^-) of the medium have been shown to affect the growth, biomass accumulation, and SM production in hairy root culture of few plant species (Shinde et al. 2010; Liu et al. 2013; Praveen and Murthy 2013; Sharafi et al. 2013; Murthy et al. 2014).

Total nitrogen concentration in the medium had significant effects on growth and alkaloid production in *A. acutangulus* (Liu et al. 2013). The highest biomass yield (4.5 g L^{-1}) and the maximum alkaloid production (9.9 mg L^{-1}) have been achieved in 90 mM nitrogen concentration with ratio of $NH_4^+/NO_3^- = 4:1$, whereas the low or high total nitrogen concentrations showed inhibitory effect on both biomass and alkaloid production (Liu et al. 2013). However, NH_4^+/NO_3^- ratio of 20:10 mM in liquid MS medium favored the growth of hairy root culture of *P. bracteatum* compared to the tested ratios of NH_4^+/NO_3^-, i.e., 0:20, 10:20, 20:20, 40:20,

60:20, 20:0, 20:10, 20:40, and 20:60 mM (Sharafi et al. 2013). Similarly, maximum biomass accumulation (13.86 g L^{-1} DW) and highest isoflavone productivity of 2.05% DW of daidzein and 0.51% DW of genistein in hairy root culture of *P. corylifolia* were achieved in MS medium supplemented with NH_4^+ and NO_3^- at a ratio of 20:10 than higher concentrations of ammonia or nitrate in a ratio of 40:20 and 60:20 or 20:40 and 20:60 mM (Shinde et al. 2010).

The growth and withanolide A production of *W. somnifera* hairy root culture were also affected by the ratio of $NH_4^+$and NO_3^-, and maximum biomass production (148.17 g L^{-1} FW and 14.79 g L^{-1} DW) was observed at NH_4^+/NO_3^- = 14.38:37.60 mM, while the ratio of NH_4^+/NO_3^- = 0.00:18.80 mM favored withanolide A production (maximum 14.68 mg g^{-1} DW) in MS medium (Praveen and Murthy 2013). The hairy root culture of *A. hypogaea* showed maximum yields of resveratrol, arachidin-1, and arachidin-3 in modified MS medium contain 7.7 mM NH_4^+ and 22.85 mM NO_3^- compared with Gamborg's basal medium (Condori et al. 2010).

Although hairy root growth has been reported sometimes to be retarded by high nitrogen content in the medium, culture medium with high-salt concentrations (DKW medium) and relatively high to medium nitrogen content (N6 medium) stimulates higher biomass accumulation in *E. chaffeyi*, *T. schmiedickeanus* subsp. *schwarzii*, *F. peninsulae*, and *T. lophophoroides*, while *M. bocasana* subsp. *bocasana* showed the best growth on WPM, which has a medium salt concentration and low nitrogen content. However, hairy root culture of *T. pseudopectinatus* preferred low nitrogen medium such as Heller medium (Carlín et al. 2015). Similar studies have been performed using selected hairy roots of *Datura* sp. that were developed by *A. rhizogenes*-mediated genetic transformation of *Datura tatula*, *D. stramonium*, *D. innoxia*, and *D. ferox*, respectively, for production of hyoscyamine (Harfi et al. 2011). The most effective root line, DT10, showed optimum biomass accumulation in full-strength MS and B5 media, whereas diluted media with lower salt content such as 1/2MS, 3/4MS, 1/2B_5, and 3/4B_5 favor hyoscyamine production (Harfi et al. 2011).

The optimum growth and SM production seemed to be affected by the total nitrogen content in medium as well as the ratio of NH_4^+ and NO_3^- in the culture medium in species-specific manner. But the actual mechanism of enhancement is still not clear and needs more systematic studies of expression of key enzymes involved in particular metabolic pathways.

2.3.4 *Influence of Phosphate Concentration*

Inorganic phosphate (Pi) is a crucial element of functional molecules in plants involved in many physiological processes (Peret et al. 2011) and generally obtained in form of the soluble phosphate from the soil. Low phosphate content in soil or culture medium inhibits growth and productivity thus determination of the amount

of phosphorus required by different plants grown in natural habitat as well as culture medium is important to maintain optimum growth and yield.

The effect of the phosphate concentration and other macroelements NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$, and KH_2PO_4 at concentrations of 0, 0.5, 1.0, 1.5, and 2.0× strengths in the MS medium on the production of biomass and withanolide A in hairy roots of *W. somnifera* has been evaluated by Praveen and Murthy (2013). The optimum biomass accumulation, i.e., 139.42 g l^{-1} FW and 13.11 g l^{-1} DW, was observed in the medium with 2.0× concentration of KH_2PO_4, whereas the highest production of withanolide A (15.27 mg g^{-1} DW) was recorded with 2.0× KNO_3 (Praveen and Murthy 2013).

In contrary, the low concentrations of phosphate favored production of isoflavones in hairy roots of *P. corylifolia* (Shinde et al. 2010) and production of phenolic acids in *Salvia miltiorrhiza* (Liu et al. 2016). Liu et al. (2016) reported that phosphate starvation increased the production of phenolic acids in hairy root culture of *S. miltiorrhiza* cultured in liquid 6,7-v medium by inducing the key enzyme genes in a positive feedback pathway that affect tyrosine-derived pathway more than the phenylalanine pathway. Four phenolic acids, i.e., salvianolic acid B (LAB), danshensu (DSU), RA, and caffeic acid (CA), were enhanced in 6,7-v medium by reducing the phosphate level at 0.0124 mM in comparison to the hairy roots grown in control phosphate concentration (1.24 mM). Even the medium containing 0.124 mM phosphate significantly inhibits LAB, DSU, and RA content by 0.19-, 0.06-, and 0.34-fold of control, respectively, except the enhancement of 3.17-fold in CA content. Culture medium with 0.0124 mM phosphate enhanced the yield of LAB, RA, and CA 2.33-, 1.68-, and 2.17-fold as compared to control (Liu et al. 2016). Shinde et al. (2010) also demonstrated that isoflavone production in hairy roots of *P. corylifolia* was favored by low concentrations of PO_4^{3-} as the highest volumetric yield of daidzein (2.06% DW) and genistein (0.37% DW) was obtained in MS medium supplemented with 0.625 mM PO_4^{3-} in comparison to different concentrations of phosphate (0.625–5 mM). In contrast, the biomass accumulation enhanced with increasing concentration of phosphate in the medium.

The phosphate concentration in the basal medium used for hairy root cultures affects optimum growth and SM production, and the optimum concentration required appears to depend on the species. The studies on different species (Shinde et al. 2010; Praveen and Murthy 2013; Liu et al. 2016) showed that the optimum concentration of phosphate that favors maximum accumulation of the SM might not be favorable for growth and optimum biomass in hairy root cultures.

2.3.5 *Influence of Phytohormones*

One amazing property of transformed hairy roots is that they can grow on phytohormone unsupplemented basal medium, unlike the NT roots that require supply of exogenous auxin in the medium. However, the relative influences of different types of auxin on the growth, morphology, and SM production in hairy root cultures have

been reported for a few plant species (Abbasi et al. 2012; Kim et al. 2012, 2017; Cheruvathur and Thomas 2014; Huang et al. 2014b; Nayak et al. 2015).

The influence of various concentrations (0.1, 0.5, and 1 mg L^{-1}) of different auxins [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA)] on growth and flavone (baicalin, baicalein, and wogonin) production in hairy root culture of *S. lateriflora* and *S. baicalensis* is reported (Kim et al. 2012, 2017). Auxin treatments stimulate flavone production with no significant effect on growth of *S. baicalensis* hairy roots. The highest level of baicalin and baicalein accumulation was observed in hairy root culture grown in 1 mg L^{-1} IAA-supplemented medium; meanwhile, the highest amount of wogonin was observed in 1 mg L^{-1} IBA-supplemented medium (Kim et al. 2012). Interestingly, hairy root culture of *S. lateriflora* showed maximum 8% higher biomass accumulation, 1.64-fold higher baicalin, and 2.92-fold higher wogonin accumulation compared to the control in 0.1 mg L^{-1} IBA-supplemented half-strength MS medium, whereas maximum baicalein content, 2.38-fold higher than of the control, was observed in 1/2 MS medium supplemented with 0.1 mg L^{-1} NAA (Kim et al. 2017).

Investigation of the individual effect of different concentrations of IBA and NAA on hairy root growth and rhinacanthin (RC) accumulation in the hairy root cultures of *Rhinacanthus nasutus* exhibited enhanced biomass and RC accumulation compared to control in the auxin supplement medium (Cheruvathur and Thomas 2014). Comparatively better biomass (10.2 g $flask^{-1}$ FW and 2.4 g $flask^{-1}$ DW) and RC accumulation (4.5 mg g^{-1} DW RC-C, 0.50 mg g^{-1} DW RC-D, and 0.25 mg g^{-1} DW RC-N) was observed in 2.5 μM IBA-supplemented medium as compared to NAA and control (Cheruvathur and Thomas 2014).

Hairy root culture of *G. scabra* was used to study the response of different plant growth regulators on the production of SMs. Hairy root cultures showed higher accumulation of loganic acid (6.6-fold) and gentiopicroside (1.8-fold) in medium containing 1 mg L^{-1} zeatin and 1 mg L^{-1} NAA, respectively, as compared to the roots of plants grown in greenhouse (Huang et al. 2014b). On the other hand, 1.4- and 2.5-fold higher gentiopicroside and swertiamarin were obtained in the presence of 1.0 mg L^{-1} NAA as compared to commercial *Gentiana* herb No. 2 (Huang et al. 2014b).

In the investigation on the effect of different auxins (namely, IAA, IBA, and NAA) at different concentrations (1 and 2 mg L^{-1}) on the biomass yield of the selected hairy root lines of *P. zeylanica* that were performed in optimized basal media (i.e., full-strength MS supplemented with 20 g L^{-1} sucrose), the result showed that transformed roots were more sensitive to the presence of exogenous auxin as compared to NT roots and application of exogenous auxin inhibits growth of hairy roots (Nayak et al. 2015).

Abbasi et al. (2012) demonstrated that the supplementation of light-grown hairy root cultures of *Echinacea purpurea* with optimized concentration of 0.025 μM gibberellic acid (GA_3) caused morphological change with enhanced biomass, SM production (caffeic acid derivatives), as well as increased phenylalanine ammonia-lyase (PAL) activity, cell viability, and free radical scavenging activity in hairy roots

in shake flask cultures. Application of lower and higher levels of GA_3 supplementation resulted in a decrease in all parameters tested compared to the control suggesting that the application of optimum GA_3 concentration can be used as an excellent strategy to optimize the production of secondary metabolites from *E. purpurea* hairy root cultures.

2.4 Optimization of the Culture Conditions for Growth and Secondary Metabolite Production

2.4.1 Influence of Inoculum Density

The effect of inoculum density on the growth, biomass accumulation, and SM production by cell suspension culture or hairy root culture in shake culture or bioreactors has been well reported (Danphitsanuparn et al. 2012; Jiao et al. 2015). Determination of optimum inoculum density is important for studies in optimizing various factors including availability of nutrients and oxygen and volume of culture flask (Jeong et al. 2009).

The influence of inoculum density on biomass accumulation and TAG content has been well documented in *A. membranaceus* hairy root cultures (Jiao et al. 2015). Biomass accumulation and TAG content increased with the increase of inoculum density from 1% to 1.6% at a fixed sucrose concentration, but further increase in inoculum size (1.6–2.0%) showed negative effect on both the parameters (Jiao et al. 2015).

Hairy roots of *Pueraria candollei* var. *candollei* grown in 50 ml of liquid B5 medium were used to evaluate the effects of inoculum size (IS) and temperature on growth and production of isoflavonoids (Danphitsanuparn et al. 2012). The result recommended that 1% IS and 32 °C culture temperature were best for optimum growth and isoflavonoid production in hairy roots because maximum 646.2 mg flask^{-1} biomass accumulation with higher GI decreased browning of hairy roots and the highest accumulation of total isoflavonoid content (31.0 mg g^{-1} DW) was obtained under the optimized culture condition (Danphitsanuparn et al. 2012).

2.4.2 Influence of Hydrogen Ion Concentration in Medium

The significant role of initial pH (the hydrogen ion concentration) of the culture medium on biomass accumulation and SM production by hairy root culture has also been studied in some plant species (Liu et al. 2013; Verma et al. 2015; Rahimi and Hasanloo 2016). Medium pH can affect nutrient uptake as well as enzymatic and hormonal activities in plant cell/organ cultures (Praveen and Murthy 2012).

Hairy roots of *Silybum marianum* grown in acidic pH (pH 5) at 25 °C favor biomass accumulation (highest 0.45 g) and silymarin production (0.26 mg g^{-1} DW) in respect to other medium pH 5.7, 6, and 7 (Rahimi and Hasanloo 2016). Moreover, the higher content of silybin (0.025 mg g^{-1} DW), isosilybin (0.024 mg g^{-1} DW), silychristin (0.061 mg g^{-1} DW), and silydianin (0.095 mg g^{-1} DW) was obtained as compared to those grown in higher pH (pH > 5). This could be due to strong lipoxygenase (LOX) activity at acidic environment of medium (Rahimi and Hasanloo 2016).

The significant roles of initial pH of the basal medium on growth and secondary metabolite production have been well documented in the selected hairy root clone of *P. kurroa* using half-strength B5 medium supplemented with 4% sucrose (Verma et al. 2015). Hairy root cultured in basal medium having initial pH 6.0 showed highest yields of biomass and two specific glycosides compared to the basal medium with different initial pH such as 3.0 ± 0.1, 4.0 ± 0.1, 5.0 ± 0.1, 7.0 ± 0.1, and 8.0 ± 0.1 (Verma et al. 2015).

Liu et al. (2013) reported that basal medium at pH 6.5 was optimum for *A. acutangulus* hairy root growth resulting in twofold higher biomass accumulation compared to basal medium at pH 4.5. However, roots cultured in medium at pH 4.5 showed maximum alkaloid yield, i.e., 7.2 mg L^{-1}. In *I. tinctoria* among the tested pH range 4–6.5, the culture medium with initial pH at 5.8 favored optimum biomass production and bioactive alkaloid accumulation in hairy roots (Gai et al. 2015a).

2.4.3 *Influence of Temperature*

There are few studies on the effect of temperature at which cultures are maintained on growth and SM accumulation in hairy root cultures. However, temperature of the culture environment can influence biomass and SM production by the hairy root culture to large extent (Jeong et al. 2009; Danphitsanuparn et al. 2012; Jiao et al. 2015; Rahimi and Hasanloo 2016).

The investigation on the effect of temperature regimes, 30 °C/25 °C, 25 °C/25 °C, and 15 °C/20 °C, in 16 h/8 h cycle with respect to the root biomass and silymarin production in hairy root cultures of *S. marianum* demonstrated that the optimum biomass accumulation and secondary metabolite production occurred at temperature 25 °/25 °C in 16 h/8 h cycle (Rahimi and Hasanloo 2016).

A. membranaceus hairy root cultures showed increase in biomass and TAG content with increase in temperature from 24 to 28 °C but decreased significantly at above 28 °C temperature (Jiao et al. 2015). Under optimized conditions (inoculum size 1.54%, culture temperature 27.8 °C, sucrose concentration 3.24%, and harvest time 36 days), hairy root cultures produced optimal biomass (15.79 g L^{-1} DW) and TAG accumulation (2.65 mg g^{-1} DW) which exhibited significant superiority to that of 3-year-old field-grown roots (Jiao et al. 2015).

The hairy roots of *P. candollei* var. *candollei* grown in liquid B5 medium showed a significantly lower percentage of browning and higher isoflavonoid production,

promoting accumulation of the daidzein when cultured at 32 °C than at 25 °C (Danphitsanuparn et al. 2012).

2.4.4 *Influence of Light Intensity and Quality*

Light irradiation and quality and intensity of light also can influence growth and secondary metabolisms in hairy root culture (Yu et al. 2005; Mukherjee et al. 2016; Pandey et al. 2016). Upon continuous illumination, hairy roots of *Daucus carota* was turned green due to accumulation of total chlorophyll ~1.2 mg g^{-1} FW as compared to negligible chlorophyll content in hairy roots cultured in darkness (Mukherjee et al. 2016). Interestingly, a metabolic shift from phenylpropanoid/benzenoid biosynthesis toward volatile isoprenoids was evidenced by 48% reduced *p*-hydroxybenzoic acid (*p*-HBA) accumulation (1.1 mg g^{-1} DW) along with subsequent increase of monoterpene and sesquiterpene accumulation in green hairy roots as compared to dark-grown hairy roots (Mukherjee et al. 2016). Suppression of *p*-hydroxybenzaldehyde dehydrogenase (HBD) activity and lower methyl salicylate content was noted in the green hairy roots compared to normal hairy root culture (Mukherjee et al. 2016). Reported that light levels lower than 10 μmol m^{-2} s^{-1} induced significant changes of root pigmentation and morphology by increasing root pigment content and root biomass.

Inhibitory effect of light on growth of four stable rhizoclones of *S. rebaudiana* was observed when cultured under light compared to root grown under dark condition with substantially higher photosynthetic pigment accumulation in only two rhizoclones (Pandey et al. 2016). Hairy rhizoclone SRA4 showed synthesis of diterpene steviol glycoside, stevioside (max. 1.72 mg g^{-1}DW in the root tissues and max. 2.12 mg L^{-1} in media), when grown under light, not detected in roots grown in dark, and a direct positive correlation between stevioside content and expression UGT85C2 gene was observed (Pandey et al. 2016).

2.5 Conclusions

Different biotechnological interventions such as meticulous screening and selection, optimization of culture conditions and chemical constituents of culture medium, precursor feeding, application of elicitor molecules, and metabolic engineering can improve production and yield of secondary metabolites in hairy root cultures. In this review, we discuss the aspects of meticulous screening and selection of high-yielding root line and optimization of culture conditions and chemical constituents of culture medium for improvement of secondary metabolite production. Traditionally, optimization of culture conditions and chemical constituents of culture medium was done by monitoring the influence of *one-variable-at-a-time* which requires a large number of experiments to study the effect of all the variables, and there was a

possibility to overlook the interactive affects among the variables studied (Amdoun et al. 2010). Therefore, an effective mathematical and statistical technique called response surface methodology (RSM) that is more effective for optimization studies where interactive factors may be involved becomes the most popular optimization method in recent years (Amdoun et al. 2010). Applying this model, 212.7% enhanced hyoscyamine production was achieved in the jasmonic acid (JA)-elicited hairy roots of *Datura stramonium* in optimized B5 medium (that contains 79.1 mM NO^{3-}, 11.4 mM Ca^{2+}, and 42.9 mg L^{-1} of sucrose) in comparison with elicited hairy roots cultured in B5 medium (Amdoun et al. 2010).

Another modern approach called artificial neural network (ANN)-based prediction of optimal in vitro culture parameters like inoculum density, pH and volume of growth medium per culture vessel, and sucrose content for maximum hairy root biomass yield of *Glycyrrhiza* was reported by Prakash et al. (2010). This kind of study could be a model system in exploitation of hairy root cultures for commercial production of pharmaceutical compounds using large bioreactors (Prakash et al. 2010). Later, ANN-based combinatorial model was proposed based on five hidden Markov models (HMMs) derived for five test culture conditions, i.e., pH of liquid growth medium, volume of medium per culture vessel, sucrose concentration (% w/v) in growth medium, nitrate concentration (g L^{-1}) in the medium, and density of initial inoculum (g FW) per culture vessel, and their corresponding fresh weight biomass and its efficiency were assessed for the prediction of optimal values of different environmental factors to achieve maximum productivity in a bioprocess in terms of high biomass in *Rauwolfia serpentina* (Mehrotra et al. 2013).

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Chapter 3
pRi-Transformed Plants as a Source of Secondary Metabolites

Ewelina Piątczak, Renata Grąbkowska, and Ewa Skała

Abstract p*Ri*-transformed plants are obtained from hairy roots by *Agrobacterium rhizogenes*-mediated transformation. The hairy roots may be an attractive alternative for obtaining material from field-cultivated plants because of their rapid growth and often higher secondary metabolite production. Another value of the hairy roots may be their ability to regenerate whole transgenic plants. These transgenic plants are characterized by morphological changes known as hairy root syndrome. Additionally, the transformed plants also accumulated valuable secondary metabolites at higher levels than nontransformed plants. These alterations are associated mainly with the co-expression of *rol*A, *rol*B, and/or *rol*C genes derived from *A. rhizogenes* plasmids. Recent interest has grown in the application of pRi-transformed plants as a potentially rich source of pharmaceutically valuable metabolites, especially those which cannot be chemically synthesized. The chapter presents the recent progress made in the production of valuable secondary metabolites by pRi-transformed plants and the limitations associated with it.

Keywords *Agrobacterium rhizogenes* · Transformed plants · Secondary metabolites

3.1 Regeneration of pRi-Transformed Plants from Hairy Roots

The regeneration of shoots from transformed roots often occurs spontaneously (Piątczak et al. 2015), or it can be light-dependent or induced by the growth regulators (Xu et al. 2006; Gangopadhyay et al. 2010). Table 3.1 presents a list of recent literature regarding the method of regenerating pRi-transformed plants from the hairy roots of various plant species, and it is a continuation of the previous

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Table 3.1 Transgenic plants obtained after *Agrobacterium rhizogenes*-mediated transformation (data published after 2000)

Method of plant obtained			Plant species (family)	*Agrobacterium rhizogenes* strain	Culture medium and culture conditions	References
Spontaneous	Light	Liquid medium	*Plumbago indica* (Plumbaginaceae)	ATCC 15834	Hormone-free MS, 2% sucrose, 16-h photoperiod, 40 μmol m^{-2} s^{-1} light intensity	Gangopadhyay et al. (2010)
			Rauwolfia serpentina (Apocynaceae)	A4	Hormone-free B5, 2% sucrose, continuous light	Mehrotra et al. (2013)
			Pelargonium graveolens cv. Hemanti (Geraniaceae)	A4 and LBA 9402	Hormone-free ½ MS, 3% sucrose	Saxena et al. (2007)
		Solid medium	*Solidago nemoralis* (Asteraceae)	R1000	Hormone-free MS, 1% sucrose, 12-h photoperiod, 45 μmol m^{-2} s^{-1} light intensity	Gunjan et al. (2013)
			Hypericum perforatum (Hypericaceae)	A4	Hormone-free MS/B5, 3% sucrose, 16-h photoperiod, 50 μmol m^{-2} s^{-1} light intensity	Tusevski et al. (2014)
			Tylophora indica (Asclepiadaceae)	A4	Hormone-free MS, 3% sucrose, 16-h photoperiod, 48 μmol m^{-2} s^{-1} light intensity	Chaudhuri et al. (2006)
			Digitalis purpurea (Plantaginaceae)	R1601	Hormone-free B5, 5% sucrose, 16-h photoperiod, 2000–3000 light intensity	Lioshina and Bulko (2014)
			Vinca minor (Apocynaceae)	R1601	Hormone-free B5, 5% sucrose, 16-h photoperiod, 2000–3000 light intensity	Lioshina and Bulko (2014)
	Dark	Liquid medium	*Centaurium erythraea* (Gentianaceae)	LBA 9402	Hormone-free WP, 3% sucrose	Piątczak et al. (2006), Piątczak and Wysokińska (2013)
			Rehmannia glutinosa (Orobanchaceae)	A4	Hormone-free WP, 3% sucrose	Piątczak et al. (2015)
		Solid medium	*Ophiorrhiza pumila* (Rubiaceae)	ATCC 15834	Hormone-free B5, 2% sucrose	Watase et al. (2004)
			Cephaelis ipecacuanha (Rubiaceae)	ATCC 15834	Hormone-free ½MS, 3% sucrose	Yoshimatsu et al. (2003)
			Rehmannia elata (Orobanchaceae)	R1000	Hormone-free MS + 200 mg L^{-1} timentin, 3% sucrose	Kim et al. (2012)

Under the influence of plant growth regulators	Direct organogenesis	Apple rootstock Jork 9 (Rosaceae)	ATCC 15834 and A4	Solid MS + 1 μM NAA + 0.1 or 1 μM TDZ, 30 g L^{-1} sorbitol, 16-h photoperiod, 33 μmol m^{-2} s^{-1} light intensity	Pawlicki-Jullian et al. (2002)
		Betula pendula (Betulaceae)	R1600	Solid WP + 0.1 μM TDZ, 3% sucrose	Piispanen et al. (2003)
		Crotalaria juncea (Fabaceae)	A13	Solid B5 + 1, 3 or 5 mg L^{-1} BA, 1% sucrose, continuous light, 60 μmol m^{-2} s^{-1}	Ohara et al. (2000)
		Crotalaria spectabilis (Fabaceae)	A13	Solid B5 + 1, 5 or 10 mg L^{-1} BA, 2% sucrose, continuous light, 60 μmol m^{-2} s^{-1}	Ohara et al. (2012)
		Dracocephalum kotschyi (Lamiaceae)	ATCC 15834	Solid MS + 0.1, 0.25, 0.5 or 1 mg L^{-1} BA +0.1 mg L^{-1} NAA, 3% sucrose, 16-h photoperiod	Sharafi et al. (2014)
		Duboisia myoporoides – *D. leichhardtii hybrid (Solanaceae)*	ATCC 15834	Solid ½ MS + 5 mg L^{-1} BA, 3% sucrose, 16-h photoperiod, 80 μE m^{-2} s^{-1} light intensity	Yoshimatsu et al. (2004)
		Lycopersicon chilense, *L. peruvianum* var. *humifusum*, *L. esculentum* x *L. peruvianum*, *L. esculentum* cv. MsK, *L. hirsutum f. hirsutum* (Solanaceae)	8196	MS + 1 mg L^{-1} zeatin, 2% sucrose, 16-h photoperiod, 40–50 μmol m^{-2} s^{-1} light intensity	Peres et al. (2001)
		Pelargonium graveolens cv. Hemanti (Geraniaceae)	A4 and LBA 9402	Semi-solid and liquid ½ MS + 0.25 or 0.5 mg L^{-1} BA+0.05 or 0.1 mg L^{-1} NAA, 1.5% sucrose	Saxena et al. (2007)
		Rehmannia elata (Orobanchaceae)	R1000	Solid MS+ 0.01, 0.05 and 0.1 mg L^{-1} BA, kinetin, IAA, IBA or NAA, 3% sucrose, 16-h photoperiod, 35 μmol m^{-2} s^{-1} light intensity	Chae et al. (2013)

(continued)

Table 3.1 (continued)

Method of plant obtained		Plant species (family)	*Agrobacterium rhizogenes* strain	Culture medium and culture conditions	References
		Solanum khasianum (Solanaceae)	A4	Liquid MS + 0.57, 1.14, 2.85, and 5.7 μM IAA or 0.46, 0.92, 2.32, and 4.64 μM kinetin, 3% sucrose, 14 h photoperiod, 35 μmol m^{-2} s^{-1} light intensity	Jacob and Malpathak (2005)
		Solidago nemoralis (Asteraceae)	R1000	Solid MS + 0.1 or 1 mg L^{-1} NAA and 0.1 or 1 mg L^{-1} NAA + 2 or 5 mg L^{-1} BA and 0.1 or 1 mg L^{-1} NAA + 2 or 5 mg L^{-1} BA, 1% sucrose, 12-h photoperiod, 45 μmol m^{-2} s^{-1} light intensity	Gunjan et al. (2013)
		Vinca minor (Apocynaceae)	R1601	Solid B5 + 0.1 or 1 mg L^{-1} NAA, 0.1 or 0.5 mg L^{-1} IBA, 1 mg L^{-1} BA or kinetin and 0.1 mg L^{-1} BA+ 1 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin+1 mg L^{-1} NAA, 0.5 mg L^{-1} BA+0.4 mg L^{-1} IBA, 1 mg L^{-1} BA+ 0.1 mg L^{-1} NAA, 5% sucrose, 16-h photoperiod, 2000–3000 light intensity	Lioshina and Bulko (2014)
		Zea mays (Poaceae)	ATCC 15834 and A4	MS + 0.2–0.4 mg L^{-1} NAA + 1.6 mg L^{-1} zeatin, 16-h photoperiod, 37 μmol m^{-2} s^{-1} light intensity	Xu et al. (2006)
Under the influence of plant growth regulators	Via callus	*Alhagi pseudalhagi* (Fabaceae)	A4	Solid MS + 3 mg L^{-1} BA, 16-h photoperiod, 60 μmol m^{-2} s^{-1} light intensity	Mei et al. (2001)
		Catharanthus roseus (Apocynaceae)	R1000	Solid MS + 13.32 or 31.08 μM BA and 5.37 or 10.74 μM NAA, 3% sucrose, 16-h photoperiod, 3 W m^{-2} light intensity	Choi et al. (2004)
		Digitalis purpurea (Plantaginaceae)	A13	Solid ½MS + 0.5, 1 or 2 mg L^{-1} zeatin, BA or kinetin, 3% sucrose, light 6000 lux for 16 h	Koga et al. (2000)
		Origanum vulgare (Lamiaceae)	ATCC 15834 and K599	Solid MS + 0.1, 0.5, 0.75or 1 mg L^{-1} 2,4-D, 3% sucrose, 16-h photoperiod	Habibi et al. (2016)

		Pogostemon cablin (Lamiaceae)	ATCC 15834	Solid MS +0.1, 0.5 or 1.0 mg L^{-1} BA+0.1 or 0.2 mg L^{-1} NAA, 3% sucrose, 12-h photoperiod, 40 μmol m^{-2} s^{-1} light intensity	Shi et al. (2011)
		Rehmannia glutinosa f. hueichingensis (Orobanchaceae)	ATCC 15834	Olid ½ MS + 0.2 mg L^{-1} kinetin +3.0 mg L^{-1} BA, 3% sucrose, 16-h photoperiod, 35 μmol m^{-2} s^{-1} light intensity	Zhou et al. (2007, 2009)
	Somatic embryogenesis	*Aralia elata* (Araliaceae)	ATCC 15834	Solid MS + 1.0 mg L^{-1} 2,4-D, 2% sucrose, dark	Kang et al. (2006)
		Codonopsis lanceolata (Campanulaceae)	R1000	Solid MS + 2 mg L^{-1} 2,4-D, 2% sucrose, dark	Kim et al. (2011)
		Coronilla varia (Fabaceae)	ATCC 15834	MS+ 0.2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} kinetin	Han et al. (2006)
		Panax ginseng (Araliaceae)	ATCC 15834	Solid MS + 1 mg L^{-1} 2, 4-D, 3% sucrose, 16-h photoperiod, 24 μmol m^{-2} s^{-1} light intensity	Yang and Choi (2000)
		Salvia miltiorrhiza (Lamiaceae)	R1601	Solid MS + 1.0 mg L^{-1} 2, 4-D+ 0.5 mg L^{-1} BA, 3% sucrose, dark	Wang et al. (2013)
		Tylophora indica (Asclepiadaceae)	A4	Solid hormone-free MS, 3% sucrose, 16-h photoperiod of 48 μmol m^{-2} s^{-1} irradiance	Chaudhuri et al. (2006)

B5 nutrient medium according to Gamborg et al. (1968), BA 6-benzyladenine, 2,4-D 2,4-dichlorophenoxyacetic acid, IAA indole-3-acetic acid, IBA indole-3-butyric acid, MS nutrient medium according to Murashige and Skoog (1962), ½ MS a half strength Murashige and Skoog medium (1962), NAA α-naphthaleneacetic acid, TDZ thidiazuron, WP Woody Plant nutrient medium according to Lloyd and McCown (1980)

studies (Christey 2001; Roychowdhury et al. 2013). The transformed plants often demonstrate hairy root syndrome which is characterized by the multiple branching of aerial parts and roots, increased biomass accumulation, and alterations in morphology, with small and wrinkled leaves and changes in flower size (Christensen et al. 2008; Roychowdhury et al. 2013). These alterations are associated mainly with the co-expression of *rol* and *aux* genes derived from the *Agrobacterium rhizogenes* plasmid.

3.2 Secondary Metabolite Accumulation in pRi-Transformed Plants

Secondary metabolites are the chemical compounds naturally present in the plants which need protection against stress conditions (Saxena et al. 2013). These compounds may also protect human health; they have been found to be able to prevent disease and demonstrate antimicrobial, antioxidant, antiinflammatory, and anticancer properties, among others (Saxena et al. 2013). Plant secondary metabolites comprise various classes of chemical compounds, for example, phenolic acids, flavonoids, alkaloids, plant steroids, and terpenes. The transformed plants obtained by biotechnological methods from hairy roots by *A. rhizogenes*-mediated transformation are known to accumulate valuable secondary metabolites, often at higher levels than nontransformed plants or wild plants, and this approach may serve as an alternative and complementary method for obtaining plant cultivars which produce high levels of bioactive compounds. Chaudhuri et al. (2006) suggest that the alterations occurring in biochemical phenotype of transformed plants may be due to changes in polyamine metabolism and cell sensitivity to auxins and that they are associated mainly with the co-expression of *rol*A, *rol*B, and *rol*C genes. The *rol* genes are powerful tools which can be used to manipulate the secondary metabolism (Roychowdhury et al. 2015). However, only a few different classes of secondary metabolites appear to be produced in pRi-transformed plants (Table 3.2).

3.2.1 Alkaloids

The largest class of bioactive compounds is that of the alkaloids. Under natural conditions, the level of these metabolites is low, constituting less than 1% (Thakkar and Ray 2014). Previous studies have described a promising alternative strategy for the production of high levels of alkaloids based on obtaining transformed plants of various species belonging to the Solanaceae, Rubiaceae, or Apocynaceae, among others (Sevón et al. 1997; Watase et al. 2004; Chaudhuri et al. 2006). The classification of alkaloids is a complex one, being based not only on their structure but also on their chemical characteristics and their biological or biogenetic origin (Kakhia

Table 3.2 Secondary metabolite production in pRi-transformed plants

Class of metabolite	Metabolite	Plant species (family)	Agrobacterium rhizogenes strain	References
Alkaloids				
Indole	Camptothecin, vincamine	*Ophiorrhiza pumila* (Rubiaceae), *Ophiorrhiza rugosa* var. *decumbens* (Rubiaceae), *Vinca minor* (Apocynaceae)	ATCC 15834, DC-AR2, LBA 9402	Kamble et al. (2011), Watase et al. (2004)
Isoquinoline	Cephaeline, emetine, protoemetine	*Cephaelis ipecacuanha* (Rubiaceae)	ATCC 15834	Tanaka et al. (1995)
Morphinan	Codeine, morphine	*Papaver somniferum* (Papaveraceae)	MAFF 03–01724	Yoshimatsu et al. (2003), Yoshimatsu and Shimomura (1992)
Phenanthroindolizidine	Tylophorine	*Tylophora indica* (Apocynaceae)	A4	Chaudhuri et al. (2006), Roychowdhury et al. (2013, 2015)
Tropane	Atropine; calystegines A3, B1, and B2; calystegine; hyoscyamine; 6β-hydroxyhyoscyamine; scopolamine; solasodine	*Atropa belladonna* (Solanaceae), *Convolvulus arvensis* (Convolvulaceae), *Duboisia myoporoides* × *D. leichhardtii* (Solanaceae), *Hyoscyamus muticus* (Solanaceae), *Solanum khasianum* (Solanaceae)	ATCC 15834, A4, LBA 9402, *A. rhizogenes* with synthetic *crypt* gene	Aoki et al. (1997), Celma et al. (2001), Chaudhuri et al. (2009), Jacob and Malpathak (2005), Oksman-Caldentey et al. (1991), Sevón et al. (1997)
Diterpenoids				
Abietane (tanshinone)	Cryptotanshinone, tanshinone I, tanshinone IIA	*Salvia miltiorrhiza* (Lamiaceae)	R1601	Wang et al. (2013)
Scopadulan	Scopadulcic acid B	*Scoparia dulcis* (Plantaginaceae)	ATCC 15834 with *bar* gene	Yamazaki et al. (1996)
Essential oil	6-Caryophyllene, citronellyl esters, citronellol, 10-epi-γ-		A4, LBA 9402, HRi	Pellegrineschi et al. (1994), Saxena et al. (2007)

(continued)

Table 3.2 (continued)

Class of metabolite	Metabolite	Plant species (family)	Agrobacterium rhizogenes strain	References
	eudesmol, geraniol, geranyl ester, guaia-6,9-diene, isomenthone, linalool, rose oxides	*Pelargonium graveolens* cv. Hemanti (Geraniaceae), *Pelargonium* sp. (Geraniaceae)		
Iridoids	Aucubin, catalpol, catalposide, harpagide, harpagoside, loganin	*Rehmannia glutinosa* (Orobanchaceae)	ATCC 15834, A4	Hwang (2006), Piątczak et al. (2015)
Secoiridoids	Gentiopicroside, sweroside, swertiamarin, gentiopicrin	*Centaurium erythraea* (Gentianaceae), *C. pulchellum* (Gentianaceae), *Gentiana macrophylla* (Gentianaceae)	LBA 9402, A4M7OGUS	Janković et al. (2002), Piątczak et al. (2006), Wu et al. (2011)
Phenolic compounds				
Flavonoids: flavonols, flavan 3-ols	Epicatechin, hyperoside, kaempferol 6-O-glucoside, kaempferol 3-O-rhamnoside, kaempferol 3-O-rutinoside, proanthocyanidin dimer, quercetin, quercetin 6-C-glucoside, quercetin 3-O-pentoside, quercitrin, rutin	*Hypericum perforatum* (Hypericaceae)	A4	Tusevski et al. (2014)
Phenolic acid	3-Caffeoylquinic acid, 3-p-coumaroylquinic acid, 3-feruloylquinic acid, quinic acid	*Hypericum perforatum* (Hypericaceae)	A4	Tusevski et al. (2014)
Phenylpropanoids	Epoxypseudoisoeugenol-2-methylbutyrate, isoverbascoside, verbascoside	*Pimpinella anisum* (Apiaceae), *Rehmannia glutinosa* (Orobanchaceae)	A4	Andarwulan and Shetty (1999), Piątczak et al. (2015)
Phloroglucinol derivatives	Adhyperforin, hyperforin	*Hypericum perforatum* (Hypericaceae)	A4	Tusevski et al. (2014)

Quinones				
Naphtodianthrones	Hypericin, protopseudohypericin, pseudohypericin	*Hypericum perforatum* (Hypericaceae)	A4, ATCC 15834	Tusevski et al. (2014)
Naphthoquinone	Plumbagin	*Plumbago indica* (Plumbaginaceae)		Gangopadhyay et al. (2010)
Xanthones	Banaxanthone E, demethyleustomin, eustomin, garcinones C and E, mangiferin, mangiferin C-prenyl isomer, γ-mangostin isomer, 1,3,6,7-tetrahydroxyxanthone dimer, tri-hydroxy-1-methoxy-C-prenyl xanthone	*Hypericum perforatum* (Hypericaceae), *Centaurium erythraea* (Gentianaceae), *Centaurium pulchellum* (Gentianaceae)	A4, A4M70GUS	Janković et al. (2002), Tusevski et al. (2014)
Steroids				
Cardenolides	α/β-Acetyldigoxin, digitalinum	*Digitalis lanata* (Plantaginaceae)	A4	Pradel et al. (1997)
	Verum, glucoevatromonoside, glucoverodoxin, deacetyllanatoside C, neoglucodigifucoside, neoodorobioside G, odorobioside G, lanatosides A and C			
Ecdysone	20-Hydroxyecdysone	*Ajuga reptans* var. *atropurpurea* (Lamiaceae)	MAFF03-01724	Tanaka and Matsumoto (1993)
Triterpenoid saponins	Aster saponin Hb, bacopasaponins D and F, bacopasides II and V, foetidissimoside A, lancemaside A	*Codonopsis lanceolata* (Campanulaceae), *Bacopa monnieri* (Plantaginaceae)	A4, R1000	Kim et al. (2011), Majumdar et al. (2011)

2012), and although all members possess a heterocyclic nitrogen atom, they are a chemically diverse group (Guirimand et al. 2010).

3.2.1.1 Indole Alkaloids

The pharmaceutically important alkaloids are the indole alkaloids, including vincamine obtained from the Apocynaceae family, which is used in cerebral insufficiencies and in dementia (Steinhäuser 1986; Fischhof et al. 1996), and camptothecin, a natural plant compound with anticancer properties (Ramesha et al. 2011), originally found in *Camptotheca acuminata* of the Nyssaceae (Watase et al. 2004). Tanaka et al. (1995) reported high levels of vincamine accumulation in transformed plants of *Vinca minor* (Apocynaceae). The leaves of plants originating from the transformed roots of the Vm-101 clone produced twice as much vincamine as nontransformed plants after 3 months of growth in the soil, in the greenhouse. The alkaloid content reached 0.42% of plant dry weight (Tanaka et al. 1995).

Due to difficulties in the synthesis of camptothecin, there is a need to identify alternative methods for the production of the compound. In vitro cultures of several plants have been studied as viable sources of camptothecin (Watase et al. 2004). Watase et al. (2004) described an efficient protocol for the regeneration of transformed *Ophiorrhiza pumila* plants from hairy roots after *A. rhizogenes* (15834 strain)-mediated transformation. These plants were able to produce camptothecin in levels of 66–111% compared with wild-grown *O. pumila*. It was found that shoots obtained from *Ophiorrhiza rugosa* var. *decumbens* transformed roots using *A. rhizogenes* strain LBA 9402 accumulated 0.012% dry weight of camptothecin (Kamble et al. 2011).

3.2.1.2 Isoquinoline Alkaloids

Isoquinoline alkaloids with emetine, protoemetine, and cephaeline were found in the families Rubiaceae, Alangiaceae, and Icacinaceae (Akinboye and Bakare 2011). Emetine is one of the major secondary metabolite present in the roots of *Cephaelis ipecacuanha,* a member of the Rubiaceae family which has shown antiparasitic, antiviral, and anticancer activity (Akinboye and Bakare 2011). Cephaeline is also found in *C. ipecacuanha* roots and, together with emetine, possesses expectorant and vomitive properties (Garcia et al. 2005). However, the content of cephaeline, emetine, and protoemetine cannot be enhanced in the pRi-transformed plants of *C. ipecacuanha*, regenerated spontaneously from transformed roots (Yoshimatsu et al. 2003).

3.2.1.3 Morphinan Alkaloids

Another pharmaceutically important group of alkaloids is that of the morphinan alkaloids, also known as benzylisoquinoline alkaloids. This group include morphine and codeine, these being two powerful analgesics used to treat moderate to severe and chronic pain in patients with terminal cancer; papaverine, known to be a muscle relaxant; and noscapine, an antitussive compound (Fossati et al. 2015). Yoshimatsu and Shimomura (1992) found the level of morphinan alkaloids to be similar in transformed shoots of *Papaver somniferum* from the Papaveraceae to that observed in the nontransformed plants (213 vs. 182 μg morphine equivalents g^{-1} of fresh weight analyzed by ELISA). Furthermore, HPLC analysis showed that while morphine was not detected in transformed shoots of *P. somniferum*, it was present at a level of 50 μg g^{-1} of dry weight in nontransformed shoots. In contrast, the level of codeine in transformed shoots was about half that of the control shoots (750 vs. 1310 μg g^{-1} of dry weight) (Yoshimatsu and Shimomura 1992).

3.2.1.4 Phenanthroindolizidine Alkaloids

Tylophorine, the phenanthroindolizidine alkaloid, a secondary metabolite present in *Tylophora indica,* is known to demonstrate a range of biological properties, including antiinflammatory, antileukemic, antitumor, immunosuppressive, antiamoebic, and anticandidal effects (Roychowdhury et al. 2013). Transformed plants of *T. indica* may be a valuable tool for the efficient production of tylophorine (Chaudhuri et al. 2006; Roychowdhury et al. 2013; Roychowdhury et al. 2015). Tylophorine was found in all parts of plants which were spontaneously regenerated from transformed roots, but much higher amounts were found in the shoots than in the roots (Chaudhuri et al. 2006). Additionally, the tylophorine content in the shoots of transformed plants was 20–60% higher than in the wild plants. In addition, transformed plants of *T. indica* obtained from transformed roots by somatic embryogenesis also showed higher levels of tylophorine than nontransformed plants (Roychowdhury et al. 2013, 2015). In 1-year-old transformed plants, it was found to be present in amounts ranging from 1.7 to 2.93 mg g^{-1} of dry weight, these being 1.4–2.3 times higher than in nontransformed plants (Roychowdhury et al. 2015). Additionally, the transformed plants of *T. indica* retained the ability to synthesize higher levels of tylophorine even after 6 years of growth in in vitro culture and acclimatization in the field (Roychowdhury et al. 2013). The content of tylophorine increased about twofold to 3.75 mg g^{-1} of dry weight in the leaves of the transformed plants following 1 year of transfer to the field.

3.2.1.5 Tropane Alkaloids

The tropane alkaloids, found in some species of the Solanaceae, are also very important for medicinal purposes. Tropane alkaloids act as parasympatholytics and have been found to competitively antagonize acetylcholine (Palazón et al. 2008). Greater tropane alkaloid production, especially hyoscyamine and scopolamine, was observed in transformed plants of *Atropa belladonna* compared to those of nontransformed plants (Aoki et al. 1997). On the other hand, the transformed plants of *Duboisia myoporoides* x *D. leichhardtii* (*A. rhizogenes* A4 strain) showed lower scopolamine and hyoscyamine content than nontransformed controls (Celma et al. 2001). Similarly, smaller amounts of scopolamine and hyoscyamine were found in different clones of transformed *Hyoscyamus muticus* plants than in controls (Sevón et al. 1997), but the transformed plants showed a higher level of another tropane alkaloids such as calystegines A3, B1, and B2 (Sevón et al. 1997). The *Convolvulus arvensis* plants obtained after transformation by *A. rhizogenes* with an introduced *crypt* gene also demonstrated greater calystegine content: about 35% in the roots and 42% in the shoots (Chaudhuri et al. 2009). Calystegines are a polyhydroxylated nortropane alkaloid known to be a glycosidase inhibitor (Molyneux et al. 1993).

3.2.2 Diterpenoids

Another valuable class of secondary metabolites are the diterpenoids, composed of four isoprene units. This class includes abietane diterpenoids, with the tanshinones, and scopadulan diterpenoids, with scopadulcic acid B.

3.2.2.1 Abietane Diterpenoids: Tanshinones

Tanshinones are characterized by the presence of *o*- or *p*-naphthoquinone and a furan ring, known as a chromophore system (Wu et al. 1991). These secondary metabolites are used for the treatment of coronary heart disease, angina pectoris, and myocardial infarction (Wang et al. 2013). Additionally, tanshinones are known to have antioxidant, antiinflammatory, and antibacterial effects and to provide cardiocerebrovascular protection (Xing et al. 2017). Tanshinones are lipophilic compounds identified in *Salvia miltiorrhiza* (Lamiaceae). The major tanshinones found in this genus are tanshinone I, tanshinone IIA, and cryptotanshinone. As tanshinones possess a wide range of biological activities, there is a great demand for effective biotechnological methods to enhance production. Despite the many reports describing the efficiency of the hairy root cultures as sources of tanshinones (Wang and Wu 2010; Wang et al. 2013), only one study has reported enhanced production of these compounds by transformed plants derived from the hairy roots of *S. miltiorrhiza* via somatic embryogenesis (Wang et al. 2013). The roots of 150-day-old transformed

plants accumulated about twice the amount of tanshinones (calculated as the sum of cryptotanshinone, tanshinone I, and tanshinone IIA) compared with wild-type plants. The content of tanshinones reached 0.44 mg g^{-1} dry weight. Of the three types of tanshinones, tanshinone I and cryptotanshinone production were enhanced in the transformed plants (Wang et al. 2013).

3.2.2.2 Scopadulan Diterpenoids: Scopadulcic Acid B

Scopadulcic acid B, a tetracyclic, scopadulan diterpenoid, possesses antiviral and antitumor activity (Mathew and Jayachandran 2009) and has been identified in *Scoparia dulcis*, a member of Plantaginaceae family. Scopadulcic acid B was produced in in vitro cultures of *S. dulcis* (Hayashi 1996) and in transformed plants obtained spontaneously from hairy roots transformed by ATCC 15834 *A. rhizogenes* strain including the *bar* gene (Yamazaki et al. 1996). Unfortunately, four clones of transformed *S. dulcis* plants demonstrated reduced scopadulcic acid B content (0.11–0.44% dry weight) when compared to nontransformed plants (0.74% dry weight) (Yamazaki et al. 1996).

3.2.3 Essential Oils

An interesting group of secondary metabolites are the essential oils, a mixture of volatile compounds with terpenes, terpenoids, and aromatic and aliphatic constituents characterized by low molecular weight (Bassolé and Juliani 2012). The essential oils are known to possess cytotoxic, antimicrobial, antioxidant, and antiinflammatory activities (Bakkali et al. 2008); their components demonstrate antagonistic, additive, or synergistic effects with each other (Bassolé and Juliani 2012), and their effects are strongly influenced by the ratios of their constituents. The chemical composition of essential oils is dependent on many factors such as climatic conditions, geographic origin, time of the collection, or the part of the plant. Therefore, biotechnology methods, with their greater potential for quality control, seem to represent a promising alternative to traditionally cultivated or naturally growing plants for obtaining essential oils. As reported by Saxena et al. (2007), the composition of the essential oils obtained from two lines of transformed plants (LZ-3 and 14TG) of *Pelargonium graveolens* cv. Hemanti (Geraniaceae) from hairy roots transformed by A4 or LBA 9402 *A. rhizogenes*, differed from that of control plants. The essential oils showed higher concentration of geraniol (9.6% and 7.9%, respectively, vs. 1.1%), geranyl esters (9% and 14.8%, respectively, vs. 2.2%), 10-epi-γ-eudesmol (6.6% and 10.3%, respectively, vs. 3.8%), and linalool (1.6% and 0.9%, respectively, vs. 0.5%) (Saxena et al. 2007).

3.2.4 Iridoids

Iridoids are secondary metabolites found in numerous plant species, typically as glycosides. Structurally they are cyclopentano[c]pyran monoterpenes represented by iridane (cis-2-oxabicyclo[4.3.0]nonane) (Tietze 1983). The basic skeletal ring of the compounds is a bicyclic H-5/H-9β, β-cis cyclopentanopyran ring, but different enantiomeric iridoids are also met in nature (Boros and Stermitz 1991; Foderaro et al. 1992; Dinda et al. 2007). Cleavage of the pyran ring leads to iridoid formation. In plants, iridoids are derived from 9-hydroxynerol by phosphorylation, cyclization, oxidation, or glycosidation (Cornforth 1970). It is believed that the precursor of iridoids in plants is iridodial or 8-epi-iridodial (Dinda et al. 2007). Iridoids exhibit cardiovascular, hypolipidemic, antitumor, and antihepatotoxic activities (Taskova et al. 2002).

They are present in several plant families, including the Plantaginaceae, Rubiaceae, Loganiaceae, Scrophulariaceae, and Orobanchaceae (Dinda et al. 2007). However, only two reports describe improved iridoid glycoside production in pRi-transformed plants (Hwang 2006; Piątczak et al. 2015). The authors reported spontaneous plant regeneration from hairy root clones of *Rehmannia glutinosa*, a Chinese medicinal plant which is able to produce several valuable iridoid glycosides, including catalpol, aucubin, catalposide, and harpagide, which exhibit antitumor, hepatoprotective, antiinflammatory, and hypoglycemic activities (Zhang et al. 2008).

Piątczak et al. (2015) used ultrahigh-pressure liquid chromatography (UHPLC) analysis to examine the production of six iridoid glycosides, i.e., catalpol, aucubin, loganin, catalposide, harpagide, and harpagoside, in pRi-transformed shoots cultured in vitro, as well as in leaves and roots of transformed plants grown for 6 and 12 months in pots. It was found that the shoots produced lower amounts of catalpol, while the leaves and roots of the pRi-regenerants accumulated similar amounts of harpagoside; higher amounts of catalposide, aucubin, and harpagide; or lower amounts of catalpol and loganin compared to nontransformed *R. glutinosa* plants. The authors also noted that the metabolite levels varied with the age of the plants and the organ analyzed. Moreover, it was shown that the yield of all analyzed compounds was higher in comparison with nontransformed plants because of increased shoot and root biomass of the transformed plants (Piątczak et al. 2015). In contrast, Hwang (2006) reported higher levels of catalpol in transformed plants (0.56%) of *R. glutinosa* than in nontransformed ones (0.43%).

3.2.5 Secoiridoids

Secoiridoids occur following the cleavage of the cyclopentane ring of iridoids (Dinda et al. 2007). They are usually connected with a sugar moiety to form glycosides. These metabolites are common in the orders Cornales, Dipsacales, and

Gentianales. They are usually used as bitter tonics in treating stomach disorders (Ghisalberti 1998). Higher levels of compounds have been noted in transformed plants from the Gentianaceae family (Janković et al. 2002; Piątczak et al. 2006; Wu et al. 2011); in particular, 72.4% greater production of one secoiridoid glycoside, and gentiopicroside was observed in the roots of regenerated transformed plants of *Gentiana macrophylla* compared with the roots of nontransformed plants (Wu et al. 2011). Similarly, Piątczak et al. (2006) reported higher secoiridoid glycoside content (expressed as the total content of gentiopicroside, sweroside, and swertiamarin) in the transformed shoots and whole plants of *Centaurium erythraea*, often used in gastrointestinal disorders (Šiler and Mišić 2016). The transformed shoots regenerated spontaneously from hairy roots infected by LBA 9402 strain of *A. rhizogenes* giving the whole transformed plants (Piątczak et al. 2006). The shoots were able to produce 280 mg g^{-1} dry weight of total secoiridoid glycoside content, which was eight times higher than observed in commercially available *C. erythraea* herb (Piątczak et al. 2006). Very interesting results were reported by Janković et al. (2002) who noted that transgenic plants of *C. erythraea,* regenerated spontaneously on several hairy root clones infected with strain A4M70GUS of *A. rhizogenes*, produced gentiopicrin and swertiamarin (1.19% and 1.40%, respectively), although hairy root clones did not produce the compounds at all.

3.2.6 Phenolic Compounds

Phenolic compounds are aromatic secondary plant metabolites which naturally occur in almost all plants (Herrmann 1989). The metabolites are a subclass of larger secondary metabolites named as "phenolics," which possess a phenol ring, bearing at least one hydroxyl substituent (Croteau et al. 2000). Phenolics are divided into two categories: simple phenols (possess one phenol subunit) and polyphenols (possess at least two phenol subunits) (Clifford 1999).

3.2.6.1 Flavonoids

3.2.6.1.1 Flavonols

Flavonols are polyphenols which are members of the flavonoids (Hollman and Arts 2000). Flavonoids and flavonols have a 15-carbon skeleton structure, with two phenyl rings (A and B) and a heterocyclic ring C. Chemically, flavonols have a double bond between positions 2 and 3 and a ketone group in position 4 of the C ring. Additionally, they have a hydroxyl group at position 3 (3-hydroxyflavone). The 3-hydroxyl group can be glycosylated and links to a sugar such as glucose, rhamnose, or galactose (Hollman and Arts 2000). Tusevski et al. (2014) demonstrated improved production of selected flavonols (quercetin 6-C-glucoside, quercetin 3-O-

pentoside, quercetin, kaempferol 6-C-glucoside, hyperoside) in transformed shoots of *Hypericum perforatum*. The authors found that the shoots produced significantly higher amounts of quercetin 6-C-glucoside in comparison with controls. Moreover, quercetin 3-O-pentoside and the flavonoid quercetin aglycone were de novo synthesized in transgenic shoots. On the other hand, kaempferol 6-C-glucoside and hyperoside were significantly decreased in transgenic shoots compared to controls. However, the total contents of identified flavonols were almost equal in transgenic and control shoots (Tusevski et al. 2014).

3.2.6.1.2 Flavan 3-ols

Flavan-3-ols are derivatives of flavans with a 2-phenyl-3,4-dihydro-2*H*-chromen-3-ol skeleton (Yang et al. 2012). Of these metabolites, epicatechin and an unidentified proanthocyanidin dimer were detected in transformed shoots of *Hypericum perforatum* (Tusevski et al. 2014). The pRi-transformed shoots of the species produced twice as much epicatechin and 26 times more of the unidentified proanthocyanidin dimer than controls. In contrast, a twofold decrease of the other proanthocyanidin dimer was found in the transgenic shoots. However, the total amount of flavan-3-ols remained unchanged in both transgenic and control shoots (Tusevski et al. 2014).

3.2.6.2 Phenolic Acids

Simple phenols, including phenolic acids, consist of one phenol ring and one carboxylic acid substituent. The phenolic acids present in plants contain two carbon frameworks, these being hydroxycinnamic and hydroxybenzoic structures (Robbins 2003); however, the considerable range of potential numbers and positions of hydroxyl groups on the aromatic rings result in a wide variety of phenolic acids being available (Robbins 2003). Phenolic acids play a key role in the synthesis of lignins, lignans, flavonoids, flavonols, and a wide range of other phenolic secondary constituents. Only one report found phenolic acid production to be improved in transformed plants (Tusevski et al. 2014). Of four phenolic acids (quinic acid, chlorogenic acid, 3-p-coumaroylquinic acid, 3-feruloylquinic acid) examined in transformed shoots of *Hypericum perforatum*, only 3-feruloylquinic acid was found to be produced in transformed shoots, but not in nontransformed controls. Moreover, the authors also showed that both the level of chlorogenic acid and the total amount of identified phenolic acids in the transformed shoots were 1.3 times higher than in control shoots (Tusevski et al. 2014).

3.2.6.3 Phenylpropanoids

The phenylpropanoids are a large group of phenolic compounds derived from L-phenylalanine or L-tyrosine. They consist of a three-carbon propene chain of cinnamic acid attached to a six-carbon, aromatic phenyl group (C6-C3 compounds). Most phenylpropanoids are formed from cinnamic or p-coumaric acids (Seigler 1998). Phenylpropanoids are biosynthesized in many plant families, usually as various alcohols, mono-, di- and trisaccharide esters. The metabolites are common in the Lamiaceae, Hypericaceae, Boraginaceae, Rubiaceae, Scrophulariaceae, and Apiaceae (Seigler 1998). Piątczak et al. (2015) noted 1.2- and 1.6-fold greater acteoside production in the leaves and roots of *Rehmannia glutinosa* derived by genetic transformation (*A. rhizogenes* strain LBA 9402) compared with nontransformed plants. In contrast, a lower content of phenylpropenyl ester – an anethole precursor – and epoxypseudoisoeugenol-2-methylbutyrate was detected in transformed shoot cultures of *Pimpinella anisum*; however, the metabolite content was higher in hairy root cultures than in nontransformed root culture (Andarwulan and Shetty 1999).

3.2.6.4 Phloroglucinol Derivatives: Hyperforin and Adhyperforin

Hyperforin and its homologue – adhyperforin – are polyprenylated phloroglucinol derivatives found in members of *Hypericum* genus. The compounds are accumulated in oil glands, pistils, and fruits, where it acts probably as plant defense against herbivory (Beerhues 2006). The metabolites exhibit antidepressant, anxiolytic, antitumor, and antibacterial (against Gram-positive bacteria) properties (Beerhues 2006). Transformed shoots of *H. perforatum* produced twice as much hyperforin than the control shoots. In contrast, adhyperforin was identified in trace amounts in transgenic shoots, while in control shoots the compound was not confirmed at all (Tusevski et al. 2014).

3.2.6.5 Quinones

3.2.6.5.1 Anthraquinones: Naphtodianthrones

Naphtodianthrones are anthraquinone derivatives. Two of the pharmaceutically important naphtodianthrones are hypericin and pseudohypericin which are the main active components of the plants from the *Hypericum* genus. They are localized in the glandular structures on flowers, stamens, leaves, and stems (Jensen et al. 1995). The compounds are photodynamic pigments which possess antidepressant activity, due to the inhibition of reuptake of several neurotransmitters (Butterweck et al. 1998). Moreover, the metabolites are also considered as antiviral agent against human immunodeficiency virus type 1 (HIV-1) (Meruelo et al. 1988). In transformed

shoots of *H. perforatum,* hypericin and pseudohypericin were identified by Tusevski et al. (2014). The authors reported that the transformed shoots produced 11- to 12-fold more hypericin and pseudohypericin than the control shoots. Moreover, transgenic shoot cultures of *H. perforatum* accumulated significantly higher levels of total naphtodianthrones than the control shoots (Tusevski et al. 2014).

3.2.6.5.2 Naphthoquinones

Naphthoquinones are phenolic compounds derived from the shikimate pathway and usually occur as glycosides. The compounds are usually biosynthesized in the Bignoniaceae, Plumbaginaceae, Juglandaceae, and Boraginaceae (Aldred 2009). One plant-derived naphthoquinone is plumbagin (5-hydroxy-2-methylnaphthalene-1,4-dione), occurring in the roots of the Plumbaginaceae (van der Vijver 1974), which is known to have valuable antitumor, anticancer, and antimicrobial activity (Didry et al. 1994; Kuo et al. 2006; Hazra et al. 2008). Significantly higher naphthoquinone content was detected in transformed plants of *Plumbago indica*, another traditional medicinal plant, after genetic transformation with *A. rhizogenes* (strain ATCC 15834). The content of plumbagin in the transformed plants ranged between 5.17 and 7.77 mg g^{-1} dry weight depending on the clone of transgenic plants, which was 3–3.4 times higher than in nontransformed plants (Gangopadhyay et al. 2010).

3.2.6.6 Xanthones

Xanthones (xanthen-9*H*-ones or dibenzo-gamma-pirone) are a class of polyphenolic oxygenated heterocycles. Xanthones are produced in several medicinal plants, including *Centaurium erythraea.* Janković et al. (2002) report that shoots of the transgenic plants of the species produced demethyleustomin and eustomin at higher levels than wild-growing plants (0.06–1.21% and 0.03–0.04%, respectively). Xanthones were also produced by transformed shoots of *Hypericum perforatum* (Tusevski et al. 2014). The authors demonstrated that the production of two xanthone derivatives (named X3 and X8) significantly increased in transformed shoots. On the other hand, the production of another eight xanthones decreased after transformation. It is worth nothing that mangiferin was the major xanthone in the transgenic shoots of *H. perforatum*, which accounted for 42% of the total xanthones. The authors reported also that the transformed shoots produced seven other xanthones, not detectable in control shoots. However, the total content of xanthones in transformed shoots of the species was lower than in control shoots (Tusevski et al. 2014).

3.2.7 Steroids

3.2.7.1 Cardenolides

Cardenolides are naturally occurring cardiac-active steroids possessing a five- or six-membered lactone ring. They are usually present as glycosides in several plant families including the Asclepiadaceae, Apocynaceae, and Scrophulariaceae (Yamane et al. 2010). Due to their inhibitory effects against Na^+/K^+-ATPase activity (or the Na^+-K^+ pump), which is involved in the maintenance of ion levels in cells and neurotransmission (Yamane et al. 2010), the compounds are typically toxic against cardiovascular and autonomic nervous systems in humans, as well as for most animals, including insects. Cardenolides (cardiac glycosides) were produced in transformed *Digitalis lanata* shoots (clone A4/2) regenerated from hairy roots (Pradel et al. 1997). Their production, determined by the inhibition of Na^+/K^+-ATPase activity, was found to be similar in transformed shoots (2.87 μmol g^{-1} dry weight) and nontransformed shoots (3.08 μmol g^{-1} dry weight) (Pradel et al. 1997). Pradel et al. (1997) also noted that no cardenolides were detected in hairy root clones, although they were found in roots derived from both transformed and nontransformed plants (0.81 and 1.30 μmol g^{-1} dry weight, respectively). The authors claim that these findings confirm that cardenolides are synthesized in the leaves of the whole plants and then transported to the roots (Christmann et al. 1993; Pradel et al. 1997). HPLC/MS analysis found the main cardenolides in the roots of nontransformed and transformed plants of *D. lanata* to be digitalinum verum, glucoverodoxin, deacetyllanatoside C, neoglucodigifucoside, and odorobioside G, while in the leaves, the dominant cardenolides were lanatosides A and C (Pradel et al. 1997).

3.2.7.2 Ecdysone

Another steroid which can be produced in pRi-transformed plants is 20-hydroxyecdysone (20-HE). It is a physiological inducer of molting and metamorphosis in arthropods (Borovsky et al. 1985) and is a naturally occurring phytoecdysteroid hormone which can be used as a pest control agent (Kubo et al. 1983). Ecdysteroids have an anabolic effect in humans (Gallo et al. 2006). 20-HE and its derivatives have been found to have antioxidant, antidiabetic, analgesic, and antidepressant activity and are known to stimulate protein synthesis (Gallo et al. 2006; Thiem et al. 2017). Dinan (2001) reported the level of 20-HE in naturally growing plant species to be low, being only 0.1% or less of dry weight. Increased 20-HE production may be obtained from plants spontaneously regenerated from *Ajuga reptans* var. *atropurpurea* hairy roots after *A. rhizogenes* MAFF03-01724 transformation (Tanaka and Matsumoto 1993). Transformation resulted in approximately twice the level of 20-HE being produced compared to nontransformed plants, ranging from 0.074% to 0.102% of dry weight (Tanaka and Matsumoto 1993).

3.2.8 *Triterpenoid Saponins*

Triterpenoid saponins are triterpenes which belong to the large group of the saponins (Hao et al. 2015). They are pentacyclic molecules synthesized from isoprene through the cytosolic mevalonate pathway, resulting in a 30-carbon compound arranged in 4 or 5 rings with several oxygens attached (Yarnell 2007). Triterpenoid saponin production can be enhanced in transformed plants of *Codonopsis lanceolata* (Campanulaceae): an Asian plant species used in traditional medicine as antiinflammatory agent against bronchitis and coughs (Lee et al. 2002; Xu et al. 2008; Kim et al. 2011). An LC-MS/MS study by Kim et al. (2011) found the triterpenoids lancemaside A, foetidissimoside A, and aster saponin Hb accumulated in the leaves, stems, and roots of transformed plants regenerated via somatic embryogenesis from hairy roots infected with *A. rhizogenes* (R1000 strain). The authors found greater total content of the three triterpenoid saponins (expressed as the sum of lancemaside A, foetidissimoside A, and aster saponin Hb) in transformed regenerants than wild-type plants. The highest total triterpenoid accumulation was detected in the stems of the regenerants than in their leaves and roots (Kim et al. 2011). Similarly, significantly higher levels of saponins in pRi-transformed plants were reported in an important Indian medicinal plant, *Bacopa monnieri* (Scrophulariaceae) (Majumdar et al. 2011). The authors noted that several saponins (bacopasaponin D, bacopasaponin F, bacopaside II, and bacopaside V) were produced at higher levels in the transformed plants than in nontransformed ones. However, the authors also observed that similar contents of other saponins, i.e., bacoside A3 and bacopasaponin C, were present in the transformed and wild-type plants of *B. monnieri* (Majumdar et al. 2011).

3.3 Conclusions

The chapter has described the tremendous potential of transformed plants regenerated from hairy roots obtained after *A. rhizogenes*-mediated transformation. The potential is not only derived from the altered morphology of the transformed plants (lateral branching, shortened internodes, more abundant flowering), which can be particularly useful for ornamental plants, but also with the fact that transformation may be an alternative and complementary strategy to achieve greater amounts of pharmaceutically important bioactive compounds, such as tanshinones, tylophorine, or camptothecin. Despite many reports describing the efficient protocols for hairy root cultures as the source of bioactive compounds, so far, comparatively few studies have demonstrated that pRi-transformed plants can be used for the production of valuable secondary metabolites. Therefore, it is very important to continue such studies to identify new plant species whose pRi-transformed regenerants display greater production of valuable secondary metabolites.

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Chapter 4
Biotechnological Interventions of Hairy Roots of Tropane Alkaloid-Bearing Plants

Guoyin Kai, Weiwei Zhao, Min Shi, and Yao Wang

Abstract As one large group of plant secondary metabolites, tropane alkaloids (TAs) can be produced by a few genera of the family *Solanaceae* including *Anisodus*, *Atropa*, *Datura*, *Hyoscyamus*, and *Scopolia*. Due to their anti-cholinergic activity, tropane alkaloids including hyoscyamine and scopolamine are widely used as antispasmodics and mydriatics. Because of low contents in tropane alkaloid-bearing plants, it is urgent to elevate the production of tropane alkaloids by means of biotechnology approaches to meet the increasing clinical demand. Hairy roots, with the characters of fast-growing, auxin-independent, and genetically stable, were considered as a promising system to produce active plant-origin compounds including tropane alkaloids. Recently, hairy root systems of some tropane alkaloid-producing plants such as *Anisodus acutangulus* have been successfully established. Meanwhile, several key enzymes involved in the TAs biosynthetic pathway have been cloned and introduced into related genetic engineered hairy root systems, which lay the foundation for production of tropane alkaloids in hairy roots by large-scale bioreactors in the future. Here, the recent advances of pharmacological activity, hairy root, biosynthesis pathway, and genetic engineering were summarized, and problems along with prospects were also discussed.

Keywords *Solanaceae* · Tropane alkaloids · Hairy roots · Biosynthesis pathway · Metabolic engineering

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4.1 Introduction

As one group of bioactive secondary metabolites, alkaloids are generally alkaline nitrogen-containing organic compounds in plants which play an important role in plant growth and development, some of them act as anti-insect compounds or phytoalexin, and some other have long been used as stimulants, narcotic sedatives, or poisons (Wink and Roberts 1998; Huang et al. 2005). Alkaloids exhibited various pharmacological activities, for example, anti-inflammatory, antibacterial, dilated blood vessels, anticancer, etc. Until now, the chemical structure of more than 100,000 alkaloids is clear. Most of alkaloids are distributed in higher plants, especially in dicotyledons, such as *Solanaceae*, *Rutaceae*, *Leguminosae*, *Menispermaceae*, and so on. According to the nitrogen-containing skeleton at the core, alkaloids can be divided into multiple types, namely, indole alkaloids, quinoline alkaloids, quinazoline alkaloids, and tropane alkaloids (Li et al. 2008).

Tropane alkaloids (TAs) are a class of alkaloids containing a tropane ring in their chemical structure which include more than structurally known 200 compounds, such as hyoscyamine, scopolamine, anisodamine, and anisodine (Fig. 4.1). These compounds have been among the oldest drugs in medicine because of their wide-ranging pharmaceutical applications. Several genera including *Anisodus*, *Datura*, *Atropa*, *Duboisia*, and *Hyoscyamus* which belong to plant family *Solanaceae* can produce tropane alkaloids (Kai et al. 2007). Nevertheless, the content of TAs is

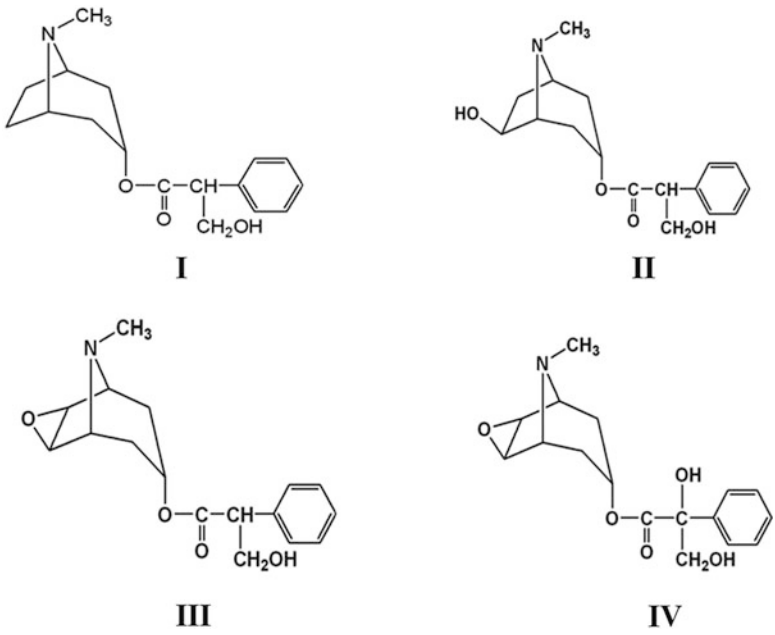

Fig. 4.1 The chemical structures of several common TAs. (**I**) Hyoscyamine (**II**) Anisodamine (**III**) Scoploamine (**IV**) Anisodine

distinguishingly distributed in different plants of *Solanaceae* (Table 4.1). In addition, field culture of traditional herbal plants exhibited several disadvantages such as long period of growth and low content, leading to the production of TAs that cannot meet the increasing demand of clinical application. Due to their anticholinergic action on the parasympathetic nervous system and other bioactivities, especially scopolamine which is the most valuable TAs because of its higher pharmacological activity, fewer side effects, and relatively limited supply, it is of importance and significance to elevate the production of TAs by multiple strategies (Häkkinen et al. 2005).

Along with the rapid progress of plant biotech methods, biosynthetic pathway of TAs has been gradually understood. Meanwhile, genetic manipulation has been a widely-used alternative for increasing the production of targeted metabolites. Here, recent advances in the understanding of pharmacological activities, biosynthesis pathway, genetic engineering with hairy root system, and various biotech approaches for more efficient production of TAs were summarized, and problems along with prospects were also discussed.

4.2 Pharmacological Activities of TAs

Hyoscyamine, scopolamine, anisodamine, and anisodine have been widely used in clinical application in different forms. For example, tiotropium bromide, a quaternary ammonium salt of scopolamine semisynthetic analogs, was approved for the treatment of chronic obstructive pulmonary disease (COPD) by FDA (Koumis and Samuel 2005). Many studies have reported different pharmacological activities of tropane alkaloids.

4.2.1 Hyoscyamine

Atropine is the racemic modification of hyoscyamine which can be used to treat the parasympathetic nervous system disease (Pan 2006). Atropine also has a certain toxicity, which can be used as an anticholinergic enzyme in agricultural organophosphorus insecticides. However, excessive absorption of atropine may lead to respiratory failure and death (Liu 2016). Hyoscyamine is an important antispasmodic drug, which has analgesic antispasmodic function. It is mainly used to relieve smooth muscle spasms, biliary tract and stomach spasm, and duodenal ulcer pain. It can also relieve syncope caused by heart conduction, arterial spasm, and other blood circulation diseases. In addition, hyoscyamine also has a certain role in peripheral vascular disease, sudden deafness, and a variety of neuralgia (Ullrich et al. 2016; Zhang 2010; Dehghan et al. 2017).

Table 4.1 The content and distribution organs of TAs in some plants of *Solanaceae* family

Plant species	Tissue	Tropane alkaloids (%)				References
		Hyoscyamine	Anisodamine	Scopolamine	Anisodine	
P.tangutica	Root	3.820	0.680	0.020	0.020	Xiao et al. (1973)
	Leaf	2.180	0.600	0.090	–	
A.tanguticus	Root	0.190	–	0.080	0.200	
	Leaf	0.800	0.460	0.270	0.320	
H.niger	Plants	–	0.070	0.410	–	Wang et al. (2002)
	Seed	0.120	–	0.010	–	Xiao et al. (1973)
A.luridus	Leaf	0.510	–	0.170	–	Xiao et al. (1973)
P.praealta	Root	0.460	–	0.160	–	
	Leaf	0.920	0.070	0.040	–	
M.officinarum	Root	0.130	–	–	–	
	Leaf	0.070	–	–	–	
D.stramonium	Seed	0.024	–	–	–	
	Stem	0.007	–	–	–	
	Leaf	0.020	–	–	–	
A.acutangulus	Root	+++	+	–	++	
A.belladonna	Root	0.159	–	0.002	–	Qiang et al. (2014)
	Older stem	0.028	–	0.004	–	
	Caulicle	0.336	–	0.060	–	
	Older leaves	0.030	–	0.008	–	
	Spire	0.152	–	0.060	–	
	Fruitlet	0.127	–	0.003	–	
	Systellophytum	0.143	–	0.103	–	

Notes: – show undetected type of tropane alkaloids; + exhibit low content of tropane alkaloids; ++ represent higher content of tropane alkaloids; +++ mean the highest content of tropane alkaloids

4.2.2 Scopolamine

Compared with hyoscyamine, scopolamine has weaker side effects and stronger pharmacological effects; therefore, there is an extraordinarily great demand for it. Importantly, it can inhibit the central nervous system and parasympathetic nerve block; therefore, scopolamine is mainly used for anesthesia, analgesia, Parkinson disease, anti-motion sickness, microcirculation improvement, pesticide poisoning, detoxification, and so on (Zhao et al. 2017; Shawwal et al. 2017).

4.2.3 Anisodamine (6β-Hydroxyhyoscyamine)

A hydroxyl group at the sixth position of the anisodamine nucleus compared with hyoscyamine endows the molecule with increasing polarity rendering it hard to penetrate through the blood-brain barrier (Sun et al. 2012). Compared with atropine, anisodamine has a slightly weaker pharmacological effect; meanwhile its toxicity and adverse reactions are relatively lower. It is mainly used for the treatment of toxic shock, smooth muscle spasm, vascular disease, and a variety of neuralgia, vertigo, and fundus diseases. Apart from the M muscarinic receptor blocking effect, anisodamine also showed some non-M receptor blocking effects. Its effect of calcium antagonists can increase the fluidity of the cell membrane and antagonize the oxygen free radicals produced by damaged cells (Eisenkraft and Falk 2016; Marín-Sáez et al. 2017).

4.2.4 Anisodine

Anisodine is one of the anticholinergic drugs that can block the M-cholinergic receptor. It is effective in treatment of anti-tremor, antispasmodic, asthma, inhibiting saliva secretion, mydriasis, acute paralysis, motion sickness, vascular headache, against the role of organic phosphorus poisoning pesticides. Anisodine can also be used as one kind of neuroprotective agents for the treatment of ischemic optic nerve retinal choroidal lesions (Wang et al. 2017; Varma and Yue 1986; Liu et al. 2015). In terms of relieving the smooth muscle spasm and inhibiting the salivary secretion, the peripheral anticholinergic effect was inferior to hyoscyamine and scopolamine. Otherwise, its effect on treatment of mydriasis was five times higher than anisodamine, and its central action was similar to that of scopolamine (Zhang 2010; Kai et al. 2011).

4.3 Hairy Root Culture System

In the last three decades, it is considered that hairy roots are a biological matrix for various biotechnological functions. The neoplastic manifestation or hairy roots ensued from the transfer of *Agrobacterium rhizogenes* transfer DNA (T-DNA) into the plant genome possess a potential for secondary metabolite production (Mehrotra et al. 2015). In 1907, Smith and Townsend firstly found that *Agrobacterium rhizogenes* can induce the occurrence of hairy roots of plants (Smith and Townsend 1907). And Chilton reported the mechanism of infection of *Agrobacterium rhizoma* into plants tissues (Ren 2003). Various aspects and applications in hairy root culture have been utilized, including phytoremediation, introduction of desirable foreign genes, phytochemicals, molecular breeding and crop improvement, recombinant protein production, rhizosphere physiology and biochemistry, bioreactor design, metabolic engineering, and general overviews of the system (Ono and Tian 2011). In recent years, hairy root cultures have attracted much attention due to their attractive feature for producing valuable metabolites, such as high genetic stability and relatively fast growth rates, auxin-independent, and no geographical limit (Guillon et al. 2006; Wu and Shi 2008). Meanwhile, hairy root system is able to synthesize a large number of secondary metabolites in liquid medium without exogenous hormone, thus becoming a kind of new resource material to facilitate the raw plants (Moyano et al. 2002). Therefore, hairy roots system is an excellent means to improve secondary metabolites of plants.

4.4 Establishment of Hairy Root System of Tropane Alkaloid-Bearing Plants

Several ways have been used to enhance the TAs production such as chemical synthesis, interspecific hybridization, and cell culture; however all of them showed some shortcomings. For instance, synthesis routes by chemical method are long period, expensive, environment-polluting, and low-yielding because of the complexity of the chemical structure (Huang et al. 2005). Conventional interspecific hybridization exhibited disadvantages of too long breeding cycle, environmental limitation, and lack of specific parent materials (Yun et al. 1992). Poor genetic stability is one of the critical defects of cell culture (Huang et al. 2005; Wu et al. 2005).

Tropane alkaloids mainly accumulate in roots of *Solanaceae* plants, which provides theoretical basis to produce TAs in hairy roots. At present, most of the medicinal plants of *Solanaceae* which produce TAs have established hairy root induction and culture system (Lei et al. 2016) (Table 4.2). Here, we took *A. acutangulus* and *A. belladonna* as examples to elaborate establishment of hairy roots of tropane alkaloid-bearing plants.

Table 4.2 Culture conditions of hairy root and tropane alkaloid content in some *Solanaceae* plants

Plant species	Medium of induction	Liquid medium	Bacterial strain	References
Brugmansia candida	1/2 B5	1/2 B5	LBA9402	Cardillo et al. (2013)
Datura inoxia	B5	B5	1855	Dechaux and Boitel-Conti (2005), Boitel-conti et al. (2000)
Datura candida	MS	MS	15834	Christen et al. (1989)
Scopolia carniolica	LS	LS	A4	Altabella et al. (1994), Knopp et al. (1988)
Hyoscyamus albus	B5	B5	A4	Christen et al. (1992)
Hyoscyamus muticus L	B50	B50	LBA9402	Jouhikainen et al. (1999)
Scopolia japonica	White	Heller	15834	Mano et al. (1986)
Duboisia myoporoides	LS	LS	HRI	Deno et al. (1987)
Anisodus luridus	MS	MS	C58C1 (Ri)	Qin et al. (2014)
Anisodus acutangulus	MS	1/2MS	C58C1	Li et al. (2008)
Scopolia lurida	–	1/2MS	C58C1	Zhao et al. (2017)
Atropa belladonna L	MS	MS	15834	Kamada et al. (1986)
	MS	MS	A4	Yang et al. (2006)
Hyoscyamus niger L	MS	1/2MS	MAFF03–01724	Shimomura et al. (1991)
	MS + B5	1/2B5	LBA9402	Lu et al. (2005)
Datura stramonium Linn	MS	MS	A4	Maldonado-Mendoza and Loyola-Vargas (1995)
	B5	B5	TR-105	Maldonado-Mendoza et al. (1993)
	B5	B5	LBA9402	Robins et al. (1991)
Anisodus tanguticus	MS	1/2MS	A4	Shimomura et al. (1991)
	LS	LS	15834	Meng et al. (2002)

4.4.1 Hairy Roots of Anisodus acutangulus

Anisodus acutangulus is a *solanaceous* perennial herbal plant that is endemic to China, and it has been used as an esthetic medicine in Yunnan Province for hundreds of years (Kai et al. 2007). It is an important source plant of TAs which has been a research hotspot in recent years (Kai et al. 2007, 2011). Aseptic *A. acutangulus* plants were grown in a greenhouse at 25 °C with 16 h light and 8 h dark periods in Murashige and Skoog (MS) basal medium with 3% sugar and 0.8% agar (pH 5.8). Different explants including leaves, petioles, or stems were isolated from 4-week-old

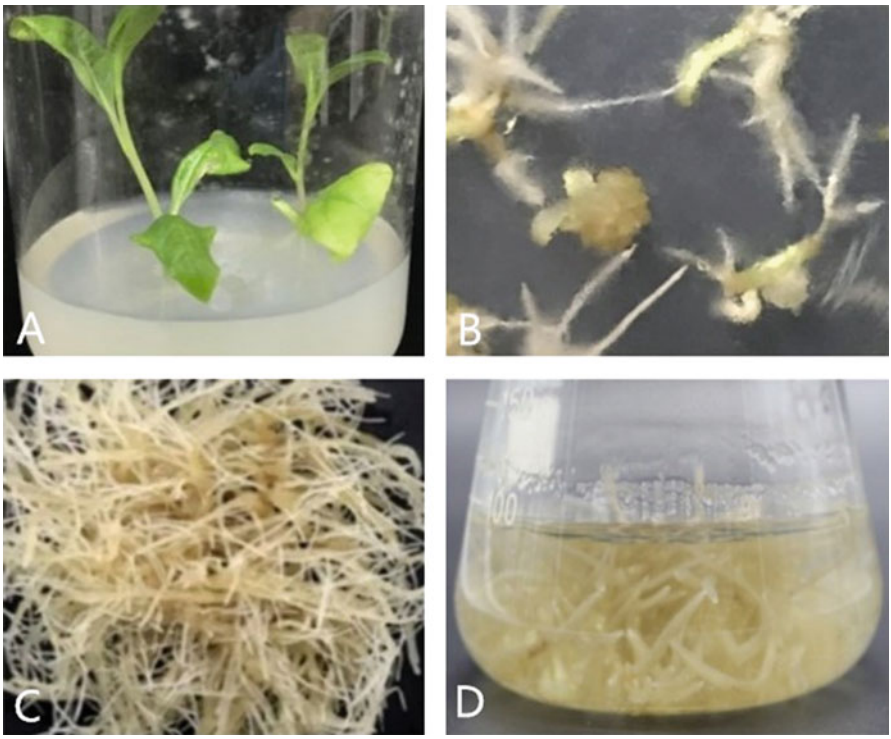

Fig. 4.2 Induction of hairy roots from explants of *A. acutangulus* with strain C58C1. (**a**) Aseptic seedlings of *A.acutangulus* (**b**) Generated hairy roots co-cultured with seedless stem of *A. acutangulus* (**c**) Monoclonal hairy roots of *A.acutangulus* (**d**) A liquid culture of hairy roots of *A. acutangulus* in shake flask

in vitro-grown sterile seedlings of *A. acutangulus*; leaves were cut into small pieces about 1 cm and cultured in hormone-free MS medium for 48 h in darkness. Disarmed *A. tumefaciens* strain C58C1 harboring both the *A. rhizogenes* Ri plasmid pRiA4 and a plasmid containing a target gene were used for genetic transformation for 15 min. C58C1 with blank vector was used as control. After coculture for 2–3 days, the infected explants were transferred to B5 medium supplemented with 500 mg/L cefotaxime sodium to kill the residual *Agrobacterium*. The concentration of cefotaxime was lowered every 2 weeks till bacterial-free, and rapidly growing lines were used to establish hairy root lines further (Kai et al. 2011). Specific primers of targeted genes and *rolB* gene were validated in the same time (Kai et al. 2011). Root fragments of approximately 3–4 cm in length from positive colonies were culture in 100 mL of 1/2 MS medium on an orbital shaker with the speed of 100 r per minute at 25 °C in darkness. The hairy roots were subcultured every 30 days and harvested after 60 days for extraction of TAs (Fig. 4.2).

4.4.2 *Transformation of* Atropa belladonna *L Hairy Roots*

A.belladonna is a perennial plant belonging to the family *Solanaceae* and widely distributed over Central and Southern Europe (Munir et al. 2014). *A.belladonna* attracted more attention because it can produce the pharmaceutical bioactive tropane alkaloids which are broadly used as antagonists of acetylcholine in both central nervous system and autonomic system. *Agrobacterium strains* have been reported to infect the explants in *A. belladonna* including *Agrobacterium rhizogenes* 15834 and GV3101 including pPCV002-ABC called *A. tumefaciens rol ABC* (Bonhomme et al. 2000; Vervliet et al. 1975). Approximately 0.8 cm diameter leaves from 5-week-old plantlets were infected with the strains. Then these explants were transformed onto LS solid medium supplemented with 0.5 g/L cefotaxime to get rid of bacteria. Five weeks later, the hairy roots can be observed at the wounded sites of explants. The hairy roots were cut out and transferred to LS liquid medium. All the culture media used for the hairy root cultures were hormone-free. When the hairy roots were 4 weeks old, they can be inoculated about 100 mg (fresh weight) into 30 mL liquid LS medium supplemented with 0.5 g/L cefotaxime, in 100 ml Erlenmeyer flasks, and maintained in the dark at 22 °C on a rotary shaker (130 rpm) (Bonhomme et al. 2000).

4.5 Biosynthesis of Tropane Alkaloids (TAs) in Plants

4.5.1 *Biosynthetic Pathway of Tropane Alkaloids (TAs)*

The biosynthesis of TAs is a complicated process and involves several catalytic steps (Fig. 4.3). Some related genes have been isolated and cloned in TAs-producing plants. L-ornithine or arginine is the biological precursor of TAs which can be converted to polyamine putrescine by ornithine decarboxylase (OrnDC) and arginine decarboxylase (ArgDC), respectively. Then putrescine N-methyltransferase (PMT) which is the first rate-limiting enzyme in the pathway of nicotine and the tropane alkaloid catalyzes the polyamine putrescine to form N-methylputrescine and afterward is oxidized by diamine oxidase (DAO) to 4-N-methylamino butanal (Heim et al. 2007; Katoh et al. 2007) which rearrange spontaneously to N-methyl-Δ1-pyrrolinium. N-methyl-Δ1-pyrrolinium is the common intermediate precursor of cocaine, nicotine, and TAs synthesis (Jirschitzka et al. 2013). It is still unknown how the N-methyl-Δ1-pyrrolinium turned to tropinone. TRI and TRII are two tropinone reductases which compose a branching point in the biosynthesis routes of tropane alkaloids (Kai et al. 2009a; Dräger 2006). TRI converts tropinone to tropine; nevertheless tropinone was converted to pseudotropine by TRII (Kai et al. 2009a). Owing to TRI as the first putative metabolite branch specific to

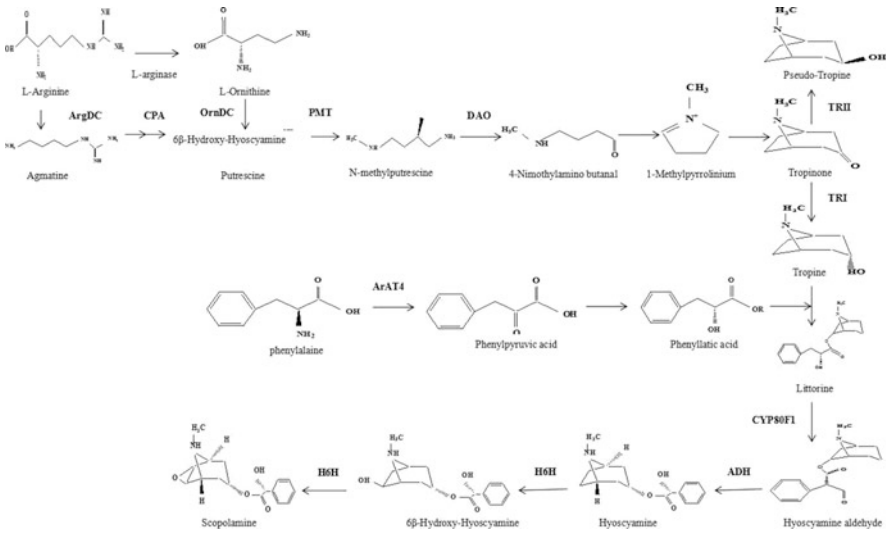

Fig. 4.3 The biosynthetic pathway of tropane alkaloids (TAs) in plants

hyoscyamine, TRI has been regarded as a significant enzyme in the TAs biosynthetic pathway. Subsequently, tropine condensed with phenyllactic acid which is derived from phenyllactic acid results in littorine. Littorine is converted to hyoscyamine by littorine mutase/monooxygenase (CYP80F1) and alcohol dehydrogenase (ADH) (Li et al. 2006). Aromatic amino acid aminotransferase (ArAT) is identified in the route that converts phenylalanine into phenyllactic acid recently (Bedewitz et al. 2014; Cui et al. 2015). Hyoscyamine 6β-hydroxylase (H6H) which was identified in the last committed step in the scopolamine biosynthetic pathway catalyzes the hydroxylation and epoxidation of hyoscyamine to produce scopolamine (Kai et al. 2012). The structure of anisodine is similar to scopolamine with an additional hydroxyl, whereas it is unclear about its biosynthetic pathway by unknown enzymes.

4.5.2 *Cloning and Characterization of Genes Related to TAs Biosynthesis*

With the increasing demand of TAs in recent years, considerable efforts have been made to excavate biosynthesis pathway of TAs. Several rate-limiting enzyme genes including *PMT*, *TRI*, *TRII*, *CYP80F1*, and *H6H* have been successfully isolated and cloned from various *solanaceous* plants (Table 4.3).

As the first rate-limiting upstream enzyme in TAs biosynthetic pathway (Kutchan 1995; Kholodenko et al. 1998; Zhang et al. 2007), putrescine N-methyltransferase (PMT) belongs to the *S*-adenosyl-methionine-dependent N-methylation transferase family that catalyzes the methylation of putrescine to form N-methylputrescine for

Table 4.3 Related genes involved in biosynthetic pathway of TAs from plants of *Solanaceae* family

Genes	Function	Plant sources	GenBank Number	References
PMT1	Catalyzing the methylation of putrescine to form N-methylputrescine	*Atropa belladonna*	AB018570.1	Suzuki et al. (1999)
PMT2		*Atropa belladonna*	AB018571.1	Suzuki et al. (1999)
PMT		*Hyoscyamus*	AB018572.1	Suzuki et al. (1999)
PMT1		*Datura inoxia*	AM177609.1	Teuber et al. (2007)
PMT2		*Datura inoxia*	AM177610.1	Teuber et al. (2007)
PMT1		*Anisodus acutangulus*	EU670745	Kai et al. (2009b)
PMT2		*Anisodus acutangulus*	EU670745	Kai et al. (2009b)
PMT		*Anisodus tanguticus*	AY690623.1	Liu et al. (2005)
TRII	Converting tropinone to tropine/pseudotropine	*Hyoscyamus niger*	L20485.1	Nakajima et al. (1993a)
TRI		*Datura stramonium*	L20473.1	Nakajima et al. (1993b)
TRII		*Datura stramonium*	L20474.1	Nakajima et al. (1993b)
TRI		*Anisodus acutangulus*	EU424321	Kai et al. (2009a)
TRII		*Anisodus acutangulus*	EU424322	Kai et al. (2009a)
TRI		*Solanum tuberosum*	AJ305841.1	Kaiser et al. (2006)
TRII		*Solanum tuberosum*	AJ245634.1	Kaiser et al. (2006)
TRI		*Scopolia lurida*	–	Zhao et al. (2017)
CYP80F1	Catalyzing (R)-littorine to form hyoscyamine aldehyde	*Hyoscyamus niger*	DQ387048.1	Li et al. (2006)
H6H	Catalyzing hyoscyamine to form scopolamine	*Atropa belladonna*	AB017153.1	Suzuki et al. (1999)
H6H		*Anisodus acutangulus*	EF187826.1	Kai et al. (2007)
H6H		*Hyoscyamus niger*	M62719.1	Matsuda et al. (1991)
H6H		*Datura arborea*	KR006981	Qiang et al. (2015)

TAs biosynthesis. Several PMT genes have been isolated from various *Solanaceae* plants (Hibi et al. 1994). For example, two *AaPMT* genes have been isolated from *Anisodus acutangulus*, namely, *AaPMT1* and *AaPMT2*, and showed high similarity with other *PMTs* from some plants. The full-length cDNA of *AaPMT1* was 1322 bp containing a 1014 bp open reading frame (ORF) encoding a polypeptide of 338 amino acids, while *AaPMT2* was 1219 bp containing a 1041 bp ORF encoding a 347-amino acid protein. *AaPMT1* and *AaPMT2* presented similar tissue expression profiles while *AaPMT2* with a weaker trend, expressing strongly in roots, weakly in stems and leaves. A full-length cDNA encoding PMT was isolated from *A. tanguticus*. Nucleotide sequence analysis showed that the cDNA contained an ORF of 1017 bp encoding 338 amino acids bearing 92% identity with both *HnPMT* (*Hyoscyamus niger*) and *AbPMT* (*Atropa belladonna*). *A. tanguticus* PMT was expressed in *Escherichia coli*, and recombinant AtPMT was purified which exhibited *S*-adenosyl-methionine-dependent N-methyltransferase activity (Liu et al. 2005).

Tropinone reductase (TR) catalyzes tropinone to downstream molecules. *TRI* (tropinone reductase I) and *TRII* (tropinone reductase II) both existed in tropane alkaloids-producing plants while function in a different way (Hashimoto et al. 1992). *TRI* converts tropinone to 3a-hydroxytropane (also known as tropine), while *TRII* converts tropinone to pseudotropine (also known as c-tropine or 3b-hydroxytropane). Hyoscyamine is derived from tropine and then converted into scopolamine by *H6H* (hyoscyamine 6β-hydroxylase), whereas calystegines are metabolites of pseudotropine which illustrates that metabolic regulation of these two genes is species-specific in the biosynthesis pathway of TAs. *TRI* has been considered as an important branch point in the TAs biosynthetic pathway (Kai et al. 2009a). *TRI* together with *TRII* were cloned from the *solanaceous* plant *A. acutangulus* (designated as *AaTRI*, *AaTRII*) exhibiting high homology with other tropinone reductases from other plants such as *Hyoscyamus niger*, *Datura stramonium*, etc., while *AaTRI* and *AaTRII* only showed identity of 60.8%. One-step RT-PCR showed that *AaTRI* and *AaTRII* were expressed in all tested tissues, and both could be induced by methyl jasmonate (Kai et al. 2009a).

Littorine is an important precursor in biosynthesis pathway for production of hyoscyamine and scopolamine (Robins et al. 1994). A cytochrome P450 enzyme was thought to be involved in the rearrangement of (R)-littorine to (S)-hyoscyamine. A full-length cDNA of *CYP80F1* with an ORF of 1533 bp of 510 amino acids was identified from *H. niger* (Li et al. 2006). Sequence alignment presented that *CYP80F1* of *H. niger* share generally conserved eukaryotic cytochrome P450 regions. Employing RNAi technique could reduce the expression level of *CYP80F1* and resulted in decrease of hyoscyamine production. In addition, hyoscyamine can be detected in *CYP80F1*-expressing tobacco hairy roots supplied with (R)-littorine. Expression in yeast confirmed that *CYP80F1* catalyzes the oxidation of (R)-littorine with rearrangement to form a putative precursor of hyoscyamine (hyoscyamine aldehyde) or without rearrangement to form 3′-hydroxylittorine (Li et al. 2006).

H6H (hyoscyamine 6β-hydroxylase) is a bifunctional enzyme which was identified as the last rate-limiting enzyme involved in scopolamine biosynthesis. It can catalyze the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and the epoxidation of 6-hydroxyhyoscyamine to scopolamine (Kai et al. 2012; Xia et al. 2016). Full-length cDNA encoding hyoscyamine 6β-hydroxylase in *A. acutangulus* (designated as *AaH6H*) is identified from young roots by rapid amplification of cDNA ends (RACE). The complete ORF of *AaH6H* was 1035 bp encoding a deduced protein of 344 amino acid residues. Sequence analyses showed that *AaH6H* had high homology with other H6Hs isolated from some other scopolamine-producing plants such as *Atropa belladonna*, *Hyoscyamus niger*, and *Datura metel* (Kai et al. 2007). The cDNA encoding hyoscyamine 6β-hydroxylase in *Atropa belladonna* (designated as *AbH6H*) and *Hyoscyamus niger* (designated as *HnH6H*) has also been identified. The ORF of *AbH6H* was 1029 bp encoding a deduced protein of 343 amino acid residues, and the ORF of *HnH6H* was 1035 bp encoding a deduced protein of 344 amino acid residues, which is the same length with *AaH6H* (Suzuki et al. 1999; Matsuda et al. 1991).

4.6 Biotechnological Approaches to Improve the Production of TAs

TAs as well as other secondary metabolites used in traditional Chinese medicine are mainly from the herbal plants. However, cultivation of medicinal plants is time-consuming and exhibited low contents in plants. Application of plant biotechnology for elevation of bioactive and desired constituents is more attractive and efficient than conventional approaches.

4.6.1 Genetic Engineering

In recent years, genetic engineering strategy has drawn a lot of attention because it provides an alternative way to improve the accumulation of some active compounds in some medical plants. This strategy has been used in many sources plants, such as *Salvia miltiorrhiza*, *Isatis indigotica*, and so on. Recently, several genes involved in TAs biosynthetic pathway have been successfully isolated from different TAs-producing plants such as *Anisodus acutangulus*, *Atropa belladonna*, *Datura metel*, *Atropa belladonna*, etc. It is more effective and feasible to apply genetic engineering for improved contents of TAs in genetic modified hairy roots.

Key enzyme genes related to TAs have been overexpressed in hairy roots derived from different plants individually or in combination. It has been reported that overexpression of the tobacco *PMT* gene under the control of CaMV 35S promoter could enhance the production of tropane alkaloids in *Hyoscyamus muticus*, *Datura*

metel, and *Scopolia parviflora* (Moyano et al. 2002). Hairy roots of *A. baetica*, *A. belladonna*, and *H. muticus* harboring the *H6H* gene can produce higher accumulation of scopolamine (Yun et al. 1992; Jouhikainen et al. 1999; Zárate et al. 2006), which illustrated that it was a viable way to increase scopolamine content by metabolic engineering. Hairy root cultures of *Scopolia lurida* were established to investigate the effect on the accumulation of tropane alkaloids by overexpressing *SlTRI*. In the *SlTRI* overexpression hairy root cultures, the content of hyoscyamine was 1.7- to 2.9-fold higher than those in control, while the scopolamine contents were likewise elevated (Zhao et al. 2017). Two full-length cDNAs encoding TRI (tropinone reductase I) and TRII (tropinone reductase II) from the solanaceous plant *Anisodus acutangulus* have been isolated by rapid amplification of cDNA ends. *AaTRI*-transformed hairy root lines were accompanied by a mean 1.87-fold higher level of hyoscyamine and a mean eightfold higher level of scopolamine compared with control roots, indicating that *AaTRI* is a promising target for genetic engineering to increase tropane alkaloid in *A. acutangulus* (Kai et al. 2009a, b).

Overexpression of single gene encoding a key enzyme may increase flux through the pathway, but other rate-committed steps may limit its effect to some extent. Hence, co-expression of two or several genes would be more suitable to increase the production of bioactive products. This may be more suitable in branched pathways in which precursors can be channeled into a variety of metabolites away from the desired products (Kai et al. 2011). Co-introduction of genes encoding the branch-controlling enzyme tropinone reductase I (*TRI*) and rate-limiting enzyme hyoscyamine-6β-hydroxylase gene in the downstream pathway (*H6H*) into hairy roots of *A. acutangulus* by *Agrobacterium*-mediated gene transfer technology can produce obviously higher level of TAs in contrast with the control and single gene-transformed hairy root lines. The best transformed lines produced 4.49-fold TAs higher than that of the control lines (Kai et al. 2012). Overexpression of putrescine N-methyltransferase gene (*PMT*) which was considered as the first rate-limiting upstream enzyme and tropinone reductase I (important branch-controlling enzyme) in *A. acutangulus* hairy roots led to significantly increased production of four kinds of TAs and showed higher antioxidant activity than control lines (Kai et al. 2011). Simultaneous overexpression of both *PMT* and *H6H* coordinately promoted biosynthesis of scopolamine and made the scopolamine content very high in transgenic hairy root cultures of *H. niger* (Zhang et al. 2004). *Atropa belladonna* is one of the plants that produced tropane alkaloids, and overexpressing of both *PMT* and *H6H* in *A. belladonna* hairy roots can significantly improve the content of hyoscyamine and scopolamine compared with wild-type lines (Yang et al. 2011). In addition, consequent to the introduction of the two key enzyme genes, the production of the alkaloids hyoscyamine and scopolamine was enhanced by co-expressing the two genes *PMT* and *H6H* in *Scopolia parviflora* (Kang et al. 2011).

4.6.2 Elicitation Treatment

Elicitation is one of the most common and effective strategies for stimulating secondary metabolite production in plant tissue cultures (Zhao et al. 2005; Wang and Wu 2013). Elicitors are some compounds which generally refer to the agents or stimuli that can induce phytoalexin synthesis and defense responses in the host plants. Therefore, the elicitation effect is based on the accumulation of most secondary metabolites in plants which is part of the defense responses of plants to pathogen infection or stresses from the environment. Elicitors can be grouped into two categories based on their source: abiotic elicitors and biotic elicitors. Biotic elicitor is some material that formed by the plants to resist the microbial abuse. It includes isolates of plant cell wall and various fungi, for instance, fungi, bacteria, viruses, or yeast. Whereas, abiotic elicitor is generally some of the physicochemical factors that are not provided by the organism such as organic compounds, methyl jasmonate (MJ), acetylsalicylic acid (ASA), high temperature, ultraviolet, and heavy metals (Ebel 1998).

A variety of biotic or abiotic elicitors have been used to enhance the TAs production in hairy roots of TAs-bearing plants. For example, salicylic acid, $AgNO_3$, $CaCl_2$, and $CdCl_2$ were used to elevate the TAs contents in *Brugmansia candida* hairy roots; the results showed that scopolamine and hyoscyamine were both increased compared with the control lines (Pitta-Alvarez et al. 2000). It has been reported that Ag^+, ethanol, and methyl jasmonate could enhance the accumulation of tropane alkaloids up to 1.08, 1.51, and 1.13 times as the control after 24 h treatment, respectively, but salicylic acid reduced the average production of tropane alkaloids in hairy root of *Anisodus acutangulus* (Kai et al. 2012). Different concentrations of iron oxide nanoparticles (FeNPs) were used to elicited hairy roots of *Hyoscyamus reticulatus* L. derived from cotyledon explants inoculated with *Agrobacterium rhizogenes*. Antioxidant enzyme activity was increased significantly in induced hairy roots than non-transgenic roots. Accumulation of hyoscyamine and scopolamine was both promoted (Moharrami et al. 2017). The methyl jasmonate (MJ) has also been used in *Datura stramonium* hairy roots to observe the change of accumulation of hyoscyamine and scopolamine, and the results showed that the increase of scopolamine was 1.36-, 1.42-, 1.17-, and 1.12-fold higher than that of the control after dealing with MJ on days 3, 6, 9, and 12, respectively. And the increase of hyoscyamine was 2.28-, 1.11-, 0.63-, and 0.70-fold higher than that of the control, respectively (Sun et al. 2013) (Table 4.4).

Table 4.4 Elicitors applied for stimulation of TAs accumulation in hairy roots

Elicitor	Plant source	References
SA, $AgNO_3$, $CdCl_2$, $CaCl_2$	*Brugmansia candida*	Pitta-Alvarez et al. (2000)
Ag+, ethanol, MJ	*Anisodus acutangulus*	Kai et al. (2012)
MJ, Quercetin	*Brugmansia suaveolens*	Zayed et al. (2004)
MJ	*Datura stramonium*	Sun et al. (2013)
FeNPs	*Hyoscyamus reticulatus L*	Moharrami et al. (2017)

4.6.3 In Combination of Genetic Engineering and Elicitation

Most previous studies focused on the production of the targeted products by the method of genetic engineering in hairy roots; the combination of transgenic technology with elicitor treatments was also an effective method to promote secondary metabolites. For example, transgenic *S. miltiorrhiza* hairy root lines co-expressing *HMGR* and *DXR* (HD lines) with increased tanshinone production exhibited higher tanshinone content after elicitation by yeast extract and/or Ag^+ than before. Tanshinone can be significantly enhanced to 5.858, 6.716, and 4.426 mg/g DW by YE, Ag^+, and YE-Ag^+ treatment compared with non-induced HD42, respectively (Shi et al. 2014). Methyl jasmonate (MJ) and salicylic acid (SA) were used to investigate their effects on tanshinone accumulation in the hairy roots of geranylgeranyl diphosphate synthase (SmGGPPS) overexpression line (G50) in *Salvia miltiorrhiza*. High-performance liquid chromatography analysis showed that total tanshinone content in G50 was obviously increased by 3.10-fold (11.33 mg/g) with MJ and 1.63 times (5.95 mg/g) after SA treatment in contrast to control (Hao et al. 2015).

For large-scale production of TAs, salicylic acid (SA), methyl jasmonate (MJ), and acetylsalicylic acid (ASA) were used to induce the transgenic *Atropa baetica* overexpressing the *H6H* gene to enhance tropane alkaloid yields. Production of scopolamine was improved after treatment with MJ and ASA. The highest enhancement of scopolamine was achieved with MJ followed by ASA dissolved in EtOH (El Jaber-Vazdekis et al. 2008).

4.6.4 Production of TAs in Bioreactors

To successfully achieve high production of bioactive constituents by hairy root-based biotechnology, the key procedure is the cultivation in optimal bioreactors on a large scale (Georgiev and Weber 2014). Because of sensitivity of hairy root cultures to shear stress, bioreactor systems suitable for the cultivation of hairy root are different from those of suspension plant cell cultures (Mishra and Ranjan 2008). A variety of reactor configurations have been used to cultivate hairy roots for desired products, and the optimization of conditions for efficient cell growth and secondary metabolite production is necessary (Stiles and Liu 2013). It has been reported that

hairy root cultures of *Hyoscyamus niger* were cultivated in a bubble-column bioreactor and a hybrid bubble-column/spray bioreactor together with elicitation and permeabilization (Jaremicz et al. 2014). Hairy root culture in a hybrid bubble-column/spray bioreactor showed the highest anisodamine content of 0.67 mg/g DW, and the bubble-column reactor gave the highest concentration of scopolamine, hyoscyamine, and cuscohygrine. Besides, elicitation with methyl jasmonate increased scopolamine productivity by 146% in roots grown in the hybrid bubble-column/spray bioreactor, whereas their permeabilization with DMSO caused an increase in scopolamine, hyoscyamine, anisodamine, and cuscohygrine concentrations in the growth medium. In situ extraction with Amberlite XAD-2 doubled scopolamine productivity in the hybrid reactor after 50 days culture.

4.7 Transcriptome Analyses

Metabolic engineering is a very useful tool for enhancing the accumulation of valuable compounds in plants. In many medicinal plants, bioengineering techniques significantly improved the production of valuable compounds with deep understanding of biosynthesis pathway (O'Connor 2012). Whereas, metabolic engineering may not achieve successful progress in some plants, due to the limited information of biosynthesis pathways. Hence, it is imperative to deeply understand the biosynthetic pathways of various compounds produced by the plants (Chen et al. 2013). Transcriptome sequencing or RNA sequencing (RNA-seq) is one of the famous high-throughput sequencing methods and can produce millions of short cDNA reads in a parallel manner. The abundance of transcripts, sequences, and even the single-cell level can be determined by RNA-seq (Tang et al. 2009). RNA-seq can provide a holistic view of a transcriptome, including novel transcriptional active regions and the precise location of transcription boundaries (Wilhelm et al. 2010).

To further understand the biosynthesis and transportation mechanism of TAs, a de novo transcriptome assembly was developed for deadly nightshade (*Atropa belladonna*) (Bedewitz et al. 2014). Excavating of the transcriptome identified a phylogenetically distinct aromatic amino acid aminotransferase (ArAT), named as Ab-*ArAT4*, co-expressed with some known tropane alkaloid biosynthesis genes in the roots of *A. belladonna*. Ab-*ArAT4* preferentially catalyzes the transamination of phenylalanine (L-Phe) to phenylpyruvate, the initial step leading to formation of littorine, a key intermediate in hyoscyamine and scopolamine biosynthesis. Cui et al. (2015) reported that transcriptome analysis with deep RNA sequencing in *A. acutangulus* roots was performed. And a series of genes related to tropane, piperidine, and pyridine alkaloid biosynthesis, distribution of arginine to TAs biosynthesis has been detected. Besides, potential transcription factors of WRKY, AP2/ERF, MYB, and bHLH families that possibly link to TAs synthesis were identified, which would be helpful to understand transcriptional regulation of secondary metabolite biosynthesis which updated the knowledge of TAs biosynthetic mechanism at the molecular level.

4.8 Conclusions and Prospects

Tropane alkaloids (TAs) such as anisodamine, anisodine, hyoscyamine, and scopolamine are extensively used in clinical practice as anticholinergic agents. All of them are mainly extracted from root tissue in the TAs-producing plants which cannot meet the increasing clinical demand. Although multiple genes involved in scopolamine biosynthesis have been cloned, the biosynthetic pathway of other TAs remains poorly understood. Large-scale culture of hairy roots has become an efficient way to improve the accumulation of TAs. However, many problems such as root distribution, the uniformity of oxygen supply, and weaker sheerness should be improved. Combination of genetic engineering, elicitation treatment, and transcriptomic and genomic analysis into hairy root culture can provide new ideas for production of TAs.

To excavate the biosynthesis mechanism of TAs, multiple techniques including metabolomics, transcriptome, proteomics, and genomic technologies should be utilized together to study the transcriptional regulation and transportation mechanism which can provide a new insight into the genetic manipulation of secondary metabolites in other medicinal plants. With the increasing demand for desired secondary products including TAs, synthetic biology has been a valid and effective strategy. *Saccharomyces cerevisiae* and *Escherichia coli* chassis, etc. have been utilized for production of various bioactive ingredients. The H6H enzyme from *Brugmansia candida* has been produced in *Saccharomyces cerevisiae* to obtain a biological catalyst for potential industrial applications. More systematic work related to synthetic biology should be conducted in TAs production.

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Chapter 5
Hairy Root Cultures for Monoterpene Indole Alkaloid Pathway: Investigation and Biotechnological Production

Shakti Mehrotra, Sonal Mishra, and Vikas Srivastava

Abstract Terpene indole alkaloids (TIAs) comprise a major group of alkaloids as more than 3000 TIAs are known with resilient and beneficial biological activities. These TIAs exhibit varied structural intricacy with a characteristically common tryptophan or tryptamine residue with a carbon tail of terpenoid origin derived from the dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) pathways. According to the number of isoprene units, monoterpene indole alkaloids (MIAs) comprise the major class of TIAs that have two isoprene units originated from secologanin. MIAs have been extensively investigated for their immense pharmaceutical importance as these compounds possess strong properties against various types of cancers, diseases of the central nervous systems, malaria, hypertension, and major cardiac ailments. Keeping in mind their immense pharmaceutical worth and ever-increasing demand from the pharmaceutical world, solicitous attention is needed on their biological production and scientific strategies to abate the demand and supply ratio. Furthermore, a holistic understanding is always required to explore intimately interconnected facts of synthesis and regulation of these metabolites. In this context, with their reasonable competence, the hairy root cultures (HRCs) have gained center-stage focus as an excellent in vitro system for different scientific investigatory objectives. This chapter provides condensed information about various MIAs, their biosynthesis in native plants, and contribution of HRCs to investigate the operational and regulatory mechanism of their in vitro and in planta biosynthesis.

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5.1 Introduction

Plants produce a vast variety of secondary metabolites that do not have any essential and direct role in processes of growth and development. Rather, they do have a certain role in defense mechanism of plants against various biotic and abiotic challenges. Primarily on the basis of structural differences, such compounds are grouped under terpenes, alkaloids, and phenolics. The terpenes are the most structurally diverse class of secondary metabolites that are derived from the five-carbon intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Alkaloids, another highly diverse group of nitrogen-containing heterocyclic molecules, are mainly biosynthesized from the amino acids like phenylalanine, tyrosine, tryptophan, lysine, and ornithine. On the basis of nitrogen-containing ring structure, the alkaloids are of various types such as tropane, indole, pyrrole, pyrrolidine, steroidal, etc. Indole alkaloids (alkaloids containing an indole skeleton) constitute the largest group of alkaloids. When indole alkaloids contain terpene (isoprene) group in their structure, then they are known as terpene/terpenoid indole alkaloids (TIAs). TIAs are known as major group of alkaloids as more than 3000 TIAs with rich biological activities have been identified (Singh and Singh 2018). These TIAs reveal varied structural complexity with a characteristically common tryptamine (tryptophan derivative) residue with a carbon tail of terpenoid origin derived from the dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) pathways. According to the botanical origin as well as on the basis of the number of isoprene units, monoterpene indole alkaloids (MIAs) comprise the major class of TIAs that have two isoprene units or C_9 and C_{10} carbon tail that originate from secologanin. Thus, these are also known as secologanin tryptamine alkaloids. It is pertinent to state here that with the number of isoprene units accumulated, the resulting terpene is classified as hemiterpene, monoterpene, sesquiterpene, diterpene, sesterterpene, triterpene, and tetraterpene. The MIAs constitute a large and diverse group with more than 2000 structurally defined molecules (Buckingham et al. 2010), thus sharing major repository. All of these molecules possess a variety of biological activities and have been thoroughly investigated for their potential pharmaceutical relevance. The biosynthetic pathways liable for the synthesis of these MIAs are mostly confined to few plant families, out of which Apocynaceae, Nyssaceae, Loganiaceae, and Rubiaceae are the dominant ones represented by plant genus like *Catharanthus*, *Rauwolfia*, *Ophiorrhiza*, *Camptotheca*, *Cinchona*, etc. (De Luca et al. 2012). Owing to the presence of these MIAs, these plants are accepted worldwide as medicinal plants (MPs) that have been characterized to contain bioactive substances in their aerial and/or underground parts capable of being used for wide therapeutic purposes against the number of human health disorders (Table 5.1). The global trade of such MPs is continuously expanding,

Table 5.1 Major plant-based monoterpene indole alkaloids and their biological activity

Plant family	Source plant	Alkaloid (MIA)	Biological activity	References
Apocynaceae	*Catharanthus roseus*	Vincristine (*leurocristine*)	Antitumor, anticancer	Arora et al. (2010), Seca and Pinto (2018)
		Vinblastine	Antitumor	
		Catharanthine	Antitumor	
		Vindolinine	Chemically lacks physiological activity alone but is contained as the pentacyclic moiety in the antineoplastic agents, vinblastine and vincristine	
		Tabersonine	Antimicrobial	
		Eldisine	Antineoplastic, antitumor	
Apocynaceae	*C. roseus* *Rauwolfia serpentina*	Serpentine	Antimicrobial	Mehrotra et al. (2015a, b)
		Ajmaline	Effective against ventricular extrasystoles, atrial/ventricular fibrillation, and Brugada syndrome	
		Ajmalicine (raubasine)	Central depressant with adrenergic blocking properties	
	R. serpentina	Yohimbine	Alpha-2-adrenergic blocking activity	Mehrotra et al. (2015a, b)
		Reserpine	Antidepressant and hypotensive Capable of inducing activity on the central nervous system	
Apocynaceae Apocynaceae	*Chonemorpha* spp. *Ervatamia* spp.	Camptothecin	Topoisomerase inhibitor, anticancer	Lorence and Nessler (2004), Thomas et al. (2004)
Nyssaceae	*Camptotheca acuminata*			
Meliaceae	*Dysoxylum binectariferum*			
Rubiaceae	*Ophiorrhiza pumila*, *O. rugosa*, *O. alata*, and *O. liukiuensis*			
Icacinaceae	*Merriliodendron* spp. *Pyrenacantha* spp. *Nothapodytes*			

(continued)

Table 5.1 (continued)

Plant family	Source plant	Alkaloid (MIA)	Biological activity	References
	foetida and *N. nimmoniana*			
Loganiaceae	*Mostuea brunonis*			
Rubiaceae	*Cinchona officinalis*	Cinchona alkaloids	Antimalarial, analgesic, and antipyretic	Kacprzak (2013), Gurung and De 2017
Apocynaceae	*Gongronema latifolium*			

and according to the International Trade Centre report, India occupies a leading position among suppliers of MP material for worldwide pharmaceutical industries. At present, India has about 315 of the 400 families of flowering MPs in the world that have spectacular perspective in plant-based global pharmaceutical industries (Kala et al. 2006). The MIAs like vincristine and vinblastine, synthesized in genera *Catharanthus* and *Vinca*, are known for their strong anticancer properties. These dimeric indole alkaloids have become valuable drugs in cancer chemotherapy due to their potent antitumor activity against various leukemia and tumors (Arora et al. 2010). Furthermore, *Catharanthus* MIAs are also used as the major compounds in the drugs used against Hodgkin's disease, Wilkins's tumor, neuroblastoma, and reticulum-cell sarcoma (Arora et al. 2010). At present, India is among the largest manufacturing countries of vinblastine and vincristine and also holds a strong position to fulfill the global demand of these alkaloids (Aslam et al. 2010). Moreover, Eldisine, which is recently introduced as drug molecule for the treatment of blood cancer, is a structural modification of vinblastine (deacetyl vinblastine amide or vindesine) (Khazir et al. 2014). High demand and low yield of these alkaloids in the plant have led the workers to search for alternative means for their production. MIAs like reserpine, ajmaline, ajmalicine, etc. which possess strong antidepressant, hypotensive properties and have the capability of inducing activity on the central nervous system (CNS) are dominant in genus *Rauwolfia*. Reserpine, the most potent alkaloid, is a strong antidepressant and is commonly used in a variety of drugs meant for hypertension, fever, colic, insomnia, giddiness, anxiety, maniacal behavior, psychosis, schizophrenia, dyspepsia, hyperglycemia, and hypochondria. Ajmaline is effective against ventricular extrasystoles, atrial/ventricular fibrillation, and Brugada syndrome. It is a class III anti-arrhythmic agent that causes the lowering of cardiac rhythm (Mehrotra et al. 2015a, b). Ajmalicine or raubasine, on the other hand, is a central depressant with adrenergic blocking properties. Similarly, another important MIA, camptothecin (CPT), which is naturally accumulated in *Camptotheca*, *Ophiorrhiza*, *Nothapodytes*, and various other plants, is a topoisomerase inhibitor, and analogues of this compound are used as anticancer agents. CPT is thought to stabilize the topoisomerase I-DNA covalent complex which acts as physical barriers to DNA synthesis, repair, and transcription. Furthermore, CPT functions as inhibitor to hypoxia-inducible factor 1 (HIF1), a regulator of cancer cell activities under low oxygen. Thus, CPT is also known to have strong anticancer

properties and is currently being used in various pharmaceutical industries worldwide (Lorence and Nessler 2004; Thomas et al. 2004; Venditto and Simanek 2010). Scientific studies on the potential of these plant-based MIAs in the treatment of problems of CNS, depression, cancer, heart arrhythmias, malaria, high fever, and other diseases are not new and have been in continuation since the past half century (Newman and Cragg 2012). Nevertheless, solicitous attention is always needed on the information flowing in with reference to their immense pharmaceutical worth, biological production, and scientific strategies to abate the demand and supply ratio. Out of several scientific methodologies that have been executed to claim the normal and enhanced biosynthetic yield of various MIAs, biotechnological interventions have proposed certain flawless systems and strategies like cell suspensions and hairy root culture in vitro systems of TIA-producing plants to explore desirable biosynthesis of these molecules.

Out of other in vitro systems, hairy root cultures (HRCs) are recognized as an "easy to establish and explore" system with unpretentious maintenance for a number of valuable biotechnological objectives (Mehrotra et al. 2015b; Srivastava et al. 2017). Not restricting the use of HRCs for secondary metabolite production in native plant species, these days, rational utilization of HRCs for elucidation of biosynthetic mechanism in terms of pathway, precursors and intermediates, rate-limiting steps, enzymatic gene sequences, and ultimately the product flux has attracted the focus of global scientific arena (Talano et al. 2012). Further, the development of genetically modified HRCs or transgenic HRCs is observed as a bonus to the system (Mehrotra et al. 2010). Several such genetic engineering studies have been done to improve the production of different kinds of pharmaceutically valuable plant-based bioactive metabolites. In this context, HRCs have proved their capabilities and competence and subsequently emerged as an excellent in vitro system for different biotechnological and metabolic engineering objectives. In this context, with their reasonable competence, the hairy root cultures (HRCs) have gained key-stage focus as excellent in vitro system for different scientific investigatory objectives. With these points in mind, this chapter provides informative minutiae about various MIAs and their biosynthesis in native plants. Further, the text also includes in brief the contribution of HRCs to investigate production and regulatory phenomenon of these pharmaceutically valuable MIAs.

5.2 An Insight on MIA Biosynthetic Pathway

On the basis of backbone structure and/or attached functional group, the MIAs are commonly divided into major subgroups which include sarpagan, corynanthe, aspidosperma, and iboga types. However, strictosidine is the common intermediate for most of the MIAs. Once the strictosidine molecule is formed, it serves as starting material for the biosynthesis of different MIAs (Fig. 5.1). The structural rearrangement in terpene unit of strictosidine leads to the formation of different MIA backbones/groups and subsequently the characteristic of MIA. The enzymes

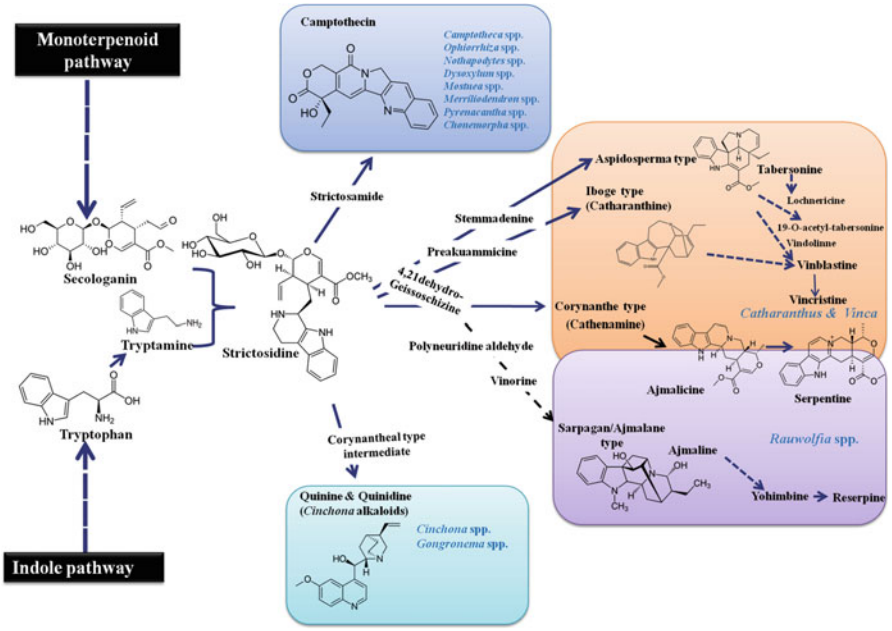

Fig. 5.1 Occurrence and synthesis of different monoterpene indole alkaloids in various plant species

catalyzing these complex rearrangements are restricted to specific genera within TIA-specific plant families (Salim and De Luca 2013). Therefore, concerned genera among these families have been characterized for occurrence of specific MIAs. Although Apocynaceae members *Catharanthus* and *Rauwolfia* share the biosynthesis of ajmaline and serpentine, the corynanthe-type MIAs, they are specifically known for strachynos/iboga, bisindole (vincristine, vinblastine), and sarpagan type (reserpine, yohimbine), respectively. The presence of vincamine, ajmalicine, and 2 yohimbine isomers along with 20 other MIAs is also detected in *Rhazya stricta* (Akhgari et al. 2015a). Similarly, accumulation of camptothecin (CPT) is largely known in genera *Ophiorrhiza* and *Camptotheca.* However, some other members of different families of related and unrelated orders are also known to accumulate CPT. CPT, the potent topoisomerase inhibitor and anticancer agent, is a well-known member of TIA family. However, since CPT molecule does not possess basic indole structure, yet it is considered as a part of TIA family as feeding experiments on *Camptotheca* plants with radiolabeled tryptamine and strictosidine have substantiated the presence of these compounds as intermediates in CPT pathway (Sirikantaramas et al. 2007a, b). Quinine, another quinoline-type MIA and a highly potent antimalarial agent derived from strictosidine is normally produced by *Cinchona* species. It is relevant to state that in spite of such discrete distribution of MIAs among related and unrelated plant families, *Catharanthus roseus* is the only known plant species to possess the biosynthetic enzymes involved in the formation

of the sarpagan, corynanthe, aspidosperma, and iboga MIA backbones (O'Connor and Maresh 2006). Thus, this trait draws the attention toward phylogenetic origin and evolution of specific secondary metabolic pathways among stated families and emergence of *Catharanthus* as a model plant to investigate synthesis of different kinds of MIA in vitro and in vivo. Providing an ease to understand natural biosynthetic machinery for the formation of complex and skeletally diverse MIAs, the biosynthesis can be divided into three phases: (1) fragment coupling, (2) cyclization, and (3) post-cyclization. The first stage comprises of early pathway which includes strictosidine formation, whereas the latter two phases are also considered as late pathway which comprises of various steps of formation of different characteristic MIAs according to different genera (Vantourout et al. 2017).

5.2.1 *Early Pathway: Synthesis of Strictosidine and the Rate-Limiting Steps*

Tryptophan is the precursor amino acid for all types of MIAs. In the presence of tryptophan decarboxylase (TDC, EC 4.1.1.28), tryptophan (Trp) is converted to tryptamine. Condensation (Pictet–Spengler) reaction of tryptamine with iridoid glucoside secologanin, in the presence of strictosidine synthase (*STR*), produces strictosidine. Strictosidine is the parent molecule for all classes of pharmaceutically active MIAs. Focusing on rate-limiting steps of MIA synthesis, conversion of tryptophan to tryptamine is important as it is the first unwavering step of MIA biosynthesis (Glenn et al. 2011). Among MIA-producing plants, TDC was first isolated from HRCs of *C. roseus*, and its relationship to tryptamine, ajmalicine, and catharanthine accumulation was investigated (Islas et al. 1994; Islas-Flores et al. 2002). Further, several plant *TDCs* were cloned and characterized from the HRCs of different MIA-producing plant species, such as *Camptotheca acuminata* (López-Meyer and Nessler 1997), *Ophiorrhiza pumila* (Yamazaki et al. 2003), *Rauvolfia verticillata* (Liu et al. 2012), *Rhazya stricta* (Akhgari et al. 2015b), etc. In *C. roseus* TDC is encoded by a single gene (*TDC*) (De Luca et al. 1989), whereas in *C. acuminata*, it is encoded by two autonomously regulated genes (López-Meyer and Nessler 1997). Significant increase in TDC activity and amount of immunoreactive TDC protein has been reported in elicited hairy roots of *C. roseus*. This indicates toward the requirement of TDC polypeptides during elicitor-induced alkaloid accumulation (Islas-Flores et al. 2002). Furthermore, hairy roots of *C. roseus* were also used to investigate the intracellular distribution of TDC using immunofluorescence and immunogold techniques. The investigation revealed the enriched presence of enzyme in cytosol and in the apoplastic region of the root meristematic cells indicating toward the high efficiency of these tissues to pursue those biochemical reactions which require Trp in this pathway. The enzyme channel tryptophan from primary metabolism into the MIA pathway thus represents a transition point from primary pathway to a secondary pathway. A constant inflow

of Trp is required for smooth MIA pathway operation. Further, the precursor-feeding studies have also indicated that MIA accumulation in hairy roots can be improved through enhanced (Trp) administration. The shikimate pathway funnels compound from erythrose 4-phosphate and phosphoenolpyruvate to chorismate. Trp is obtained from chorismate in a five-step reaction in which the first two critical steps that produce anthranilate are catalyzed by alpha and beta subunits of single tetramer enzyme complex anthranilate synthase (AS, EC 4.1.3.27). This conversion is considered as rate-limiting step for further reactions including Trp synthesis and subsequent MIA pathway, because AS-α subunit is subjected to feedback inhibition by Trp. Therefore this feedback inhibition of AS must be overcome to accumulate and maintain constant influx of Trp for tryptamine production and consecutive MIA synthesis. Experimental evidence suggests the tight regulation of consecutive MIA synthesis steps in *C. roseus* HRCs having glucocorticoid-inducible promoter regulating the expression of an *Arabidopsis* feedback-resistant AS-α subunit (Hughes et al. 2004a). The *Arabidopsis* AS-α subunit was found compatible with the native β subunit, and AS activity was observed more resistant to tryptophan feedback inhibition. The result supported greater accumulation of Trp and tryptamine in engineered hairy root tissues.

The other counterpart for strictosidine synthesis is secologanin (a secoiridoid monoterpene) which combines with tryptamine to produce strictosidine. The secologanin is synthesized from its precursor dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) via mevalonate pathway which remains active in cytosol and responsible for triterpenes and sesquiterpenes production. Moreover, these precursor molecules are also produced from mevalonate-independent pathway (non-mevalonate pathway), viz., 1-deoxy-D-xylulose-5-phosphate pathway (DXP or DOXP pathway) or the 2C-methyl-D-erythritol-4-phosphate pathway (MEP pathway). The MEP pathway remains active in plastids and leads to the production of mono-, di-, and tetraterpenes (Oudin et al. 2007). In this multistep synthesis of secologanin, geraniol, iridotrial, deoxyloganin, and loganin are the main intermediate compounds (Fig. 5.1).

Conversion of geraniol to 10-hydroxygeraniol by geraniol 10-hydroxylase (G10H) is considered as the first committed step in secologanin biosynthesis. G10H activities have been found to be higher in native root tissues as compared to vitro cell cultures indicating the basic difference in biosynthetic potential of two systems. The two main enzymes geraniol 10-hydroxylase (G10H) and a cytochrome P450 secologanin synthase (SLS) have been well documented for the conversion of IPP to geraniol and loganin to secologanin, respectively. Therefore, regulated expression of these two important enzymes G10H and SLS in MIA-producing plants could potentially improve the yield of secologanin-derived alkaloids. The role of SLS is also studied in CPT-producing HRCs of *O. pumila*. To identify the intermediates in CPT biosynthesis, expression of genes encoding tryptophan decarboxylase (TDC) and secologanin synthase (SLS), the two enzymes catalyzing the early steps in CPT biosynthesis, was suppressed in the hairy roots of *O. pumila* by RNA interference (RNAi), and metabolite changes were investigated (Asano et al. 2013). In most TDC- and SLS-suppressed lines, accumulation of CPT and related

alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside, was reduced suggesting their possible involvement in CPT biosynthesis.

The stereoselective condensation of strictosidine, which is a product of Pictet–Spengler condensation of tryptamine with secologanin, requires the presence of enzyme strictosidine synthase (STR). STR has been isolated and characterized from in vitro cultures of a number of MIA-bearing plants of Apocynaceae, Rubiaceae, Loganiaceae, and Nyssaceae and is considered as the enzyme of prime importance for the biosynthetic pathway of the indole alkaloids. Moreover, STR initiates all biosynthetic pathways leading to the entire MIA family. The analysis of crystalline structure of STR and its chemical properties have been well characterized in *R. serpentina* (Ma et al. 2006). Cloning and characterization of cDNAs encoding strictosidine synthase (OpSTR, EC 4.3.3.2) and tryptophan decarboxylase (OpTDC, EC 4.1.1.28) have also been reported from CPT accumulating hairy roots of *O. pumila* (Yamazaki et al. 2003). Suppression of *OpTDC* and *OpSLS* in HRCs of *O. pumila* by RNAi resulted in reduced accumulation of CPT and related alkaloids like strictosidine, strictosamide, pumiloside, and deoxypumiloside. Such results suggested the crucial involvement of these enzymes in CPT biosynthesis. The accumulation levels of secologanin exhibited a strong negative correlation with the expression level of *TDC*, and that of loganin exhibited a negative correlation with the expression level of *SLS*. These studies indicate an intricate regulatory mechanism behind CPT biosynthesis.

The chemistry behind the downstream fate of strictosidine molecule and formation of different types of MIAs is though comprehensively uncovered up to a major extent but still has loopholes. Different members of MIA-producing plant group possess their characteristic processing of strictosidine. This processing includes ring fragmentation in backbone moieties and cascade rearrangements in intermediate molecules. This consequently enables an access to the large structural diversity of this family of alkaloids including historic "type I" alkaloids (e.g., corynan, akuammilan, strychnan, secocuran, sarpagan, and alstophyllan types), "type II" alkaloids (e.g., aspidosperma and eburnan types), and "type III" alkaloids (e.g., ibogan type) (Benayad et al. 2016).

5.2.2 Late Pathway

5.2.2.1 Sarpagan Type

The genus *Rauwolfia* is distinguished by the presence of some important sarpagan-ajmalan class of MIAs like reserpine, ajmaline, vomiline, yohimbine, etc. (Wu et al. 2016; Mehrotra et al. 2015a, b and reference therein). HRCs of *R. serpentina* have been meticulously investigated for MIA synthesis. Ajmaline biosynthesis in *Rauwolfia* species is well characterized, and various ajmaline biosynthetic steps and their enzymes have been purified and isolated. The glycosylation of strictosidine and subsequent formation of 4,21-dehydrogeissoschizine are the preliminary steps of

ajmaline biosynthesis. The 4,21-dehydrogeissoschizine is a major intermediate product, and the reaction is catalyzed by strictosidine glucosidase (*SGD*), the key early gene which plays an important role in the biosynthesis of various classes of MIAs (Barleben et al. 2007). In *R. serpentina*, this enzyme is characterized by heterologous expression of its cDNA in *E. coli*, and its comparison with *Catharanthus SGD* was made (Gerasimenko et al. 2002). The intermediate dehydrogeissoschizine is liable to produce sarpagan-type intermediate polyneuridine aldehyde in ajmaline pathway of which further fate is well characterized. At least eight enzymatic reactions are known in which a central reaction is catalyzed by enzyme polyneuridine-aldehyde esterase (PNAE). This step is important as it yields the immediate precursor epi-vellosimine which serves as a substrate for the next step in the pathway which delivers the alkaloid vinorine (ajmalane backbone) in the presence of vinorine synthase (VS). PNAE and VS, purified from of *R. serpentina*, play key roles in the production of ajmalane type of MIAs at this step (Ma et al. 2004). Ensuing reactions produce subsequent intermediate products like vinorine, vomilenine, and norajmaline. The enzyme liable for the formation of norajmaline (acetylesterase, hydrolyzing acetylnorajmaline) has been purified, and its full-length clone was isolated from a cDNA library prepared from *R. serpentina*. In the final step of ajmaline biosynthesis, an *N*-methyl transferase introduces a methyl group at the indole nitrogen of norajmaline to produce ajmaline (Fig. 5.1).

The enzymes that convert deglycosylated strictosidine (dehydrogeissoschizine) to yohimbine have not been identified. However, a direct biosynthetic route may involve homoallylic isomerization of the keto dehydrogeissoschizine followed by 1,4-conjugate addition. Reserpine is the most valuable MIA of *Rauwolfia* species and is a 3,4,5-trimethyl benzoic acid ester of reserpic acid, an indole derivative of 18-hydroxyl yohimbine. The formation of reserpine from their precursor molecule is still in need of scientific focus.

5.2.2.2 Corynanthe Type

The geissoschizine and 4,21-dehydrogeissoschizine that are formed by deglycosylation of strictosidine have basic corynanthe-type backbone structure that serves as key molecules for further biosynthetic reactions. A single-step reversible conversion of 4,21-dehydrogeissoschizine in the presence of geissoschizine dehydrogenase yields heteroyohimbine cathenamine. Further, the fate of cathenamine depends upon the NADP reductase which produces ajmalicine and subsequent oxidation which produces serpentine. Though these pathways have been elucidated in *C. roseus*, ajmalicine and serpentine have also been isolated from *Rauwolfia* species and are presumably produced by similar mechanisms.

5.2.2.3 Strychnos, Aspidosperma, and Iboga Type

The *Strychnos*, *Aspidosperma*, and *Iboga* MIAs are considered as structurally more complex than corynanthe type. As stated above, the corynanthe alkaloid geissoschizine, an intermediate key product of deglycosylation of strictosidine, constitutes the other key intermediates in the formation of the structurally more complex *Strychnos*, *Aspidosperma*, and *Iboga* alkaloids. *Catharanthus*, which possesses enzymes for different MIA synthesis, is observed to accumulate corynanthe-type MIAs early in its lifetime in comparison with *Strychnos*, *Aspidosperma*, and *Iboga* types that generally accumulate in older plants. This is in agreement with the fact that the latter (three) types are derivatives of corynanthe-type precursor molecule. The 4,21-geissoschizine undergoes chemical conversions (mechanism still not clear) and forms preakuammicine which is a *Strychnos*-type derivative (Benayad et al. 2016; Fig. 5.1). Reduction of preakuammicine yields stemmadenine, a productive intermediate in the pathway of *Aspidosperma*- and *Iboga*-type MIAs. Thus, preakuammicine holds a crucial role in the biosynthesis of the latter two. This is why in some literatures, the latter two are also referred as type II and type III MIAs (Benayad et al. 2016). Major examples of *Aspidosperma* are tabersonine and vindoline, whereas common *Iboga* alkaloids include catharanthine, ibogaine, harmaline, tabernanthine, coronaridine, voacangine, ibogamine, etc. The hairy root cultures of Apocynaceae members are known to produce these MIAs in detectable amount. Conversion of tabersonine to vindoline is rather well characterized in terms of enzymes and intermediates. In a six-step pathway, tabersonine subsequently produces vindoline through intermediates like hydroxy- and methoxytabersonine molecule and deacetylvindoline (Geerlings et al. 2000; Levac et al. 2008; Costa et al. 2008; Fig. 5.1).

5.2.2.4 Bisindole Type

The bisindole alkaloids (majorly vinblastine and vincristine) are derived from dimerization of vindoline and catharanthine. This step is believed to proceed via the formation of an intermediate anhydrovinblastine, a naturally occurring compound in *C. roseus* plants. In support of this mechanism, anhydrovinblastine is incorporated into vinblastine and vincristine in feeding studies with cell-free extracts.

5.2.2.5 Quinoline Type

CPT has been found in at least 16 different plant species belonging to 3, 5, and 13 unrelated plant orders, families, and genera, respectively, across the plant kingdom and also in endophytic fungi associated with these CPT-producing plants (Raveendran 2015). Two well-known and much explored plant species for CPT

production are *Ophiorrhiza pumila* and *Camptotheca acuminata*. As stated above CPT does not possess indole structure and hence considered as modified MIA. It is one of the most promising natural plant-derived antitumor agents, and its biosynthetic pathway is not very well known. The biosynthesis is a little different from other MIAs as in this case strictosidine is not immediately deglycosylated; rather, it forms strictosamide (a cyclic amide). Integration of radiolabeled strictosamide into CPT molecule confirmed that strictosamide is a productive intermediate in CPT pathway. Although biosynthetic steps following strictosamide are not much clear yet, the presence of 3(*S*)-pumiloside and 3(*S*)-deoxypulminoside as pathway intermediates is evident, and the two have been isolated from *O. pumila*. The 3(*S*)-pumiloside was also detected in *C. acuminata*.

Quinine is a highly potential antimalarial compound and naturally synthesized in *Cinchona* species. Feeding studies in *C. robusta* and *C. ledgeriana* with radiolabeled tryptophan and strictosidine indicate that the *Cinchona* quinoline alkaloids are derived from strictosidine via corynanthe intermediate. Strictosidine synthase has been purified from cell cultures of *C. robusta.* Two isoforms of the enzyme, involved in the later stages of the quinine biosynthetic pathway, have been purified from cell suspension cultures of *C. ledgeriana.* One isoform of this NADPH-dependent oxidoreductase catalyzes the reduction of cinchoninone (which equilibrates with its epimer cinchonidinone) to give a mixture of cinchonine and cinchonidine, while second isoform catalyzes the reduction of both cinchoninone and quinidinone (O'Connor and Maresh 2006).

5.3 Hairy Root Cultures for MIA Pathway Investigations and Biotechnological Production

Contemporary techniques have been developed to repress hitches associated with the production and extraction of plant-derived natural products. In this context, advances of biotechnological methods particularly in vitro cultures have paved the way to procure natural compounds in desired amounts in a process similar to their production in plants. HRCs, out of other in vitro systems, have attained superiority in metabolite production as well as proven their worth as excellent experimental system to investigate operational and regulatory characteristics of biosynthetic pathways. This has been evidenced by literature repositories where myriad reports are continuously accruing with reference to the incisive utilization of HRCs for various objectives. HRCs of MIA-bearing plants represent a rich source of variety of their representative alkaloids. Since the past few decades, MIA biosynthesis, production, and other related issues like MIA pathway elucidation and manipulation have been meticulously investigated in HRCs of several MIA-bearing plants. Ensuing text provides compiled information of contribution of HRCs in MIA biosynthesis investigation and various strategies opted for a better understanding of ultimately increased alkaloid flux.

Catharanthus HRCs are the much explored hairy root system to study the biosynthesis of various MIAs (Guillon et al. 2008). These HRCs have been reported to produce high level of MIAs, mainly catharanthine, serpentine, and ajmalicine. The growth kinetic studies of *C. roseus* HRCs have established a linear correlation between the kinetics of biomass growth and accumulation of alkaloids like ajmalicine and catharanthine (Benyammi et al. 2016). However, neither vincristine nor vinblastine is known to be reported from roots possibly due to the absence of vindoline which is exclusively synthesized and accumulated in chloroplasts (Ferreres et al. 2011). With the establishment of *Catharanthus* HRCs as a potential system for production of alkaloids like ajmalicine, serpentine, catharanthine, tabersonine, and vindolinine (Parr et al. 1988; Toivonen et al. 1989; Bhadra et al. 1993), gradually the research focus transited towards the optimization of yield enhancement strategies like culture media optimization (Toivonen et al. 1991; Bhadra and Shanks 1997; Morgan et al. 2000; Li et al. 2011), growth kinetics (Leduc et al. 2006), precursor feeding (Morgan and Shanks 2000), elicitation with biotic/abiotic molecules and stress (Vazquez-Flota et al. 1994; Rijhwani and Shanks 1998; Ruiz-May et al. 2009; Binder et al. 2009), and ultimately the product extraction (Sim et al. 1994; Tikhomiroff and Jolicoeur 2002; Moreno-Valenzuela et al. 2003). Various hairy root lines of *C. roseus* were also compared for growth and alkaloid production in relation to their morphology and pattern of *rol* gene (s) integration. The hairy root lines did not differ significantly for their total alkaloid content which was in the range of 1.10–1.30% dry weight but showed significant variations in relative ajmalicine content (0.007–0.08% dry weight), serpentine (0.01–0.08% dry weight), and catharanthine (0.01–0.04% dry weight). The practical feasibility of upscaling of *Catharanthus* HRCs in various types and configuration of bioreactors and their alkaloid production potential has also been investigated (Verma et al. 2012; Thakore et al. 2017).

Establishment of hairy root cultures of genus *Rauwolfia* has been attempted with different strains of *Agrobacterium* for the synthesis of sarpagan-type alkaloids (Benjamin et al. 1993; Falkenhagen et al. 1993; Sudha et al. 2003). HRCs of *Rauwolfia* species have proven a rich repository of a range of MIAs (Mehrotra et al. 2015a), and various species of the genus such as *R. serpentina*, *R. verticillata*, *R. tetraphylla*, *R. vomitoria*, *R. micrantha*, etc. have been explored to produce pharmaceutically important MIAs. Nevertheless, out of all, hairy roots of *R. serpentina* have been investigated methodically for alkaloid biosynthesis. Considering the pharmaceutical importance of reserpine, at present HRCs of *R. serpentina* have been developed showing a range of reserpine accumulation (0.0064–0.088% dry weight). This is comparatively much higher than that of field-grown plants of an improved variety (cim-sheel) of *R. serpentina* (Mehrotra et al. 2015a). Further, various biotechnological developments, such as scaling up in bioreactors and pathway engineering, have also been explored to improve metabolite production potential of *R. serpentina* HRCs (Mehrotra et al. 2015a, b, 2016).

Several native plant families have been explored for in vitro production of camptothecin (CPT). CPT was first isolated from family Nyssaceae of genus *C. acuminata*, a deciduous tree species. However, later on it was isolated from

families like Rubiaceae (*Ophiorrhiza* sp.), Meliaceae (*Dysoxylum binectariferum*), Icacinaceae (*Merriliodendron*, *Pyrenacantha*, and *Nothapodytes*), Apocynaceae (*Chonemorpha* and *Ervatamia*), and Loganiaceae (*Mostuea brunonis*) (Namdeo and Sharma 2012). Out of these plant species, maximum concentration of CPT has been reported from two species of *Nothapodytes*, viz., *N. foetida* and *N. nimmoniana*. Interestingly, synthesis and accumulation of CPT are known in various related and unrelated orders of angiosperms. The discrete occurrence of genes in CPT biosynthesis has led to the assumption of their early evolution among different families. However, in due course of time and gradual process of adaption, they have lost their functionality in some plant families. Initially, suspension cultures of *N. nimmoniana* were reported to secrete CPT and 9-methoxy camptothecin in its growth medium. However, in recent years, HRCs of this plant species have been explored for CPT production. It was observed that approximately 93% of total alkaloid produced by HRCs was excreted into the medium (Chang et al. 2014). In relevant reviews, challenges and status of CPT production in *N. nimmoniana* by application of in vitro techniques have been reviewed (Isah and Mujib 2015). Moreover, considering the plant as a potent natural source of CPT, such reviews discuss conservation and production of CPT, identification of high-yielding individuals and molecular profiling, and the possibility of biotechnological intervention for higher production of CPT without destructive harvesting of natural population (Rajasekharan et al. 2011). HRCs of *Chonemorpha fragrans* are also reported to produce CPT in comparable amounts (0.024–0.030% dry weight) to that of intact roots (0.033% dry weight) (Kedari and Malpathak 2013). Various species of genus *Ophiorrhiza*, i.e., *O. pumila*, *O. rugosa*, *O. alata*, and *O. liukiuensis*, are reported to produce CPT (Roja 2006; Ya-ut et al. 2011; Kamble et al. 2011). However, HRCs *O. pumila* and *O. rugosa* have only been thoroughly explored for biosynthesis of CPT and related alkaloids, like pumiloside, deoxypumilosides, and strictosamide (Kitajima et al. 2002; Kamble et al. 2011). Although the callus cultures of *O. pumila* do not produce CPT, HRCs accumulate substantial amounts of CPT in cells as well as in culture medium. A practically and commercially feasible method was developed by the use of polystyrene resins as specific absorbents to extract the CPT from growth medium (Sudo et al. 2004). In a comparative study of CPT production from indigenous plants *N. foetida*, *O. mungos*, and *O. rugosa*, it was *N. foetida* which resulted in the highest yields of CPT and 9-methoxy camptothecin. The other two plants *O. mungos* and *O. rugosa* contained low levels of alkaloids (Roja 2006). Likewise, HRCs of *Camptotheca* also produce and secrete CPT along with another more potent and less toxic natural derivative, 10-hydroxycamptothecin (HCPT), into the medium. These cultures were able to synthesize the alkaloids at levels equal to, and sometimes greater than, the native intact roots.

The genus *Cinchona officinalis* 'Ledgeriana,' belonging to the family Rubiaceae, is known for the production of *Cinchona* bark, the raw material for the alkaloids quinine and quinidine. *Cinchona* alkaloids are in extensive uses, not only for drugs but also for soft drink industries. Major *Cinchona* alkaloids include stereoisomers cinchonine and cinchonidine, quinine and quinidine, and dihydroquinine and dihydroquinidine. All these compounds contain strong antimalarial and antipyretic

activities. Besides *C. officinalis*, another medicinally well-known milkweed *Gongronema latifolium* is also known to produce cinchonidine. Attempts have been made to produce cinchona alkaloids from in vitro systems including suspensions and hairy root cultures.

5.3.1 Transgenic Hairy Roots and Biosynthesis of MIA

In planta low accumulation of valuable MIAs and the results obtained from all these reports have laid the ground to explore the production of significant MIAs through other unconventional strategies. Additionally, the search of precise molecular mechanism behind MIA synthesis and large-scale production had surfaced the need to opt various biotechnological approaches such as overexpression and pathway engineering utilizing HRCs of MIA bearing plant species. Progressively, MIA pathway engineering through overexpression of related genes took thoughtful scientific attention, and numerous efforts have been made in this direction. In this regard, *Catharanthus* hairy root cultures have taken center-stage position and emerged as a model system for majority of TIA-related studies. This is not only because of the easy development of HRCs but also due to chemotaxonomical importance of this genus between the members of MIA-producing families. Additionally, the inconsistent production coupled with high rising industrial demand of *Catharanthus* alkaloids imposed the unification of various advanced molecular approaches to the existing production technology. To sum up the hitherto studies in *Catharanthus* HRCs to elucidate terpene indole alkaloid pathway genes and their regulatory mechanism, in a recent report, terpene indole alkaloid pathway engineering has been reviewed (Sun and Peebles 2016). Pertinent to this, various significant experimentations have been performed to investigate overexpression of *TDC* and subsequent alkaloid production. *TDC* overexpression study in crown-gall callus of *C. roseus* revealed increased tryptamine levels but not over all alkaloid level (Goddijn et al. 1995). In another study, expression of TDC gene of *C. roseus* in non-TIA-harboring plants such as tobacco was examined which resulted in increased tryptamine (Goddijn et al. 1994). The transgenic hairy roots of *C. roseus* were generated to investigate glucocorticoid-inducible *TDC* expression alone or in combination with inducible expression of a feedback-resistant anthranilate synthase-alpha subunit (AS-α) from *Arabidopsis*. Though no significant increase in tryptamine levels was observed in *TDC* lines, the root lines with *TDC* + AS-α resulted in increases in tryptamine levels. Downstream effects on alkaloids were noted only in the *TDC* lines with increased serpentine, and *TDC* + AS-α did not show any effects on measured alkaloids (Hughes et al. 2004a, b). This report suggested the contribution of the indole pathway in terpene indole alkaloid biosynthesis. In an attempt to more successfully engineer the indole pathway, *Arabidopsis* AS-β subunit cDNA was constitutively expressed along with the inducible expression of *AS-α* and *TDC* in *C. roseus* hairy roots. Transgenic hairy roots expressing both AS-α and AS-β show a significantly greater resistance to feedback inhibition of AS activity by

tryptophan than plants expressing only AS-α (Hong et al. 2006). Precursor-feeding studies have also directed that enhanced tryptophan availability can lead to increased alkaloid accumulation in HRCs. This relationship and the role of tryptophan in TIA biosynthesis were verified with the expression of an *Arabidopsis* feedback-resistant AS-α subunit under the control of glucocorticoid-inducible promoter in transgenic HRCs of *C. roseus* (Hughes et al. 2004b). Showing the compatibility with native AS-β subunit, the expressed *Arabidopsis* AS-α subunit resulted to more resistance to tryptophan inhibition. This led to a very high increase in tryptophan and tryptamine levels albeit, the large increases, the TIA levels not significantly altered, with the exception of lochnericine. In another similar kind of study, the effects of overexpressing the AS-α and α + β subunits in combination with feeding with the terpenoid precursors 1-deoxy-D-xylulose, loganin, and secologanin were investigated (Peebles et al. 2006). The hairy root lines expressing the AS-α subunit fed with 1-deoxy-D-xylulose showed increased accumulation of hörhammericine, whereas those fed with loganin showed increased accumulation of catharanthine. Catharanthine along with ajmalicine, lochnericine, and tabersonine was accumulated in high levels in hairy root lines expressing AS-α + β subunits. These results although ratify the pivotal role of indole pathway and tryptophan in terpenoid alkaloid biosynthesis yet also indicate toward constricted regulation of the pathway at various levels. Overexpression of *Catharanthus TDC* (*CrTDC*) has also been studied in HRCs of *R. serpentina* (Mehrotra et al. 2013). Gene expression analysis indicated that *CrTDC* was expressed in transgenic root lines, which could be correlated with enhanced reserpine and ajmalicine accumulation in roots. A wide array of variation in relative reserpine content was observed that could be attributed to the number of factors including insertion site, orientation, and differential expression of inserted genes (Mehrotra et al. 2013). Possibilities of enhanced biosynthesis of quinine and quinidine alkaloids have also been observed by overexpression of *CrTDC* and *STR* in *Cinchona officinalis* hairy roots. The HRCs were initiated containing constitutive-expression constructs of cDNAs encoding the enzymes *TDC* and *STR* from *C. roseus*. The products of *TDC* and *STR*, tryptamine and strictosidine, respectively, were found in high amounts and accordingly the quinine and quinidine levels also (Geerlings et al. 1999). The results show that genetic engineering with multiple genes is well possible in hairy roots of *C. officinalis*.

Engineering of isoprenoid pathway was also conducted to investigate its role in terpene indole alkaloid biosynthesis in *C. roseus* hairy roots. In a study, hairy roots were generated with inducible overexpression of 1-deoxy-D-xylulose synthase (DXS) or geraniol 10-hydroxylase (G10H). Additionally, hairy root lines were also generated with inducible expression of *DXS* and AS-α subunit (ASA) or *DXS* and *G10H*. *DXS* overexpression resulted in a significant increase in ajmalicine, serpentine, and lochnericine and a significant decrease in tabersonine and hörhammericine. Co-overexpression of *DXS* and *G10H* resulted in notable increase in ajmalicine, lochnericine, and tabersonine. Likewise, a noticeable increment in hörhammericine, lochnericine, and tabersonine was observed due to overexpression of *DXS* and AS-α. These results indicated toward the need for overexpressing multiple genes within the pathway to increase the ultimate flux (Peebles et al. 2011).

The role of *G10H* in CPT biosynthesis in *O. pumila* has also been investigated (Cui et al. 2015). In HRCs of *O. pumila*, genes from *C. roseus* encoding *STR* and *G10H* were separately and simultaneously introduced. Hairy root lines having individual *G10H* have shown significantly increased CPT production as compared to non-transgenic hairy root cultures and single *STR* overexpressing hairy root lines. Furthermore, a synergistic effect of co-overexpression of *G10H* and *STR* genes was also observed as it caused about 56% increase in the yields of CPT compared to non-transgenic and single-gene transgenic lines. Various experiments on precursor feeding have revealed that the biosynthesis of secologanin and possibly the conversion of loganin to secologanin are the rate-limiting steps in indole alkaloid biosynthesis and the production of important MIAs is partly controlled at transcriptional stage of these enzymes. In a study of *C. roseus* HRCs elicited with methyl jasmonate, regulation on MIA biosynthesis was investigated through precursor feeding and gene expression profiling (Goklany et al. 2009). The transcript levels of three key alkaloid pathway genes *G10H*, *TDC*, and *STR* were investigated along with metabolite levels of tryptamine, loganin, secologanin, strictosidine, ajmalicine, serpentine, and tabersonine. It was observed that elicitation improved the expression of three genes differently and this led to the increase in strictosidine, ajmalicine, and tabersonine levels. However, feeding of loganin and tryptamine as precursor TIA production was not enhanced.

The biosynthesis of vindoline in HRCs of *C. roseus* is not clear, and possibly these cells do not produce vindoline due to the lack of expression of the seven-step pathway from tabersonine to vindoline. Furthermore, complex spatiotemporal developmental regulation of terpenoid alkaloid biosynthesis has restricted the use of HRCs for the production of highly important anticancer molecules vinblastine and vincristine. To investigate possibilities of vindoline pathway, the induced overexpression of *T16H* and *16OMT* was done in order to ensure tabersonine availability (Sun et al. 2018). In this recent study, possible alteration in the pathway was done through genetic engineering of the first two genes tabersonine 16-hydroxylase (*T16H*) and 16-O-methyl transferase (*16OMT*). The glucocorticoid-inducible promoter was used to control the gene expression (Sun et al. 2018). The proper availability of vindoline and its channeling towards vinblastine and vincristine biosynthesis was supposed in *C. roseus* hairy roots. In comparison with normal hairy root lines, accumulation of two vindoline pathway metabolites 16-hydroxytabersonine and 16-methoxytabersonine was observed in transgenic root lines. However, the levels of other root-specific terpenoid alkaloids, lochnericine, 19-hydroxytabersonine, and hörhammericine, significantly decreased. Additionally, accumulation of two new metabolites in HRCs has led to conclusion that proper channeling of tabersonine is required for vindoline pathway. Furthermore, complex transcriptional changes in terpenoid alkaloid pathway genes and regulators indicated toward the tight regulation of the MIA pathway in response to *T16H* and *16OMT* engineering in *C. roseus* hairy roots. In a study, the *O. pumila* HRCs with RNAi-mediated *TDC* and *SLS* gene suppression accumulated reduced levels of CPT and related alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside (Asano et al. 2013). Further, secologanin and loganin levels were

negatively correlated with *TDC* and *SLS* expressions, respectively. The study proposed the possibilities of combined transgenic and metabolomic approach in identification of pathway intermediates. A combined strategy was also used to analyze transcriptome and metabolome of hairy root cultures of *O. pumila* to identify potential candidate genes that are involved in biosynthesis of CPT and other related alkaloids along with anthraquinones as well (Yamazaki et al. 2013). A hybrid transcriptome assembly of *O. pumila* hairy roots was constructed using the Illumina-derived short read sequences and Sanger-derived expressed sequence tag clones. The study resulted in the identification of potential genes involved in CPT, anthraquinones, and chlorogenic acid biosynthesis.

A possible role for 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) in MIA biosynthesis was concluded in *C. roseus* hairy roots expressing hamster (*HMGR*) cDNA without membrane-binding domain that was also evaluated for terpenoid alkaloid accumulation (Ayora et al. 2002). Significant variation in metabolite accumulation was observed with transgenic hairy root lines. Hairy root lines with the highest hybridization signal had the lowest soluble and microsomal HMGR activity and produced more ajmalicine and catharanthine than the control but had reduced campesterol concentration. Another line with low hybridization signal had high soluble *HMGR* activity and produced high levels of campesterol and is five to seven times more serpentine than the control but has a low level of ajmalicine and no accumulation of catharanthine. These results suggest a potential role for *HMGR* in MIA biosynthesis and a possible co-suppression of both the endogenous and foreign *HMGR* genes in selected lines.

5.3.2 Regulation of MIA Pathway: Transcription Factors

Since the past few years, researchers have been meticulously trying to figure out complete control network of terpene indole alkaloid biosynthetic pathway, and myriad evidence indicates the involvement of transcription factors that target key structural genes of this pathway. A tightly regulated process of MIA synthesis involves a number of transcriptional activators and repressors. At present, seven putative activators (ORCA2, ORCA3, CrBPF1, CrMYC1, CrMYC2, CrWRKY1, and CrWRKY2) and five putative repressors (ZCT1, ZCT2, ZCT3, GBF1, and GBF2) have been identified as regulators of the terpenoid alkaloid pathway (Liu et al. 2017 and reference therein). Transcription factors ORCA2 and ORCA3 of AP2/ERF family have been well characterized for their role in regulation of MIA synthesis. ORCAs or octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins have their expression induced by jasmonates, the major signaling molecules for MIA pathway. These proteins used to bind to the 42 bp region of *STR* promoter which is identified as necessary for jasmonates and elicitor-responsive expression (JERE) (Li et al. 2013). To identify the role of *ORCA2* and *ORCA3* in regulation of MIA biosynthetic genes other than *STR*, transgenic hairy roots overexpressing *ORCA2* under the control of an ethanol-inducible

promoter of *C. roseus* were generated. The upregulated expression of *ORCA2* significantly altered the transcripts of many structural genes in MIA biosynthesis, such as *AS*, *TDC*, *G10H*, *LAMT*, *STR*, *T16H*, *PRX1*, *D4H*, *SGD*, and *DAT*; moreover, the induced *ORCA2* also caused the changes of the expressions of several TF-encoding genes, such as *ORCA3*, *ZCT1*, *ZCT2*, *ZCT3*, and *CrMYC2*. The accumulation of catharanthine, ajmalicine, serpentine, and tabersonine was also changed dramatically after *ORCA2* induction which was correlated with the change in activities of concerned genes. In another study, average content of catharanthine and vindoline in *C. roseus* hairy root lines overexpressing *ORCA2* was found to increase in comparison with control lines (Liu et al. 2011). In a little different scenario, JA elicitation and overexpression of *ORCA3* in hairy roots induced the expression levels of *AS*, *DXS*, *SLS*, and *STR* but decreased the expression of *SGD*. However *TDC*, *G10H*, *CPR*, *GBFs*, and *ORCA2* remain unaffected. In a study, overexpression of ORCA3 alone although resulted in upregulation of many known TIA genes but no change in total amount of TIAs is measured in *C. roseus* hairy roots. However, co-overexpression of ORCA3 and SGD resulted in a significant increase of serpentine, ajmalicine, catharanthine, tabersonine, and other TIAs (Sun and Peebles 2016). Functional studies of the AP2/ERF transcription factors reveal their pivotal role in CPT biosynthesis in HRCs of *O. pumila* also. In a recent study, five genes (OpERF1 to OpERF5) that encode for AP2/ERF TFs have been isolated form HRCs of *O. pumila* (Udomsom et al. 2016). The analyses reveal close evolutionary relationship of *Op*ERF1 with stress-responsive ERF factors in *Arabidopsis* and of *Op*ERF2 with ERF factors such as ORCA3 in *C. roseus*, *NIC2* locus ERF in tobacco, and JRE4 in tomato that are known to regulate production of terpenoid and other alkaloids. Furthermore, transgenic hairy root lines with suppressed expression of *OpERF1* and *OpERF2* were also analyzed for their transcriptome and metabolomes. The suppression of *OpERF2* resulted in reduced expression of genes in the 2-*C*-methyl-D-erythritol 4-phosphate and secologanin-strictosidine pathways, which supply a precursor, strictosidine, for CPT biosynthesis, but no significant change was observed in metabolites. In *C. roseus* HRCs, overexpression of *ORCA3* caused an increase of ajmalicine and serpentine and a significant decrease in other alkaloids including tabersonine and lochnericine (Peebles et al. 2009). In a multigene overexpression experiment when *ORCA3* in combination with *G8O* was overexpressed in hairy roots of *C. roseus*, as compared to non-transgenic clones, the transgenic root clones revealed higher accumulation level of catharanthine (Wang et al. 2010). On the basis of previous studies where overexpression of *G10H* in HRCs improved alkaloid production alone as well as when combined with precursors, etc., the *G10H* integrated with *ORCA3* was co-overexpressed. This resulted in increased accumulation of MIAs like strictosidine, vindoline, catharanthine, etc. both in plants and hairy roots (Pan et al. 2012). WRKY TFs are among the largest families of transcriptional regulators in plants and are well documented for their role in various stress-induced biochemical processes including terpenoid indole alkaloid biosynthesis. These WRKY TFs identify and bind to the W-box cis-regulatory element in the promoters of the number of terpenoid alkaloid pathway genes. The binding of WRKYs to the

W-box elements of genes is a part of biotic and abiotic stress responses as well as other biological processes. In *Catharanthus* WRKY TF has been identified and overexpressed in hairy roots (Suttipanta et al. 2011). This overexpression of *CrWRKY* upregulated a number of terpenoid alkaloid pathway genes including *TDC* and transcriptional repressors like *ZCT1*, *ZCT2*, and *ZCT3*. The overexpression was further repressed by *ORCA2*, *ORCA3*, and *CrMYC2*. *TDC* upregulation led to increased *TDC* activity and tryptamine concentration and threefold increases in serpentine in *CrWRKY1* hairy roots. Besides ORCAs and WRKYs, the other TFs proven to have pivotal roles in terpenoid alkaloid biosynthesis include the *ZCTs* (*ZCT1*, *ZCT2*, and *ZCT3*; zinc-finger-binding protein family) which are known to bind promoters of *TDC* and *STR*. In a recent study, interaction between expression of various TFs, terpenoid alkaloid pathway genes, and accumulation of major terpenoid alkaloids was investigated in *C. roseus* hairy roots elicited with methyl jasmonate (Goklany et al. 2013). Transcript level of ORCA along with MIA biosynthetic genes was dramatically increased even at moderate dosage of MJ. Accordingly, the MIA production was also increased. However, with an increase in MJ dose, the alkaloid accumulation lowers down. This was supposed to be due to elevated transcript levels of transcriptional repressor *ZCT* which was about 40-fold as compared to its levels (2- to 7-fold) at low MJ dose. Minimal induction of pathway genes and low transcript level of *ORCA* were also observed at high MJ dosage. Another transcriptional activator BPF1 (box P-binding factor for box P cis-element of various plant defense-related genes) has been isolated from *Catharanthus* that has a significant influence of biosynthetic gene *STR*. Transgenic hairy root line of *C. roseus* was developed with overexpression of *CrBPF1* (Li et al. 2015). Overexpression of *CrBPF1* caused not only an increase in transcript levels of indole and terpenoid as well as MIA pathway genes but also in 11 genes that were supposed to act as transcriptional regulators of genes of MIA and associated pathways. Furthermore, *CrBPF1* overexpression also caused increased transcript levels of MIA transcriptional activators and repressors. Interestingly, the MIA levels which were expected to be high due to an increase in transcript levels of various genes of the pathway had minimal or no effects.

5.4 Conclusion and Future Perspective

Two differently compartmentalize and independent pathway precursors in cell are responsible to provide five-carbon building blocks of all terpenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAP). These two pathways are (1) methylerythritol phosphate (MEP or non-mevalonate) pathway localized in plastids and (2) mevalonate pathway in cytosol. Several studies related to localization, transportation, and accumulation of end products have revealed that one directional plastid to cytosol trafficking of precursor IPP from the two pathways results its channeling to alkaloid biosynthesis (Sun and Peebles 2016). Large emphasis on the cross talk between the two pathways has been given in the research

focused on MIA biosynthesis. The simultaneous occurrence of two entirely different pathways for isoprenoid formation in plant cells is astonishing as nowhere (such instances are rare) else the similar situation occurs for any other metabolic route. Besides, the better understanding of stepwise down processing of precursors for secologanin synthesis and, likewise, synthesis of tryptamine in indole pathway is undoubtedly a remarkable hallmark discovery of plant secondary metabolism. Furthermore, application of modern biology, biochemistry, and biotechnological concepts in unraveling the MIA flux in an intricate and tightly regulated pathway has advanced the knowledge of biotechnological production of MIAs. The role of plant hairy root cultures has definitely proven their worth in investigating all sorts of radical and peripheral issues related to MIA secondary metabolism. Be it the elucidation of biosynthetic pathway or the enhancement of metabolite flux through various basic or engineering approaches, plant HRCs have emerged as flawless biological system. Not only because of their simplicity in establishment but also because of their rigid genetic and biochemical features. Utilizing hairy root system for genetic manipulations in geraniol, secologanin, tryptamine, tabersonine/vindoline, and catharanthine producing subways that ultimately focused on enhanced MIA production has elevated the understanding of MIA metabolism up to an anticipated level. The operational and regulatory complexities behind the MIA pathway have been uncovered up to a great extent; nevertheless certain issues are still in need of thoughtful scientific attention. These complexities are in terms of rigid interplay of differential intercellular localization of MIA pathway components. Secondly, intense understanding is also required to unravel the exact regulatory mechanism behind the transportation of precursor and intermediates at intracellular level. Another major point of judicious attention is the absence of expression of seven-step pathway from tabersonine to vindoline in root tissues. Coupled with this, the intricate spatial and temporal developmental regulation of MIA biosynthesis has restricted the use of HRCs for the production of highly important anticancer molecules vinblastine and vincristine. However, efforts for induced expression of *T16H* and *16OMT* genes of vindoline pathway and resultant accumulation of two important pathway metabolites have shown the direction of induced vindoline biosynthesis and ultimately its channeling toward vincristine and vinblastine synthesis in root tissues. Many unresolved queries exist regarding the intracellular and intercellular transport of MIA intermediates and end products. The molecular components that are involved in transportation and trafficking of precursors and intermediates are in need of serious investigation. With the use of appropriate strategies to functionally characterize these, transporter components will significantly exemplify the dynamic synthesis and trafficking processes in MIA-producing tissues. This may help to draw a whole picture of subcellular and intercellular MIA biosynthesis.

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Chapter 6
Stress-Induced Metabolite Production Utilizing Plant Hairy Roots

Kulwinder Kaur and Pratap Kumar Pati

Abstract Plant secondary metabolites comprise a diverse variety of organic compounds that facilitates defense response against various biotic and abiotic factors present in the environment. Many of these secondary metabolites are used in pharmaceutical, food, confectionary, cosmetics, insecticide, rubber, and agrochemical industries. In the last two decades, various attempts have therefore been undertaken to increase the production and accumulation of valuable secondary metabolites utilizing several approaches in cell, tissue, and organ culture systems. Cell and tissue culture systems, being undifferentiated, have limited potential to increase the production of secondary metabolites. Among different organ culture systems, hairy root has gained considerable interest due to its high rate of growth in minimal media, genetic and biochemical stability, non-dependence on phytohormones, and higher accumulation of secondary metabolites in short time period. Manipulations with hairy root culture medium and concentrations of carbon and nitrogen source have resulted in limited success. However, as plant secondary metabolites are produced under stress conditions, use of elicitors (elicitation) is one of the most promising strategies to increase secondary metabolite production. The present chapter briefly discusses the various extrinsic factors and their role in valuable secondary metabolite production in hairy root culture system.

Keywords Hairy root · Secondary metabolites · Elicitor · Elicitation · Stress

6.1 Introduction

Secondary metabolites are a diverse group of low molecular weight organic compounds produced by either cultivated or wild plants. These secondary metabolites do not have direct role in functioning of plants, but they provide defense against various biotic and abiotic factors and hence facilitate the adaptation of plants during its

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interaction with environment. Approximately 100,000 secondary metabolites have been recognized (Lajayer et al. 2017). Plant secondary metabolites confer specific tastes, odors, natural colors, and toxins in plants and thereby are widely used in food, textile, confectionary, cosmetics, insecticide, rubber, and agrochemical industries (Vasconsuelo and Boland 2007; Hussain et al. 2012; Ramirez-Estrada et al. 2016). Beside these, many of plant secondary metabolites are pharmaceutically important and contributed immensely in the success stories of various therapeutic agents: serpentine isolated from *Rauwolfia serpentina* is widely used in the treatment of hypertension (Mallick et al. 2012; Mehrotra et al. 2015a, b). Ajmalicine, atropine, caffeine, digitoxin, and quinine are obtained from *Catharanthus roseus*, *Atropa belladonna*, *Camellia sinensis*, *Digitalis purpurea*, and *Cinchona ledgeriana* and used for circulatory disorders as anticholinergic, CNS stimulant, cardiotonic, and antimalaria drugs, respectively (Fabricant and Farnsworth 2001). However, the production of these important secondary metabolites is very low (less than 1% of the dry weight) under cultivated or natural conditions (Verpoorte et al. 2002; Kaur et al. 2017). Their yield and composition are easily affected by developmental and physiological state of the plant, growing conditions, climate, light, temperature, mineral elements, heavy metals, and genetic makeup of plant, thus creating major obstacles during standardization of drug formulations. Furthermore, chemical synthesis of most of the secondary metabolites is difficult owing to multiple chiral centers in complicated stereochemical rings (Gai et al. 2017). All these challenges present a major hindrance for large-scale industrial use of plant secondary metabolites. Therefore, in the last two decades, several attempts have been made to increase the production and accumulation of plant secondary metabolites through biotechnological interventions (Singh et al. 2017; Kaur et al. 2017). Biotechnological tools such as cell and tissue culture systems have limited potential to increase secondary metabolite production mainly due to their undifferentiated nature. Among different organ culture systems, hairy roots have gained much interest from researchers owing to its potential to accumulate high amount of secondary metabolites in short time span. Furthermore, many of the plant secondary metabolites are produced and/or accumulated in roots, and harvesting of plant roots for medicinally important secondary metabolites is destructive for the plant; therefore, hairy root system is an attractive option for exploiting the potential of plant roots.

Hairy roots are produced by infection of explants with *Agrobacterium rhizogenes* and subsequent transfer of T-DNA of Ti plasmid from *A. rhizogenes* to the explants (Chandra 2012). Hairy roots are characterized by high branching, absence of geotropism, genetic and biochemical stability, high rate of growth in minimal medium, and non-dependence on phytohormones. Additionally, the ability of hairy roots to produce secondary metabolites for an extended period of time presents hairy root culture system as an excellent alternative for continuous production of pharmaceutically important secondary metabolites (Shanks and Morgan 1999; Cai et al. 2012).

Secondary metabolite production in hairy root culture system can be increased by manipulating the composition of nutrient medium, source of nitrogen, and type and concentration of carbon source (Sivanesan and Murugesan 2008; Mehrotra et al. 2015a, b). Wealth of literature also suggests that the use of elicitors and various other

treatments and environmental factors can affect the production of secondary metabolites in hairy root culture system. The present chapter discusses some of these issues which are very critical in production of valuable secondary metabolites using hairy root culture system.

6.2 Elicitors

Elicitor is any stress factor capable of triggering inducible defense responses in plants, including production and accumulation of secondary metabolites that have important roles in adaptation to the stressful conditions (Naik and Al-Khayri 2016). The process of inducing the production of secondary metabolites by using elicitor is known as elicitation. On the basis of their nature, elicitors are broadly classified into two major categories, abiotic and biotic elicitors (Fig. 6.1). Abiotic elicitors have nonbiological origin and are further divided into hormonal, physical, and chemical factors. Biotic elicitors are the substances having biological origin such as polysaccharides derived from plant cell wall and microorganisms.

Plants synthesize secondary metabolites as a response to the attack of pathogens, herbivores, insects, and other biotic and abiotic stresses. Work on induction of phytoalexin accumulation in *Glycine max* by *Phytophthora megasperma* divulged that just like pathogen itself, pathogen-derived small molecules can initiate the same response in the plant (Keen 1975). With further studies on the effect of microbe-derived oligosaccharides on plants, the role of elicitors on plant secondary metabolism was evident (Vasconsuelo and Boland 2007). The elicitor-mediated secondary metabolite production has opened new sectors of research that could have important economical benefits for various industries. However, the selection and optimization of different parameters like elicitor type and concentration, duration of exposure, type and age of in vitro culture system, and composition of nutrient medium are much needed investigations to utilize the full potential of elicitor-induced secondary metabolite production in hairy root culture system (Namdeo 2007).

6.2.1 Abiotic Elicitors

Plant growth and development are strongly influenced by environment. Abiotic factors such as light, temperature, pH, salt, heavy metals, and osmoticum have been shown to induce multigene responses leading to alterations in several proteins and accumulation of primary and secondary metabolites and hence enable the plant to ameliorate abiotic stress in the environment (Rodziewicz et al. 2014). In cell suspension and callus cultures of various plants, abiotic stress causes a drastic increase in valuable secondary metabolites (Naik and Al-Khayri 2016). The current section presents an overview of recent work directed toward abiotic elicitor-mediated production of secondary metabolites in hairy root culture system.

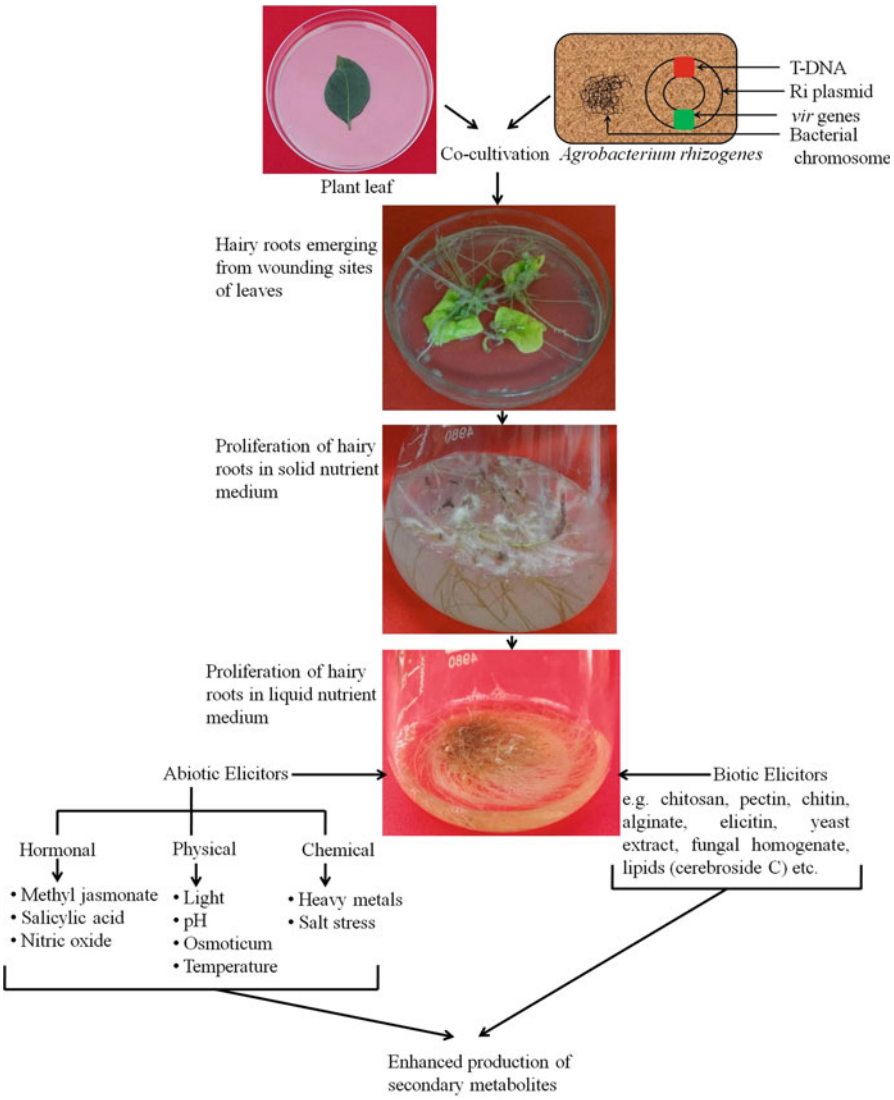

Fig. 6.1 Schematic representation of hairy root induction from leaf explant and its subsequent proliferation. Elicitation treatment is given to hairy roots proliferating in liquid nutrient medium

6.2.1.1 Hormonal Abiotic Elicitors

Methyl Jasmonate Jasmonic acid (JA) and its ester derivative methyl jasmonate (MeJA) are derived from catabolism of linolenic acid (Yendo et al. 2010). It acts as secondary messenger in several physiological processes and also triggers the defense responses (including biosynthesis of secondary metabolites) against various

pathogens and herbivores' attack, hence facilitating plant adaptation in challenging environment. In a recent study, Staswick (2008) demonstrated that the active form of JA is JA-Ile, which is actually a complex of JA with isoleucine. JA-Ile regulates the specific proteolysis of transcription repressor JAZ that blocks the MYC2 transcription factor which in turn activates the genes responsive to JA. JA and its ester derivatives are most commonly used elicitors in plant tissue culture studies to enhance the production of valuable secondary metabolites; as when applied exogenously, jasmonates stimulate the production of secondary metabolites in intact plant and also in cell suspension culture (Akula and Ravishankar 2011). In hairy root cultures of several plants, treatment with MJ and its derivatives resulted in significant increase in valuable secondary metabolites (Table 6.1).

In *Salvia sclarea* hairy root, 7-day elicitation with MeJA (100 μM) and phytotoxin coronatine (Cor, 0.1 μM) induced transcriptional reprogramming, resulting in significant increased accumulation of aethiopinone, a diterpene capable of arresting human melanoma cells at G_2/S phase (Vaccaro et al. 2017). MeJA was more effective in enhancing the production of aethiopinone (25-fold; 9.72 ± 0.08 mg g^{-1}DW) as compared to Cor (sevenfold; 2.57 ± 0.15 mg g^{-1}DW) over untreated hairy roots (0.38 ± 0.07 mg g^{-1}DW). The elicitor-induced higher accumulation of aethiopinone was due to transcriptional activation of several genes involved in 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol-4-phosphate (MEP)-derived isoprenoid pathway. In order to get insights into MeJa- and Cor-mediated enhancement in secondary metabolite production, transcript analysis was conducted at 0, 12, 24, and 48 h of elicitor treatment. Maximum elicitation was at 24 h of treatment and thereafter a decline in the next 24 h. The transcript analysis revealed approximately 60- and 20-fold upregulation in 1-deoxy-D-xylulose 5-phosphate synthase 2 (*DXS2*) after treatment of hairy roots with MeJa and Cor, respectively. Similarly, 4-(cytidine 50-diphospho)-2-C-methyl-D-erythritol kinase (*CMK*) and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (*MCS*) genes showed 6- and 12-fold enhancement in the transcript abundance upon elicitation with MeJA and Cor. Among other highly upregulated genes were 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (*HDR1*) (30-fold and 8-fold higher transcript level with MeJa and Cor, respectively, than control) and copalyl diphosphate synthase (*CPPS1*) (60-fold higher transcript level with MeJa than that of Cor (eightfold)). Further, treatment with MeJa was more effective than Cor in enhancing the gene expression. Interestingly, longer elicitation treatment (28 days) with MeJA caused significant growth inhibition of hairy roots, whereas Cor did not show any detrimental effects on biomass accumulation. Consequently 1 L of hairy root culture elicited with Cor for 28 days yielded 24-fold (103.32 ± 2.10 mg L^{-1}) more aethiopinone than control hairy roots (4.40 ± 0.13 mg L^{-1}), and this content was 16-fold higher than that produced after prolonged elicitation with MeJA (73.29 ± 0.11 mg L^{-1}).

Salicylic Acid. Salicylic acid (SA) is a small molecule that induces systemic acquired resistance (SAR) against several pathogens and hence plays a pivotal role in plant defense system. During the interaction of plant with pathogen, accumulation

Table 6.1 Selected examples of elicitor-mediated secondary metabolites production in hairy root culture system

Type of elicitors	Hairy root system	Secondary metabolite produced	References
Jasmonic acid and its derivatives	*Salvia sclarea*	Aethiopinone	Vaccaro et al. (2017)
	Withania somnifera	Withanolides	Saxena et al. (2017)
	Gentiana dinarica	Xanthone	Krstić-Milošević et al. (2017)
	Silybum marianum	Silymarin	Gabr et al. (2016)
	Papaver orientale	Thebaine morphine and codeine	Hashmi and Naghavi (2016)
	Salvia castanea	Cryptotanshinone, tanshinone I, and tanshinone IIA	Li et al. (2016)
	Arachis hypogaea	Arachidin-1, arachidin-3, piceatannol, and resveratrol	Yang et al. (2015)
	Catharanthus roseus	Ajmalicine	Thakore et al. (2015)
	Solanum trilobatum	Solasodine	Shilpha et al. (2015)
	Artemisia annua	Artemisinin	Ahlawat et al. (2014)
	Glycine max	Isoflavones	Theboral et al. (2014)
	Salvia miltiorrhiiza	Cryptotanshinone, dihydrotanshinone I	Cheng et al. (2013)
	W. somnifera	Withanolides	Doma et al. (2012)
			Sivanandhan et al. (2013)
	Sinapis alba	Glucosinolates	Kastell et al. (2013)
	Brassica rapa	Glucosinolates	Kastell et al. (2013)
	S. miltiorrhiza	Cryptotanshinone, tanshinone I, tanshinone IIA, and dihydrotanshinone I	Liang et al. (2012)
	Anisodus acutangulus	Tropane alkaloid	Kai et al. (2012)
	Pueraria candollei	Isoflavonoid	Udomsuk et al. (2011)
Salicylic acid	*Rehmannia glutinosa*	Acteoside	Wang et al. (2017)
	Gentiana dinarica	Xanthone	Krstić-Milošević et al. (2017)

(continued)

Table 6.1 (continued)

Type of elicitors	Hairy root system	Secondary metabolite produced	References
	P. orientale	Thebaine morphine and codeine	Hashemi and Naghavi (2016)
	S. marianum	Silymarin	Gabr et al. (2016)
	C. roseus	Ajmalicine	Thakore et al. (2015)
	G. max	Isoflavones	Theboral et al. (2014)
	W. somnifera	Withanolides	Doma et al. (2012)
			Sivanandhan et al. (2013)
	P. candollei	Isoflavonoid	Udomsuk et al. (2011)
NO	*W. somnifera*	Withanolides	Doma et al. (2012)
	S. miltiorrhiza	Cryptotanshinone, tanshinone I, tanshinone IIA, and dihydrotanshinone I	Liang et al. (2012)
	A. annua	Artemisinin	Wang et al. (2009)
			Zheng et al. (2008)
Ag^+	*S. castanea*	Cryptotanshinone, tanshinone I, and tanshinone IIA	Li et al. (2016)
	S. miltiorrhiza	Rosmarinic acid, caffeic acid, and ferulic acid	Xing et al. (2014)
		Cryptotanshinone and dihydrotanshinone I	Cheng et al. (2013)
		Rosmarinic acid	Yan et al. (2006)
		Total tanshinone	Zhang et al. (2004)
	Datura metel	Atropine	Shakeran et al. (2015)
	A. acutangulus	Tropane alkaloid	Kai et al. (2012)
	S. marianum	Silymarin	Khalili et al. (2010)
Biotic elicitors			
Piriformospora indica	*W. somnifera*	Withanolides	Saxena et al. (2017)
	A. annua	Artemisinin	Ahlawat et al. (2014)
	Linum album	Lignan	Kumar et al. (2012)
	D. metel	Atropine	

(continued)

Table 6.1 (continued)

Type of elicitors	Hairy root system	Secondary metabolite produced	References
Bacillus cereus, *Staphylococcus aureus*			Shakeran et al. (2015)
Alternaria alternata, *Curvularia limata*, *Fusarium solani*	*A. annua*	Artemisinin	Ahlawat et al. (2014)
Trichoderma atroviride	*S. miltiorrhiza*	Tanshinone I, tanshinone IIA, dihydrotanshinone I, and cryptotanshinone	Ming et al. (2013)
Yeast extract	*S. miltiorrhiza*	Cryptotanshinone, dihydrotanshinone I	Cheng et al. (2013)
		Rosmarinic acid	Yan et al. (2006)
	G. dinarica	Xanthone	Krstić-Milošević et al. (2017)
	S. castanea	Cryptotanshinone, tanshinone I, and tanshinone IIA	Li et al. (2016)
	P. candollei	Isoflavonoid	Udomsuk et al. (2011)
Chitosan	*W. somnifera*	Withanolides	Doma et al. (2012)
	S. marianum	Silymarin	Gabr et al. (2016)
	G. dinarica	Xanthone	Krstić-Milošević et al. (2017)
	P. candollei	Isoflavonoid	Udomsuk et al. (2011)
	A. annua	Artemisinin	Putalun et al. (2007)
Miscellaneous			
Cyclodextrin	*A. hypogaea*	Arachidin-1, arachidin-3, piceatannol, and resveratrol	Yang et al. (2015)
KCl	*C. roseus*	Ajmalicine	Thakore et al. (2015)
Tween-80	*Panax ginseng*	Ginsenoside	Liang et al. (2015)
Ethanol	*A. acutangulus*	Tropane alkaloid	Kai et al. (2012)
Agrobacterium rhizogenes	*P. candollei*	Isoflavonoid	Udomsuk et al. (2011)

of SA in the infected site induces a hypersensitive response. The signal thus triggered metastasizes to other parts of the plant and induces an array of defense responses that involves secondary metabolite production. However, SA induces accumulation of only some classes of secondary metabolites as it is not a global elicitor (Ramirez-Estrada et al. 2016). At whole plant level, SA foliar spray causes modulation of secondary metabolites (Dučaiová et al. 2013). Similar effects have

been demonstrated in cell suspension and hairy root culture systems of many plants (Kang et al. 2006; Yousefzadi et al. 2010; Hashemi and Naghavi 2016).

Rehmannia glutinosa is an important herb used in traditional Chinese medicine (Wang et al. 2017). After 10-day treatment of *R. glutinosa* hairy roots with SA (25 μmol/L), 2.28-fold enhancement in the content of acteoside was recorded than control hairy roots. As genome sequence of *R. glutinosa* is not available, detailed investigation was conducted by transcriptome analysis using Illumina HiSeq 2000 platform. The samples were harvested at 0, 12, and 24 h after treatment with SA. 3716 and 4018 differentially expressed transcripts (DETs) were identified in 0 h vs. 12 h and 0 h vs. 24 h, respectively. Further, 2715 DETs were found in 12 h vs. 24 h. The 2401 DETs were upregulated, and 1617 DETs were downregulated at 24 h treatment with SA in comparison to 0 h. On the other hand, 1470 upregulated and 1245 downregulated DETs were present at 24 h as compared to 12 h. In silico differential analysis was confirmed by qRT-PCR performed on ten randomly selected transcripts. The expression analysis through qRT-PCR correlates with DETs indentified by RNA-seq. This work has provided a platform to understand the molecular basis of biosynthesis of acteoside in *R. glutinosa.*

In case of *Papaver orientale*, hairy roots were treated with 100 μM SA, and samples were harvested after 6, 12, 24, and 48 h (Hashemi and Naghavi 2016). HPLC analysis of morphinan alkaloids revealed that longer exposure of elicitors (24 and 48 h) led to higher accumulation of thebaine, morphine, and codeine. Codeine was maximum accumulated at 24 h, whereas thebaine and morphine showed highest content at 48 h. SA elicitation for 48 h increased the content of thebaine by 2-fold (1.66 mg g^{-1}) and morphine by 4.22-fold (2.87 mg g^{-1}). Further, 2.59-fold (1.61 mg g^{-1}) increase was seen in the content of codeine after 24 h treatment with SA. The relative expression of key genes such as salutaridine synthase (*Salsyn*), salutaridine reductase (*SalR*), salutaridinol 7-O-acetyltransferase (*SalAT*), codeine O-demethylase (*CODM*), thebaine 6-O-demethylase (*T6ODM*), and codeinone reductase (*COR*) involved in biosynthesis of morphinan alkaloids was analyzed at 6, 12, 24, and 48 h. Upon treatment with SA for 48 h, *Salsyn*, *T6ODM*, and *CODM* showed 13.39, 9.6, and 2.98 times upregulation, respectively.

Nitric Oxide. Nitric oxide (NO) is a nontraditional phytohormone having wide physiological implications in both plants and animals. It also acts as a signal molecule in various stress responses (Zhang et al. 2012). Artificial NO donors, viz., S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione, and sodium nitroprusside (SNP), are added in the nutrient medium to study the effect of NO in various culture systems. Due to its longest half-life, SNP in its crystalline form, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$, is mostly used in tissue culture of medicinal plants. It is used in the concentrations ranging from 10 μM to 100 mM; the wide range indicates the species-specific effect of NO. Intriguingly, NO released from SNP is photosensitive, and NO emission is fully inhibited in dark (Floryszak-Wieczorek et al. 2006). At the level of plants, NO (SNP) increases the growth of plant as well as secondary metabolite production (Wang et al. 2011). NO has been shown to cross talk with

reactive oxygen species (ROS) and phytohormones such as SA and JA (Zhang et al. 2012; Mishra et al. 2017).

Cerebrosides are glycosphingolipids and act as potential elicitors. Cerebroside C (CE) was isolated from *Fusarium* sp. NO potentiates the CE-induced production of artemisinin in hairy root cultures of *A. annua* (Wang et al. 2009). NO-donor SNP (10, 50, and 100 μM) enhanced the hairy root growth. However, it did not alter the content of artemisinin (Zheng et al. 2008). However, 2 days of combined treatment of hairy roots with CE (30 μg/ml) and SNP (10 μM) led to enhanced artemisinin than hairy roots treated with CE alone. The 4-day treatment of hairy roots with CE and SNP showed 36% higher artemisinin content than control (CE only). The potentiating effect of NO on CE-induced accumulation of artemisinin was further divulged by expression studies of key genes (*HMGR* and DXS) involved in artemisinin biosynthesis. Samples treated with CE alone showed 9.3 and 6.6 times higher expression of *HMGR* and *DXS*, respectively, than control (without CE). Interestingly, SNP alone did not enhance the expression of these genes, but a combined treatment of CE and SNP caused significant increase in the expression of *HMGR* and *DXS*. The role of NO in potentiating the CE-induced accumulation of artemisinin was confirmed by using inhibitors such as Nω-nitro-L-arginine methyl ester (L-NAME) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) as NO synthase (NOS) inhibitor and NO scavenger, respectively. cPTIO- and NOS-inhibited artemisinin production was reinitiated by supplying SNP, suggesting that these inhibitors inhibited artemisinin production by reducing the level of NO. In another study, Zheng et al. (2008) showed that NO can further enhance the oligosaccharide-induced production of artemisinin in hairy root cultures of *A. annua*.

6.2.1.2 Physical Abiotic Elicitors

Light. Light has tremendous effect on plant growth and biosynthesis of secondary metabolites. In addition to providing a source of energy, light is perceived as a signal by the photoreceptors of plants and regulates plant growth, differentiation, and metabolism. Maximum light absorption by the plant is in blue and red region of the spectrum having wavelength from 600 to 700 nm, and the maximum reflectance is in green and far red region having wavelength from 700 to 800 nm. Ultraviolet (UV)-B indicates radiations of wavelength 280–315 nm. In plants, UV-B light is absorbed by specific UV-B photoreceptors (Zu et al. 2010). UV-B radiations cause growth reduction and also influence plant secondary metabolite production by regulating the key genes (phenyl ammonia lyase and chalcone synthase) of phenylpropanoid pathway.

In plants, flavonoids are produced and accumulated as protective substances against UV-B stress. In callus and cell suspension culture, light affects the accumulation of secondary metabolites such as flavonoids, anthocyanins, zingiberene, and gingerol (Krewzaler and Hahlbrock 1973; Anasori and Asghari 2008). Similar studies have also been conducted to enhance secondary metabolite production in hairy root

cultures of various plants. Hairy roots of *Fagopyrum tataricum* Gaertn. were exposed to UV light for 30 min (3 days). The UV-C light was blocked from UV lamp by wrapping it in cellular diacetate filter. After UV treatment of hairy roots (302 nm was the maximum radiation peak, intensity of UV-B light on the surface of sample was 1.26 μW/cm^2), the content of rutin was 5.18-fold higher (increased from 0.93 to 4.82 mg g^{-1}) than wild-type (WT) roots (Huang et al. 2016). Following UV treatment, the maximum level of rutin was observed in leaves (9.35-fold) followed by hairy roots (5.18-fold), stem (3.5-fold), and non-transformed roots (2.95-fold). The least rutin was in flowers (2.66-fold). The quercetin content was increased from 0.02 to 0.04 mg g^{-1} in hairy roots upon exposure to UV-B stress. To further investigate the flavonoid biosynthetic pathway, transcript abundance of key genes, viz., phenylalanine ammonia lyase (*ftPAL*), cinnamic-4-hydroxylases (*FtC4H*), 4-coumaroyl-CoA-ligase (*Ft4CL*), chalcone synthase (*FtCHS*), chalcone isomerases (*FtCHI*), flavanone 3-hydroxylase (*FtF3H*), flavonoid 3′-hydroxylase (*FtF3'H-1*), *FtF3'H-2*, flavonol synthase (*FtFLS-1*), *FtFLS-2*, dihydroflavonol reductase (*FtDFR*), and anthocyanidin synthase (*FtANS*), was analyzed by qRT-PCR. *FtFLS*-1 gene showed 30–40-fold high transcript level than WT. *FtCHI* and *FtCHS* showed 20–30-fold high expression. Contrary to this, only slight enhancement was seen in transcript level of *FtF3'H-1*, *FtF3'H-2*, *FtFLS-2*, *FtDFR*, and *FtANS* genes upon exposure to UV-B light. UV-B stress-induced increased expression of flavonoid biosynthetic pathway was due to the presence of UV-B stress-sensitive domains in the promoter region of UV-induced upregulated genes.

In *Catharanthus roseus*, 32-day-old hairy roots were exposed to UV-B radiations (intensity of UV-B light on the surface of the sample was 9,000 uW/cm^2) for 0, 5, 10, and 20 min and harvested at 72 h (Binder et al. 2009). The most significant changes in the content of ajmalicine, serpentine, lochnericine, tabersonine, and hörhammericine were observed after exposure for 20 min. During further experiments, UV-B treatment of 20 min was followed by sample harvesting at 0, 6, 12, 24, 36, 48, 72, and 168 h. The level of hörhammericine decreases for the first 48 h of UV-B exposure and returned to normal after 168 h, while lochnericine content continuously increased for initial 48 h and then returned to normal after 168 h. However, no significant change was seen in the content of ajmalicine, serpentine, tabersonine, and catharanthine. The analysis of transcript abundance of geraniol 10-hydroxylase (*G10H*), tryptophan decarboxylase (*TDC*), and strictosidine synthase (*STR*) showed the maximum accumulation of transcript of *G10H* at 36 h, whereas *TDC* and *STR* showed minor changes after UV-B stress.

pH. In plants, the optimum pH varies considerably in different plant species. The pH influences the uptake of nutrients and enzymatic and hormonal activity. In most of the plant tissue culture practices, pH 5.8 is maintained initially. However, with the growth of cultures, pH tends to change, thereby affecting the plant growth and development. Interestingly, in case of plant roots, pH is considered as a major modulator of transcriptome (Lager et al. 2010). Surprisingly, as compared to other abiotic factors, only few reports are available on attempts to enhance secondary metabolites production by altering medium pH in hairy root culture system.

Hairy roots (six pieces of 1 cm length of 30-day-old culture) of *Silybum marianum* were allowed to grow for 30 days in nutrient medium initially set at different pH such as pH 5, 5.7, 6, and 7 (Rahimi and Hasanloo 2016). The maximum dry biomass (0.50 g) was favored by pH 5, whereas nutrient medium adjusted to pH 5.7, 6, and 7 led to 0.24, 0.27, and 0.21 g dry biomass, respectively. Highest accumulation of silymarin (0.25 mg/g DW) was achieved at pH 5. On the other hand, silymarin content at pH 5.7, 6, and 7 was 0.18, 0.15, and 0.18 mg/g DW, respectively.

The level of silybin, isosilybin, silydianin, and silychristin was also highest at pH 5. Furthermore, antioxidant enzyme activity and lipoxygenase activity were conducted in hairy roots exposed to different pH. The enzymatic activity of guaiacol peroxidase (G-POD) and ascorbate peroxidase (APX) was highest at pH 5.7 as compared to other pH. Interestingly, lipoxygenase (LOX) activity was maximum at pH 5, indicating that LOX initiates lipid peroxidation under acidic conditions and fatty acids were converted to corresponding hydroperoxides resulting to silymarin accumulation.

In *Withania somnifera* hairy root culture system, a range of pH (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, and 6.5) was tested for its effect on accumulation of biomass and pharmaceutically important secondary metabolite, withanolide A (Praveen and Murthy 2012). The maximum biomass accumulation (12.1 g l^{-1} DW) was supported by pH 5.8, whereas optimum pH for withanolide A production (13.84 mg g^{-1} DW) was 6.0. A similar work that involves testing of pH range (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) on hairy root culture *of Picrorhiza kurroa* revealed that accumulation of biomass and important secondary metabolites such as kutkoside and picroside I was optimum at pH 6.0, whereas lowest biomass and specific glycoside content was at pH 3.0 (Verma et al. 2015). The pH 4.0 and 8.0 facilitated intermediate accumulation of hairy root biomass and secondary metabolites.

Nutrient medium having pH 2.0, 3.0, and 4.0 alters the permeability of *B. vulgaris* hairy roots to different extent and resulting to 70, 15, and 10% of betalains released into the culture medium within 30 min, respectively (Thimmaraju et al. 2003). This strategy may be employed for cost-effective industrial production of valuable secondary metabolites.

Osmoticum. Osmotic stress is a potent elicitor for increasing the production of secondary metabolites. Sorbitol and PEG are metabolically inert and thus frequently used for osmoticum studies. Biosynthesis of tanshinone in hairy roots of *Salvia miltiorrhiza* is induced by hyperosmotic stress (Shi et al. 2007). Culture medium supplemented with different concentrations of sorbitol (30–100 g/l) was tested for 6 days for its potential to increase the production of tanshinone. Surprisingly, at all the tested concentrations of sorbitol, the content of total tanshinone was higher than control. The maximum yield of tanshinone was 4.5-fold (162.0 μg/g DW) higher than control and was achieved at 70 g/l sorbitol. In contrary to this, the maximum volumetric yield of total tanshinone (8.18 mg/l; 4.8-fold higher than control) was obtained when hairy roots were cultured in medium amended with 50 g/l sorbitol. This is due to increase in dry weight at 70 g/l sorbitol. Further, since the production of reactive oxygen species (ROS) is one of the earliest responses of the plant cells

against any kind of stress, hence SOD and CAT activities in sorbitol-treated hairy roots were checked. The SOD activity in treated hairy roots reached maximum in 2–3 days, and CAT activity reached to its peak in 1–2 days. However, there was no correlation between tanshinone accumulation and enzyme activity, suggesting that ROS was not the signal molecule in sorbitol-induced higher accumulation of tanshinone.

Upon PEG treatment, the content of bioactives of *S. miltiorrhiza*, viz., tanshinone I, tanshinone II A, cryptotanshinone, and dihydrotanshinone I, was 0.9-fold (49.2 μg g^{-1}), 1.0-fold (736.2 μg g^{-1}), 1.4-fold (106.8 μg g^{-1}), and 0.7-fold (935.0 μg g^{-1}) higher than control, respectively (Yang et al. 2012). PEG-induced production of tanshinone was completely abolished by ibuprofen (IBU, MJ biosynthesis inhibitor), providing a clue for endogenous MJ signaling in PEG-induced production of tanshinone. It was noticed that MJ content was 1.8-fold (168 ng g^{-1}) higher in PEG-treated hairy roots than control. MJ content in hairy roots treated with PEG + IBU was reduced to 27.3 ng g^{-1}. Further gene expression studies of *HMGR*, *DXR*, and *DXS* as well as protein activity of HMGR and DXS were conducted. In PEG-treated samples, *HMGR*, *DXR*, and *DXS* showed 25.3-, 12.1-, and 15.7-fold upregulation than control. The enzymatic activities of HMGR and DXS were significantly enhanced, suggesting that both mevalonate (MVA) and MEP pathways were stimulated by PEG treatment. However, the gene expressions and protein activities were significantly suppressed by IBU, pointing that PEG-induced tanshinone production is dependent on MJ signaling.

Temperature. Temperature fluctuations exert multiple effects on metabolic regulations and rate of intracellular reactions in plants. Temperature has significant effects on membrane permeability by altering the composition of membrane lipid (Ramakrishna and Ravishankar 2011; Wang and Wu 2013). Generally, 25 °C temperature is maintained for hairy root cultures; lowering the temperature to 19.5 °C increases the content of linolenic acid and indole alkaloids in hairy root cultures of *Catharanthus roseus* (Toivonen et al. 1992). In case of *Silybum marianum*, maximum silymarin production (0.18 mg/g DW) was achieved when hairy root cultures were incubated at 25 °C/25 °C in 16 h/8 h light/dark cycle as compared to 30 °C/25 °C (0.13 mg/g DW) and 15 °C/20 °C (0.0.12 mg/g DW) (Rahimi and Hasanloo 2016). Furthermore, the accumulation of silybin, silydianin, and taxifolin was also highest at 25 °C/25 °C.

Secondary metabolites are sequestered in the vacuoles. Reverse sequestration of valuable secondary metabolites into cell exterior has recently been employed to enhance the yield of secondary metabolites and also to decrease the cost of downstream extraction process, thus facilitating cost-effective production of secondary metabolites. The same strategy has been employed to efflux the pigments of *Beta vulgaris* (Thimmaraju et al. 2003). Twenty-day-old hairy roots of *B. vulgaris* exposed to different temperatures such as 40, 45, and 50 °C for 30 min release approximately 5.4, 31, and 47% pigments, respectively, in the culture medium. An exposure of 60 min resulted in 13.4, 40.2, and 47.5% efflux of pigments.

6.2.1.3 Chemical Abiotic Elicitors

Heavy Metals. Heavy metals such as nickel (Ni), selenium (Se), and iron (Fe) are the essential trace elements required for plant growth and development, as these heavy metals act as cofactor for many metalloenzymes. In plant tissue culture, heavy metals have tremendous potential to stimulate the production and accumulation of valuable secondary metabolites (Lajayer et al. 2017).

Hairy root culture of *S. miltiorrhiza* supplemented with Ag^+ ($Ag_2S_2O_3$) for 2–3 days showed that the content of total tanshinone was increased by twofold (after 2 weeks) as compared to control (Zhang et al. 2004). The increase in total tanshinone content was at the expense of root growth, which was suppressed to approximately 30%. The increased tanshinone may correlate with stimulatory effect of Ag^+ on key enzymes (HMGR and DXS) involved in tanshinone production (Ge and Wu 2005). Moreover Ag^+ elicitation can cause exudation of valuable secondary metabolites into the culture medium. Treatment of hairy roots of *Brugmansia candida* with 1.0 mM $AgNo_3$ for 24 h decreased the content of hyoscyamine and scopolamine in hairy roots, but it released significant amount of scopolamine into the culture medium. The exudated scopolamine may attribute to the cell lysis induced by osmotic stress and toxicity of Ag^+ (Pitta-Alvarez et al. 2000).

Hairy roots of *Hyoscyamus reticulatus* elicited with different concentrations (0, 450, 900, 1800, and 3600 mg/L) of iron oxide nanoparticles (FeNPs) for different time periods (24, 48, and 72 h) accumulated maximum hyoscyamine and scopolamine content (fivefold increase as compared to control) when nutrient medium was supplemented with 900 and 450 mg/L FeNPs for 24 and 48 h, respectively (Moharrami et al. 2017). The higher production of hyoscyamine and scopolamine in FeNP-treated hairy roots is due to availability of sufficient Fe^{2+} required for the enzyme hyoscyamine-6-hydroxylase catalyzing the conversion of hyoscyamine to scopolamine through hydroxylation. However, exposure of hairy roots to FeNPs for longer durations led to decreased production of hyoscyamine and scopolamine mainly due to toxic effects exerted by nanoparticles on mitotic index and DNA.

Salt Stress. Plants have evolved several adaptations to survive in high salt hostile environment that induces osmotic as well as ionic stress in plants (Naik and Al-Khayri 2016). The adaptations here involve reconfiguration of metabolic network to maintain metabolic homeostasis and hence mitigating the salt stress by increasing the production and accumulation of specific metabolites (Ni et al. 2015). *Plantago ovata* in hydroponic culture system exposed to NaCl led to significant increased accumulation of proline, total saponin, and total flavonoids. The increased accumulation of proline is due to its ability to act as compatible solute and thus help in osmotic adjustments and protect the enzymes by stabilizing the structure of organelles and macromolecules. On the other hand, flavonoids and saponins protect the plant from oxidative stress induced by salt ions (Haghighi et al. 2012).

The effect of salt stress (KCl and $CaCl_2$) was also explored in hairy roots of three species of *Datura*, viz., *D. tatula* (LDT), *D. stramonium* (LDS), and *D. innoxia* (LDI) (Harfi et al. 2016). Both the salts were used in varying concentrations (0.5,

1, 2, and 3 g/l) for different elicitation time (10, 24, and 48 h). All the three species of *Datura* accumulated maximum hyoscyamine at 2 g/l KCl. The highest content of hyoscyamine recorded after 24 h elicitation with KCl was 2.32-fold (12.074 ± 0.138 mg g^{-1} DW) and 1.85-fold (12.651 ± 0.342 mg g^{-1} DW) for LDS and LDI, respectively, as compared to control. However, for LDT, hyoscyamine level reached maximum to 1.99-fold (16.289 ± 0.382 mg g^{-1} DW) after 10 h elicitation with KCl. In case of $CaCl_2$, the corresponding hyoscyamine content with 24 h elicitation with 2 g/l $CaCl_2$ and 1 g/l $CaCl_2$ was 2.07-fold (16.978 ± 0.380 mg g^{-1} DW) and 1.85-fold (12.697 ± 0.242 mg g^{-1} DW) higher in LDT and LDI, respectively, than control. On the other hand, 10 h elicitation with 2 g/l $CaCl_2$ yielded 2.08-fold (10.828 ± 0.261 mg g^{-1} DW) higher hyoscyamine for LDS.

6.2.2 Biotic Elicitors

Biotic elicitors, derived from pathogens or the plants itself, are either of defined composition (molecular structure is known) such as chitosan, pectin, chitin, alginate, and elicitin or of complex composition (having various different molecular classes) like yeast extract and fungal homogenate (Vasconsuelo and Boland 2007). The fungal elicitors used for treatment of hairy roots are mostly the crude extracts of fungal mycelia or culture filtrates derived from pathogenic or endophytic fungi (Wang and Wu 2013). Recently a new approach of using fungal elicitor was developed by immobilizing the fungus in Ca-alginate gel (CAG). This strategy was used to enhance pharmaceutically important secondary metabolites in *Astragalus membranaceus* (Gai et al. 2017).

Astragalosides (AGs) are triterpene saponins produced by roots of *A. membranaceus.* Among various AGs such as astragaloside I (AG I), astragaloside II (AG II), isoastragaloside II (IAG II), astragaloside III (AG III), and astragaloside IV(AG IV), AG IV has multiple pharmaceutical properties (Gai et al. 2017). AG I, AG II, and IAG II have low bioactivity and share structural similarity with AG IV, except that they have extra acetyl group at position C-3. Microbial transformation has recently gained considerable interest as an alternative to conventional chemical processes for hydrolysis of unwanted acetyl groups in the precursors to generate AG IV. Utilizing a cocultivation system of *A. membranaceus* hairy root cultures (AMHRCs) with CAG facilitated immobilized endophytic fungus *Penicillium canescens* (IPC), and elevated production of AG IV was achieved. IPC-treated AMHRCs accumulated high level of AG IV (1.585 ± 0.0106 mg/g DW) as compared to control AMHRCs (0.187 ± 0.014 mg/g DW) and CAG-treated AMHRCs (0.196 ± 0.009 mg/g DW). Further, the content of acetylated precursors (AG I, AG II, IAG II) of AG IV in IPC-treated AMHRCs was significantly low when compared to untreated AMHRCs, pointing toward deacetylation potential of IPC (due to the secretion of acetyl esterase) and thereby enhancing the content of AG IV. The expression analysis of 11 key genes of AG IV biosynthetic pathway was analyzed at 24, 48, 60, and 96 h after cocultivation with IPC. All the tested genes,

viz., acetoacetyl-coenzyme A thiolase (*AACT*), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (*HMGS*), HMG-CoA reductase (*HMGR*), mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), squalene synthase (*SS*), squalene epoxidase (*SE*), and cycloartenol synthase (*CAS*), showed upregulation from 24 to 60 h posttreatment suggesting transcriptional activation by IPC. However, expression level of some of the genes such as mevalonate diphosphate decarboxylase (*MVD*), farnesyl diphosphate synthase (*FPS*), *SE*, and *CAS* was decreased at 96 h as compared to control. The decline was due to prolonged cocultivation that could lead to metabolic damage or death of hairy roots in extreme cases.

Hairy roots of *Withania somnifera* were treated with different concentrations (1%, 3%, and 5%) of cell homogenates of *Piriformospora indica* (CHP) for varying time periods (24, 48, and 72 h) (Saxena et al. 2017). *P. indica* is a root endophytic fungus which is used as biofertilizer, bioregulator, and bioprotector against stress conditions. It stimulates secondary metabolite production in various medicinal plants. For preparing cell homogenate of CHP, the fungus was grown at 30 ± 1 °C on Hill and Kafer medium solidified with agar. After 8 days of incubation, fully grown fungus was inoculated in 100 ml Hill medium (liquid). At the mid-log phase (5 days), the fungus culture was subjected to autoclave at 121 °C, 15 lb. in^{-2} for 15 min. Following filtration, cell residue was washed and homogenized with sterile water, and finally the volume was maintained to 50 ml with autoclaved double-distilled water. The cell homogenate thus prepared was used to treat hairy roots of *W. somnifera*. CHP (3%) for 48 h led to 1.15-fold (34.04 ± 0.17 g FW) higher biomass accumulation than control hairy roots. The content of withanolide A, withaferin A, withanoside IV, and withanoside V increased by 2.7-fold (6.37 ± 0.116 mg/g DW), 2.5-fold (3.28 ± 0.07 mg/g DW), 2.34-fold (0.171 ± 0.006 mg/g DW), and 2.9-fold (0.147 ± 0.003 mg/g DW), respectively, than control hairy roots. Further, gene expression studies revealed the upregulation of genes involved in MVA, MEP, and the key genes of sterol biosynthetic pathway. Treatment of *W. somnifera* hairy roots for 48 h with 3% CHP upregulated the expression of *HMGR*, *FPPS*, *SS*, *SE*, *CAS*, obtusifoliol-14-demethylase (*ODM*), sterol methyltransferase 1 (*SMT*-1), and sterol-22-desaturase (*SDS*) genes by 3.2-, 3.49-, 2.87-, 3.25-, 3.08-, 4.42-, 4.81-, and 5.024-fold, respectively, than untreated hairy roots. Genes associated with MEP pathway such as 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) and 1-deoxy-D-xylulose-5-phosphate reductase (*DXR*) were also upregulated (6.5-fold) by CHP.

Chitosan is acetylated β-1, 4-linked D-glucosamine polymer and is a structural component of many fungal pathogens such as *Fusarium* sp. Chitosan has been exploited as an important elicitor to enhance secondary metabolite production in plants. The effect of different concentrations of chitosan (50, 100, and 150 mg/l) was studied on artemisinin production potential of 21-day-old hairy root cultures of *A. annua* (Putalun et al. 2007). The samples were harvested at different time periods from 2 to 6 days. After 6 days of incubation, artemisinin content was sixfold higher (1.84 ± 0.02 μg mg^{-1} DW) than control hairy roots. Further, there was a direct correlation between incubation period (2–6 days) and artemisinin yield.

6.3 Mechanism of Action of Elicitors

Elicitors belong to several classes of compounds that do not share a common chemical structure. A compound that can act as elicitor by eliciting plant secondary metabolite production in one species may be inactive in another plant species. In contrast, different plant species/genus may respond to same elicitor. The molecular basis of elicitation is the ability of plants to recognize an array of structurally diverse molecules as signals due to the presence of elicitor-specific receptors in the cell membrane (Vasconsuelo and Boland 2007).

Elicitation starts with signal perception. Elicitors act as signal, and it is perceived by the receptor on the plant cell membrane followed by initiation of signal transduction cascade (Fig. 6.2) (Zhao et al. 2005; Kurosaki 2012; Mishra et al. 2012). Several elicitor-binding sites are recognized as potential receptors. The transmembrane receptor-like kinases (RLKs) are the most studied category of the receptors capable of receiving wide range of stimuli. Flagellin receptors of leucine-rich repeats (LRR) class are the best characterized RLKs. Plant R-proteins are another class of receptor that respond to elicitor produced by *Avr* genes. The specificity of R-protein is due to the presence of nucleotide-binding site as well as leucine-rich repeat domain (NBS-LRR). The high specificity between plant R-protein and *Avr* product can explain the species-specific nature of some elicitors.

Elicitor perception is associated with activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins). Studies involving activators and inhibitors of G-proteins implicated its undisputable role in transmitting the signals to phospholipases, ion channels, NADPH oxidase, molecules such as GTPase, and signaling pathways activated in response to biotic and abiotic stresses (Goel et al. 2011). Inhibitor of G-proteins such as suramin inhibits the activation of receptor-coupled G-proteins, and subsequently the production of phytoalexin suggested an important role of G-proteins in signal transduction within the cell. NADPH oxidase along with apoplastic peroxidases led to generation of reactive oxygen species, primarily superoxide anion and hydrogen peroxide leading to oxidative burst, an earliest response of plant cells against elicitor treatment or pathogen attack (Zhao et al. 2005).

Another earliest response of plant cells after recognition of elicitors is rapid ion fluxes such as Ca^{2+} influx, K^+/H^+ exchange, and Cl^- effluxes (Zhao et al. 2005). Among these, Ca^{2+} influx is most relevant due to its participation as key second messenger in various physiological processes. After elicitor recognition, within 2–5 min, the level of Ca^{2+} increases from 50–100 nM to 1–5 μM. In response to most of elicitors, two $[Ca^{2+}]_{cyt}$ peaks were produced. First $[Ca^{2+}]_{cyt}$ peak is due to the influx of extracellular Ca^{2+}, while the second $[Ca^{2+}]_{cyt}$ peak is attributed to activation of phospholipase C (PLC) that causes hydrolysis of membrane phospholipids such as phosphatidylinositol 4,5-diphosphate (PIP_2) into 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to Ca^{2+} release from inner Ca^{2+} stores like Golgi apparatus, vacuole, and endoplasmic reticulum. The $[Ca^{2+}]_{cyt}$ spiking either directly or by Ca^{2+} sensors such as calmodulin can activate

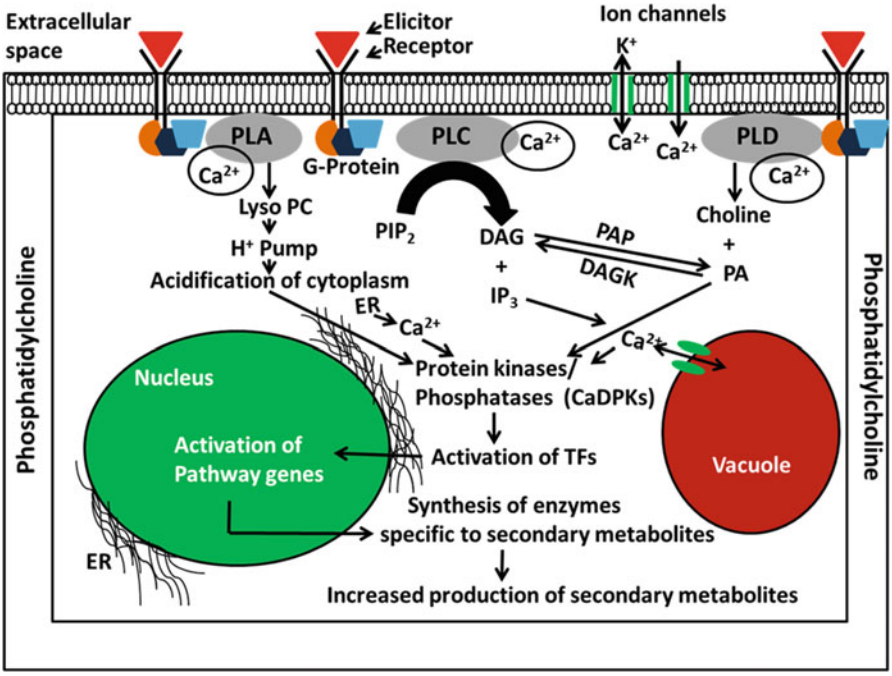

Fig. 6.2 A schematic illustration of molecular mechanism of elicitor-induced biosynthesis of secondary metabolites using lipid messengers derived from plasma membrane hydrolysis. Plasma membrane itself and the receptors present in the plasma membrane can perceive the signal. Phospholipase gets activated by several biotic and abiotic stresses and hydrolyzes the phospholipids such as phosphatidylcholine (PC) and phosphatidylinositol 4,5-diphosphate (PIP_2) and results in production of signal molecules. Lysophosphatidylcholine (lysoPC) released from the hydrolysis of PC by phospholipase A (PLA) activates proton pumping in the tonoplast and expels H^+ to the cytoplasm. The resulted cytoplasmic acidification induced biosynthesis of secondary metabolites. Phospholipase C (PLC) causes hydrolysis of PIP_2 and yield diacylglycerol (DAG) and 1,4,5-trisphosphate (IP_3). IP_3 mobilizes Ca^{2+} from intracellular Ca^{2+} reservoirs. The released Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinases (CaDPKs), which in turn differentially activates the transcription factors involved in biosynthesis of secondary metabolites. Phospholipase C (PLD) hydrolyzes phospholipids to phosphatidic acid (PA). PA and DAG are interconvertible through PA phosphatase (PAP) and DAG kinase (DAGK). Along with Ca^{2+}, PA is an emerging messenger and either directly or indirectly induced secondary metabolite production

several cellular processes. Ca^{2+} and Ca^{2+}/calmodulin subsequently activate protein phosphatase and Ca^{2+}/calmodulin-dependent protein kinases. It also causes differential activation of transcription factors (TFs) that transfer the elicitor signal to downstream reactions leading to production of secondary metabolites. Furthermore, phospholipases such as phospholipase A (PLA), phospholipase C (PLC), and phospholipase D (PLD) responsible for biosynthesis of other messengers, viz., phosphatidic acid (PA), IP_3, and DAG, are also regulated by Ca^{2+}.

PLA hydrolyzes phosphatidylcholine (PC) to lysophosphatidylcholine (lysoPC) that activates H^+-ATPase in the tonoplast and causes acidification of cytoplasm

(Zhao et al. 2005). Cytoplasmic acidification is necessary for signal transduction resulting to oxidative burst and synthesis of secondary metabolites. PLD causes hydrolysis of PC into choline and PA. PA is a messenger molecule involved in several cellular processes (including secondary metabolite biosynthesis) through activation of protein kinase cascade.

6.4 Conclusions

In recent years, the production of secondary metabolites from plants has gained momentum. Various chemical and biotechnological approaches were used to identify and understand biosynthesis of important secondary metabolites and their modulation. Production of secondary metabolites using hairy roots is an exciting prospect owing to its safe, continuous, and higher production of metabolites. In recent years, various biotechnological interventions to enhance the accumulation of secondary metabolites have been tried by researchers across the world. Inputs from these works suggest that regulation of production of valuable secondary metabolites in hairy roots vis-á-vis normal roots still needs to be properly understood. However, it provides a promising option for production of important secondary metabolites for pharmaceutical, cosmetics, food, textile, rubber, insecticide, and agrochemical industries. In the future, efforts for screening of small molecules and their role in triggering valuable secondary metabolites production in hairy roots need to be addressed.

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Chapter 7
Bioreactor Design and Analysis for Large-Scale Plant Cell and Hairy Root Cultivation

Chitra Srikantan and Smita Srivastava

Abstract Over the years, plant cells and hairy roots have been established as a successful and viable alternative for production of bioactive secondary metabolites and recombinant proteins, replacing the use of whole plants. Bioreactors are used for continuous and consistent in vitro production of these low-volume high-value bioactive/therapeutic molecules from plant cells and hairy roots at large scale. The design and operation of bioreactors for plant cell and hairy root cultivation differs from well-established microbial cultivation due to their size, aggregation, sensitivity to hydrodynamic stress, and viscous nature of the culture broth. The choice of bioreactor and nutrient feeding strategies to overcome substrate limitation and inhibition can be instrumental in enhancing the biomass and product productivity in plant cell and hairy root cultivations at large scale. Hence, this chapter deals briefly with the design and development of bioreactors to achieve maximum productivity in plant cell and hairy root cultivations. The overview of reactor operating parameters considered while designing bioreactors for plant cells and hairy roots are discussed. The chapter also includes application of mathematical modeling to optimize the design of bioreactors and in silico prediction of nutrient feeding strategies during fed-batch and continuous mode of bioreactor cultivation.

Keywords Bioreactors · Plant cell and hairy root cultures · Secondary metabolites · Bioreactor operating parameters · Mathematical modeling

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7.1 Introduction

The last 60 years have seen a steep increase in demand for production of biologically active molecules from cellular sources (Georgiev 2014). Living cells are capable of synthesizing the biomolecules in their most effective and stereo- and regiospecific form, giving an edge over the chemical synthesis (where the products are produced as racemic mixtures). The capacity to produce such biomolecules has been exploited in both prokaryotic (microorganisms) and eukaryotic (yeast, plant, and animal cells) systems. While microorganisms are most efficient in production of primary metabolites (e.g., ethanol, acetic acid, lactic acid, etc.) and less-structurally complex biomolecules, eukaryotic systems are required for production of specialized and chemically complex molecules as they have the cellular mechanism for posttranslational modifications (Huang and McDonald 2012).

Plants are known sources for secondary metabolites which are used for pharmaceuticals, flavors, fragrances, coloring agents, food additives, and agrochemicals (Wang et al. 2017). They are the major sources of medicinally active compounds, which have been used since ancient times and new ones being discovered for growing diseases and ailments. These plants have specific secondary metabolites (low volume, high value) which are produced mostly as defense-related compounds for survival of the plants against insects, pests and predators, etc. (Wink 2015). These are also non-growth associated and are not produced in large amounts, and their yield not only varies in different plants but also in different tissues of the same plant (Atanasov et al. 2015). These specific plants and trees are poached for extraction of very less amount of these biomolecules, making most of these plants endangered (Joe et al. 2015). As most of these biomolecules of interest in plants are defense-related compounds and with the development of plant cell and organ culture as successful alternative, it has led to the development of bioreactors for plant cell and organ cultures. Plant cells can also be engineered for recombinant protein production (they provide adequate posttranslational modification, being a eukaryotic system) and are advantageous over animal cell-based production systems due to lower production costs, easy scalability, and the absence of human pathogens (Kaldis et al. 2013).

Plant in vitro cultures are emerging as alternatives to replace whole plants, as a production platform for various biomolecules due to:

- Shorter production cycles (days or weeks) compared to months/years in natural and transgenic whole plants
- Consistency in product yield and quality and free of contamination
- Safer production platform in a closed bioreactor system, avoiding gene flow in the environment and contamination of the food chains
- Ease of compliance with cGMP (current good manufacturing practices) requirements, product registration process, etc.

As in vitro plant culture has been established as an efficient platform for biologically active molecule production, process optimization and engineering considerations for the factors affecting plant cells are needed for scaling it up to large bioreactor volumes (Fulzele 2000; De Muynck et al. 2010; Lienard et al. 2007; Franconi et al. 2010; Huang and McDonald 2012).

The bioreactors are suitably modified for cultivation of plant cell and hairy root cultures with low shear stress, adequate mixing, support system for organ cultures, and ease in scale-up (Honda et al. 2001). Plant cell suspensions grown in sterile bioreactors having guaranteed batch consistency in biomass and product productivity, and are more likely to proceed successfully and quickly through the regulatory approval system (Fischer et al. 2012). The enzyme taliglucerase alfa (for treatment of type I Gaucher's disease), produced as the drug Elelyso, became the first biological drug approved by the US Food and Drug Administration for human use that is manufactured in a genetically modified carrot cell suspension culture by the company Protalix (Fox 2012; Grabowski et al. 2014).

The goal of a plant cell/tissue-based bioprocess is to achieve high productivity (g product/l/day), high product yield (g product/g substrate), and high product concentration (g product/l) by selecting cell lines, optimum media, and bioreactor operating conditions (Srivastava and Srivastava 2007). This chapter describes the different factors influencing the bioreactor operating strategies and various types of bioreactors for plant cell and hairy root cultivation which can be chosen to commercialize the plant cell-based bioprocess.

7.2 Factors Influencing Plant Cells and Hairy Root Cultivation in Bioreactors

Bioreactors for plant cell and hairy roots have operating conditions similar to microbial bioreactors with modifications/features to aid in efficient growth of plant cell and hairy roots, owing to its characteristics (Table 7.1) (Chattopadhyay et al. 2002a, b, c). Plant cell cultures require aerobic bioreactors with low shear and good mixing. As plant cells are bigger than microbial cultures and form aggregates (cell suspension cultures) or organs (hairy root cultures), this makes the sampling of biomass from the bioreactor at constant intervals difficult. Measurement of the medium conductivity is an indirect way of estimating the biomass growth in the bioreactor (Hahlbrock et al. 1974; Madhusudhan et al. 1995, Maschke et al. 2015). Plant cell culture medium conductivity decreases continuously with the growth of plant cells inside the bioreactor as the growing cells take up the salts from the

medium (Eibl and Eibl 2002). Bioreactor operating parameters to be considered for designing of bioreactor for plant cell and hairy roots are as described briefly.

7.2.1 Aggregation and Adhesion

The plant cells and hairy roots are bigger in size compared to microbes (Table 7.1) and tend to grow in clumps (aggregates) as new cells need support to grow and are unable to separate after cell division. Aggregation is also due to the production of extracellular polysaccharides by the plant cells which help in the cell-cell adhesion (Sims and Bacic 1995). These polysaccharides also store the signaling molecules and other metabolites which are required for cell-to-cell communication. The cell-cell adhesion is also linked with secondary metabolite biosynthesis (Chattopadhyay et al. 2002a, b, c). Aggregated growth results in insufficient oxygen transfer, inefficient mixing, and sedimentation of cells in the bioreactor. Aggregates of large size (2–10 mm) make it difficult for oxygen to reach till the innermost cell and as a result cause death of cells in the core (Doran 1993). Aggregation maybe caused due to adhesion of cells on the bioreactor walls. At higher agitation speed, cells get deposited on the bioreactor walls when the biomass increases (Eibl and Eibl 2009). Bubbles coalescing on the walls are also responsible for cell adhesion to walls. Aggregation cannot be admonished completely as it leads to loss of viability (plant cells are unable to survive as single cell, like microbes) and product formation is related to aggregation of cells (Chattopadhyay et al. 2002a, b, c).

Table 7.1 Characteristics of plant, animal, and microbial cells for bioreactors

Characteristics	Microbial cells	Plant cells	Animal cells
Size	1–10 μm	40–200 μm	10–100 μm
Growth pattern	Individual cells/small aggregates	Small/large aggregates	Support required for growth
Doubling time	Hours (2–4 h)	Days (2–5 days)	Hours (12–20 h)
Shear sensitivity	Low	High	Very high
Product accumulation	Extracellular	Intracellular	Intracellular/extracellular
Posttranslational modifications	No	Yes	Yes
Contamination	Other microbes	Bacterial and fungal	Bacterial, fungal, and viral (human pathogens)
Culture medium components	Complex/synthetic, defined	Synthetic, defined	Complex (animal sources), not defined
Inoculum size	Low (1–2%)	High (5–10%)	High (5–10%)
Aeration rate	High	Low	Very low
Damage by aeration	Very less	Less	High
Cultivation time	Days	Weeks	Weeks
Oxygen demand	Very high	Low	Low

The high-value biologically important compounds produced by plant cells are generally defense related and in response to stress to the cells; which are produced, transported, and communicated among other cells in the aggregate (Gaurav and Roberts 2011). The product content and cell viability decreases when the culture is made to exist as single cells or smaller aggregates. Hence control of cell aggregation is an important parameter while designing large-scale plant cell/tissue culture systems (Chattopadhyay et al. 2005). To prevent the cells from sedimentation, the aggregate size should not exceed 1–2 mm having specific gravity of range 1.002–1.02. When the specific gravity increases above 1.03 and aggregate size is 0.5–1 cm, the plant cells sediment in the bioreactor (Takayama 2014). Aggregation can be reduced by addition of pectinase (enzyme) and polyvinylpyrrolidone with some loss in biomass (as the cells are more viable as aggregates due to adherence and cell-cell communication, separating them causes loss in cell viability), but overall increase in volumetric productivity of the product (Chattopadhyay et al. 2002a, b, c). Reduction in $CaCl_2.2H_2O$ concentration was found to decrease wall adhesion and retain the plant cells in bioreactor (Takayama 1991).

The aggregate sizes were correlated with paclitaxel production by Kolewe et al. (2011) and observed that smaller aggregates contained higher content of paclitaxel compared to bigger clumps. A population balance model was proposed, and the model was simulated to find an optimal breakage rate with minimal biomass loss to increase the paclitaxel concentration in cell suspension cultures of *Taxus* sp. in bioreactors.

Kolewe et al. (2012) developed a population balance equation to predict the aggregate formation in *Taxus* suspension cultures:

$$\frac{\partial n(v,t)}{\partial t} + \frac{\partial [g(v,S')n(v,t)]}{\partial v} + \Gamma(v)n(v,t) = 2(1-b)\int_v^\infty p(v,v')\Gamma(v')n(v',t)dv'$$

where $n(v,t)$ is the continuous number density function, $n(v,t)\mathrm{d}v$ is the number of aggregates in size range v to $v + \mathrm{d}v$ at time t, $g(v, S')$ is the growth rate for aggregates of size v and effective intracellular concentration of total sugar S', $\Gamma(v)$ is the breakage frequency for aggregates of size v, and $p(v,v')$ is the partitioning function describing the distribution of daughter aggregates of size v resulting from the breakage of mother aggregates of size v', assuming each breakage event results in two daughter aggregates b, representing the fraction of biomass which does not partition into daughter particles upon a breakage event.

The above equation was combined with the following equations which accounted for substrate depletion upon cell growth:

$$\frac{dS}{dt} = -\int_0^\infty \frac{g(v,S')}{Y} n(v,t)dv$$

$$\frac{dS'}{dt} = -\alpha(S - S')$$

where S is the total extracellular sugar concentration, S' is the intracellular sugar concentration, Y is a constant yield coefficient, and α is the rate constant for these lumped processes and describes how quickly cells respond to environmental changes. These equations were then used to predict the aggregate sizes to paclitaxel production by the authors.

7.2.2 Mixing and Viscosity

Mixing is required for effective transfer of nutrients and oxygen from liquid and gaseous phase to the cells without biochemical limitations. Mixing is achieved in a bioreactor, with either mechanically moving parts (shafts and impellers in an STR) or by sparging air at a high flow rate (airlift bioreactors). Agitation speed used for plant cell and hairy root cultivations (100–150 rpm) is lesser than microbial cultivation (>200 rpm) and is a major limiting factor for plant cell cultures (Doran 1999). Although plant cells have higher tensile strength in comparison to microbial cells, their large size, rigid cellulosic wall, extensive vacuole, and organ structure make them sensitive to shear stress, restricting the use of high agitation for efficient mixing (Bhojwani and Razdan 1996). Plant cells are, therefore, often grown in modified stirred-tank bioreactors at low agitation speeds due to their shear sensitivity to hydrodynamic stress (Meijer et al. 1993; Bronnenmeier and Märkl 1982).

Plant cell cultures tend to follow non-Newtonian rheological pattern, the change in viscosity, which affects the homogeneity in the culture (Raposo et al. 2010). Plant cells occupy 40–60% of the bioreactor volume under no limiting nutrient condition (Takayama 1991). At these high cell concentrations, rheological properties change as viscosity increases, and the plant cell culture starts to behave like non-Newtonian fluids (Jolicoeur et al. 1992). This behavior of culture affects effective heat and mass transfer in the bioreactor resulting in nonuniform maintenance of parameters (temperature, pH, and oxygen concentration in the bioreactor) and formation of dead pockets (no mixing/no air zone) (Bhojwani and Razdan 1996). Polysaccharide secretion (for aggregation) by the plant cells at the later stages of cultivation period also increases the viscosity rapidly. The apparent viscosity was observed to rise steeply after 10 g/l concentration of biomass (Tanaka 1982). By modifying the impeller design, adequate mixing can be achieved without the loss of viability in biomass.

Doran (1993) reviewed about the relationship between mixing time and circulation time in bioreactors for plant cells. Mixing in an STR can be expressed as a function of circulation time as follows (T_m is the mixing time, and T_c is the circulation time, i.e., time required for liquid to complete one full circulation in the bioreactor):

$$T_m = 4T_c$$

Time taken for mixing in airlift bioreactor is as follows:

$$\text{Internal loop} \quad T_m = 3.5T_c\left(\frac{A_d}{A_r}\right)^{0.5}$$

$$\text{External loop} \quad T_m = 5.2T_c\left(\frac{A_d}{A_r}\right)^{0.5}$$

where A_d is the downcomer cross-sectional area and A_r is the riser cross-sectional area in the airlift bioreactor.

For pneumatically driven bioreactors (bubble column bioreactor, airlift bioreactor, etc.), mixing is achieved by passing sterile air at a high flow rates (4–10 vvm) (Doran 2013). This air flow is responsible for providing oxygen to the cells and at the same time provides mixing due to the higher air flow rate.

7.2.3 Aeration Effects and Shear

Plant cells and hairy root cultivation require oxygen for growth, and if the culture is mixotrophic (uses energy from light and carbon source for growth)/phototrophic (uses only light as a source for energy), they also require CO_2 for photosynthesis (Bhojwani and Razdan 1996). Plant cell and hairy roots require oxygen (1–3 mmol O_2 g^{-1} h^{-1}) lesser than microorganisms (10–100 mmol O_2 g^{-1} h^{-1}) because of their slow metabolism (Bhojwani and Razdan 1996).

Plant cells are not damaged by aeration or air bubbles (unlike mammalian cells), so the bioreactor system for the plant cells is selected based on effective oxygen transfer characteristics and can be effectively grown in pneumatically driven bioreactors (Table 7.2) (Kieran et al. 2000; Takayama 2014). Effect of aeration and agitation is directly seen on the mass transfer coefficient, k_La (oxygen transfer

Table 7.2 Bioreactor configurations for plant cell cultures

Mechanically driven bioreactors	Hydraulically driven bioreactors	Pneumatically driven bioreactors	Immobilized bioreactors	Perfusion bioreactors
Stirred tank	Radial flow bioreactor	Bubble column bioreactor	Fluidized bed bioreactor	Filtration stirred tank bioreactor
Rotating drum	Jet-loop bioreactor	Airlift bioreactor	Trickle bed bioreactor	Spin filter bioreactor
Vibromixer bioreactor	Membrane bioreactor	Balloon-type bubble bioreactor	Mist bioreactor	Filtration bubble column

Adapted from Eibl and Eibl (2002), Eibl and Eibl (2009), Su (1995)

coefficient) values. It is a direct measure of effective oxygenation in the bioreactor to the plant cell cultures (Baldi et al. 2008a). To achieve a balance in good biomass and product yield, the k_La value has to be optimized for the cultures in the bioreactor. Initial k_La value was a key factor in cell suspension cultures of *Panax notoginseng* for production of ginseng saponin and polysaccharides in a 3 l STR with centrifugal impeller. At a k_La value of 30.2 h^{-1}, highest productivity of ginseng saponin, polysaccharide, and biomass dry weight (DW) was obtained. Increase in k_La increased the biomass yield, but caused a decrease in the ginseng saponin and polysaccharide yield (Zhang and Zhong 2004).

The oxygen transfer rate in a bioreactor can be estimated as:

$$OTR = k_La(C^* - C_L)$$

where OTR is oxygen transfer rate, kg m^{-3} s^{-1}; k_L is liquid-film mass transfer coefficient, m s^{-1}; a is interfacial area per unit volume of unaerated liquid, m^{-1}; C* is equilibrium concentration of oxygen in the liquid, kg m^{-3}; and C_L is actual oxygen concentration in the liquid, kg m^{-3}.

Higher air flow rates in bubble column/airlift bioreactor can lead to foaming in the bioreactor, which affects oxygen transfer, reduces homogeneity of culture, and reduces biomass (as cells carried by the foam bubbles settle on the walls). Foaming was reduced successfully by modifying a bubble column bioreactor to a balloon type (Paek et al. 2005). Addition of antifoam is effective, but frequent and higher use reduces the oxygen transfer efficiency (Kawase and Moo-Young 1990).

Aeration and agitation in the mechanically driven bioreactor also cause hydrodynamic stress to the plant cell cultures. The cells experience the stress and shear due to their bigger size, thick cell wall, and large vacuoles (Chattopadhyay et al. 2005). Impact of shear on cells can be observed by cell damage, loss of productivity, and change in cell morphology (Zhong et al. 1994; Kieran et al. 2000). Bioreactors operating without moving parts are favorable for shear-sensitive cultures as only the air bubbles cause the mixing. Shear for STR having flat blade turbine impeller is generalized as:

$\gamma_{av} = kN_i$ Metzner and Otto (1957)

where γ_{av} is the average shear, N_i is the number of impellers, and k is the proportionality constant. Many other empirical equations have been devised and used for calculating shear in a STR.

$\gamma = 4.2N\left(\frac{d_i}{d_T}\right)^{0.3}\frac{d_i}{W}$ Bowen (1986)

$\gamma = k_i\left(\frac{4n}{3n+1}\right)^{n/n-1N}$ Calderbank and Moo-young (1959)

$\gamma = \frac{0.367}{\mu}\left(\frac{P}{V}\left(\frac{V}{V_sN_p}\right)^{0.42}\right)^{0.55}$ Hoffmann et al. (1995)

$\gamma = \left(\frac{p}{V\mu_s}\right)^{0.5}$ Henzler and Kauling (1985)

$$\gamma_{max} = 9.7N\left(\frac{d_i}{d_T}\right)^{0.3}\frac{d_i}{W} \quad \text{Bowen (1986)}$$

$$\gamma_{max} = 3.3N^{1.5}d_i\left(\frac{\rho}{\mu}\right)^{0.5} \quad \text{Robertson and Ulbrecht (1987)}$$

$$\gamma_{max} = N(1+5.3n)^{1/n}\left(\frac{N^{2-n}d_i^2\rho}{K}\right)^{1/(1+n)} \quad \text{Robertson and Ulbrecht (1987)}$$

The empirical correlations used in literature to relate shear with bioreactor design parameters in a bubble column bioreactor are given below:

$$\gamma = \left(\frac{1}{K}g\rho U_g\right)^{1/(n+1)} \quad \text{Sánchez Pérez et al. (2006)}$$

$$\gamma = \left(\frac{\rho\varepsilon}{K}\right)^{\frac{1}{n+1}} \text{where } \varepsilon = \rho U_g \quad \text{Henzler and Kauling (1985)}$$

where γ_{av} is the average shear; N_i is the number of impellers; k is the proportionality constant; a, gas-liquid interfacial area per unit volume of liquid in bubble column (m^{-1}); d_i, diameter of the impeller (m); d_T, diameter of tank (m); H, height of fluid in tank (m); k_i, impeller constant; K, consistency index (Pa s^n); M, torque (N m); n, flow index; N, agitation speed (s^{-1}); N_p, power number; P, power input (W); Re, impeller Reynolds number; V, volume of fluid (m^3); V_s, volume swept by the impeller (m^3); W, width of impeller blade (m); ε, energy input per unit mass (W kg^{-1}); γ, average shear rate (s^{-1}); γ_{max}, maximum shear rate (s^{-1}); μ, viscosity (Pa s); μ_a, apparent viscosity (Pa s); and ρ, density of fluid (kg m^{-3}). Many other empirical equations have been devised and used for calculating shear in a STR.

Varying the aeration rate also enhanced production in STR with setric impeller with DO at 30%, and 176.3 mg/l of lignan were produced in a 5 l bioreactor for cell culture of *Linum album* (Baldi et al. 2008a).

7.2.4 Impellers

To achieve high density in plant cell cultivations, STRs are the most commonly used bioreactors due to their efficient nutrient mixing and aeration. Impellers are used in bioreactor cultivations to sustain mass homogeneity and oxygen dispersion (Doran 2013). The bioreactors used for microorganisms use high agitation speed and flat blade impellers for cultivation (Lawford and Rousseau 1991). Impellers used in microbial cultures have higher power input with great impeller tip speed to prevent formation of dead pockets in the bioreactor (Doran 2010). However, higher power input to impeller causes hydrodynamic shear on the cells. Microbial cultures due to their small size can withstand the high shear and grow, while the bigger sized, shear-sensitive plant cells experience stress under high hydrodynamic shear (Baldi et al. 2008b). The high-powered impellers used in microbial cultures are not suitable for the shear-sensitive plant cell cultures. Impellers for plant cell cultivations should have the following characteristics: (i) to transfer power over a large volume in the bioreactor, (ii) low impeller tip speed, and (iii) large surface area (Eibl and Eibl

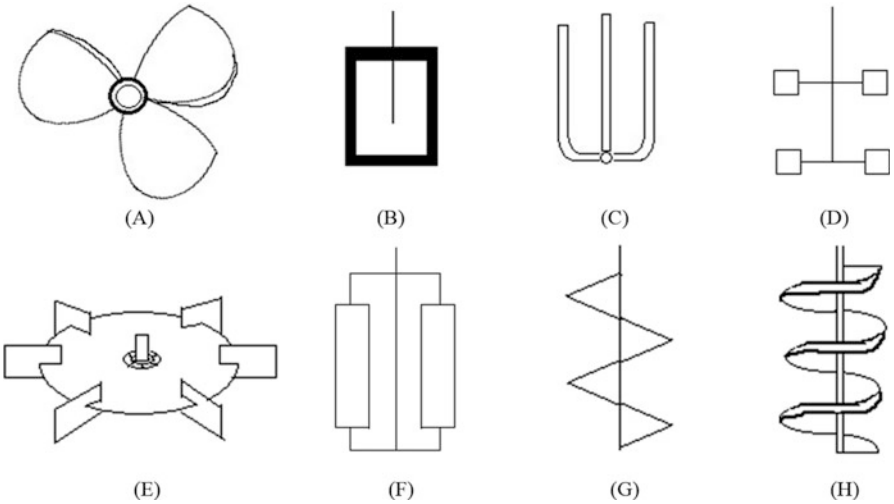

Fig. 7.1 Impellers used for plant cell cultivation: (**a**) marine propeller, (**b**) paddle, (**c**) anchor, (**d**) bladed, (**e**) rushton turbine, (**f**) spin, (**g**) helical, (**h**) helical screw

2002). Doran (1999) has deduced by analyzing various impellers for plant cell cultures that upward-pumping axial-flow turbine design of impellers is efficient in gas transfer and offers low shear to the plant cells.

Low-shear impellers have been developed by modifying an existing impeller used for microbial cell cultures or by designing a completely new one. Various impellers used for plant cell cultivations are shown in Fig. 7.1.

A low-shear helical impeller was designed and used for cell suspension cultures of *Catharanthus roseus* in a 100 l STR resulting in a very high accumulation of biomass (320 g/l of biomass in 16 days from an initial inoculum of 42.6 g/l cells) (Fulzele 2000). A novel low-shear setric impeller was used for cell suspension cultures of *Podophyllum hexandrum* and hairy root cultures of *Azadirachta indica* in STR successfully with no cell death (Chattopadhyay et al. 2002a, b, c; Srivastava and Srivastava 2012a). Cell suspension culture of *Harpagophytum procumbens* was cultivated in 3 l STR for production of anti-inflammatory phenylethanoid glycosides with a low-shear propeller impeller yielding highest biomass accumulation of 18.4 g/l (Georgiev et al. 2012).

7.2.5 Support System

Hairy root and organ cultures require a support system to be attached to while cultivated in a STR as the damage done by the impeller is high on hairy roots and organs than cell suspension cultures. Stainless steel attachment has been provided to retain the roots in a nutrient spray bioreactor and to reduce the liquid holdup by the hairy roots (Srivastava and Srivastava 2012b). The hairy root cultures can also be

separated by polyurethane foam in a STR to prevent shear from the impeller (Steingroewer et al. 2013). Autoclavable nylon mesh and baskets have also been used to separate the roots from the impellers and also to support the roots (Angelini et al. 2011; Gangopadhyay et al. 2011). A plastic nylon mesh was placed around the baffles in a zigzag fashion to provide more surface for the hairy roots of *Brugmansia candida* to grow in a 1.5 l STR for production of tropane alkaloids (Cardillo et al. 2010). Phytoremediation studies using *Brassica napus* hairy roots for removal of 2,4-dichlorophenol was studied in a 3 l STR by covering the hairy roots by an autoclavable nylon mesh covering the rushton turbine impeller (Angelini et al. 2011). *Plumbago indica* hairy roots were used for enhanced production of plumbagin in a 3 l STR, modified by addition of an autoclavable perforated basket 4 cm above the sparger (Gangopadhyay et al. 2011).

7.3 Mass Cultivation of Plant Cells and Hairy Roots in Bioreactors

Bioreactors were developed for cultivation of living cells under controlled conditions for production of biomass/biomolecules when supplied with required nutrients. Each system (microorganisms, plant, and animal) has varying characteristics which are to be considered while designing a bioreactor for production (Table 7.1). Characteristics of plant cells like larger cell size and shape, shear sensitivity, aggregation, slow growth rates, less oxygen requirement, increased mass transfer limitation, and product formation are to be considered while designing the bioreactor (Panda et al. 1989; Bisaria and Panda 1991). The following factors have to be considered while developing bioreactors for plant cells (Scragg 1995; Kieran et al. 1997):

- Homogeneous mixing for efficient nutrient transport, air-bubble dispersion, and optimum shear maintenance
- Aeration optimized for efficient oxygen uptake
- Maintenance of aseptic conditions for longer time (days/weeks)
- Light supply for phototrophic and mixotrophic cultures
- Control of physical parameters like temperature, pH, nutrients, and cell aggregate size
- Efficient mass transfers as the rheological characteristics tend to follow non-Newtonian pattern at high density.

7.3.1 Classification of Bioreactors

Since the demand for biotechnological products increased due to its low cost and high specificity, bioreactor technology has also emerged as a most sought after field for large-scale production of these products (Doran 2013). Various configurations of bioreactors have been developed to assist the biological system with efficient growth

and better product yield (Sharma and Shahzad 2013). For the cultivation of organized plant structures like hairy roots, somatic embryos, and micropropagation of plantlets, the bioreactors are modified (e.g., addition of mist spray, temporary immersion, mesh/basket) (Paek et al. 2005; Srivastava and Srivastava 2012a). Based on the energy input, plant cell bioreactors are operated as mechanically driven, hydraulically driven, and pneumatically driven bioreactors (Eibl and Eibl 2009). Few other configurations include the bed bioreactors and perfusion bioreactors (Table 7.2).

7.3.1.1 Mechanically Driven Bioreactors

Mechanically driven bioreactors (Fig. 7.2) use moving parts (impellers) inside the bioreactors which help in effective mixing and oxygen transfer. These bioreactors provide better control of temperature, pH, dissolved oxygen, and dissolved nutrients compared to other types of bioreactors (Choi et al. 2000). STR is the most used bioreactor (around 90%) in industries as its design, scale-up, and operation are well established. Though plant cells are sensitive to hydrodynamic stress due to powerful mixing, STR with modified impellers and low agitation speed have been successfully used for plant cell cultures to enhance biomass and product productivity (Fulzele 2000; Sharma and Shahzad 2013).

Rotating drum bioreactors vary in their oxygen supply mechanism and use a rotating vessel. Air is sent through the headspace of the bioreactor compared to sending it through the liquid medium in STR (Mitchell et al. 2006). The bioreactor is fitted with baffles in addition to impellers which can enhance the mixing process (Chattopadhyay et al. 2002a, b, c; Mitchell et al. 2006). Compared to other types of bioreactors, surface area to volume ratios are significantly higher in rotary drum bioreactors (Paek et al. 2005). The moving mechanical parts consist of rollers inside the bioreactor vessel, parallel to the rotating surface of the bioreactor vessel, which cause less shear stress to the plant cells. This bioreactor, owing to its low-shear, high-oxygen transfer characteristic, was suitable for high density and highly viscous cell suspension cultures of *C. roseus* (Tanaka et al. 1983). Kondo et al. (1989) were able

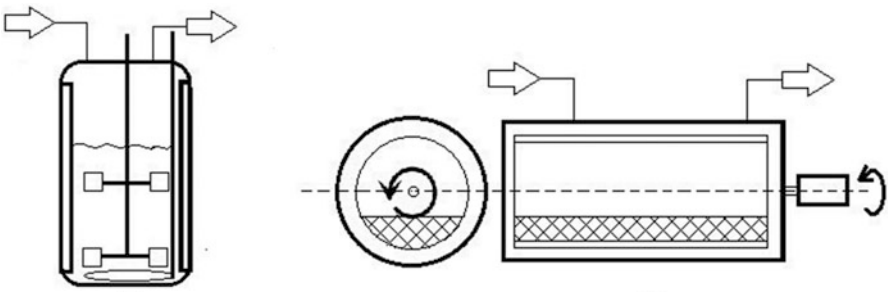

Fig. 7.2 Mechanically driven bioreactors: (**a**) stirred tank bioreactor, (**b**) rotating drum bioreactor

to achieve a maximum growth rate of 0.61 gl^{-1} d^{-1} after 30 days of cultivation of hairy roots of carrot in a glass vessel-based rotating drum bioreactor. The rotating drum bioreactors are not suitable for all types of plant cell/organ cultures and are difficult to scale up to higher volumes due to their vertical design and rotation. It also consumes much higher power than other bioreactors, due to which its use has reduced over the course of years (Sambamurthy and Kar 2006).

7.3.1.2 Pneumatically Driven Bioreactors

Pneumatically driven bioreactors (Fig. 7.3) use pressurized gas through a distributor (like nozzles, perforated plates, diffuser rings, injectors, etc.) to aid in mixing and aeration (Paek et al. 2005). Variation is observed for fluid mixing and dynamics in these bioreactors due to density differences between viscous liquid medium, bubble size and gas holdup (Eibl and Eibl 2009).

The design and operation are optimized for efficient gas holdup, which is the main criterion for designing and using pneumatically driven bioreactors. Gas holdup helps in understanding both mixing and mass transfer in these bioreactors (Takayama and Akita 1998). Variations in biomass, viscosity, and surface tension lead to foaming, floatation, and coalescence in the pneumatically driven bioreactors (Eibl and Eibl 2002).

Bubble column bioreactor has a simple design with a bioreactor vessel, gas sparger, and no moving parts (Kim et al. 2001). Gas sparging provides the necessary mixing and oxygen transfer to the plant cell/organs (Georgiev et al. 2012). The capital cost is lesser and can maintain better aseptic conditions than STR (Doran

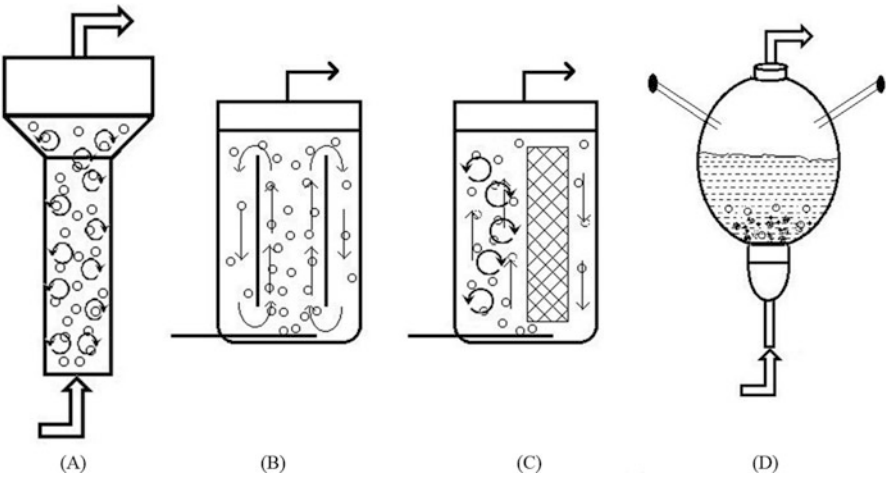

Fig. 7.3 Pneumatically driven bioreactors: (**a**) bubble column bioreactor, (**b**) airlift bioreactor (inner loop), (**c**) airlift bioreactor (outer loop), (**d**) balloon-type bubble bioreactor

2013). Airlift bioreactor is a modification of bubble column bioreactor with an addition of draught tube which aids in better mixing (Doran 2013). The flow gets divided in riser and downcomer in the draught tube, the density difference of which causes better mixing (Chattopadhyay et al. 2002a, b, c). Internal loop airlift bioreactors have the draught tube inside the system where the culture medium rises and falls inside. In an external loop airlift bioreactor, the downcomer is physically separated as an attachment to the main bioreactor vessel (Doran 2013). Mixing is achieved better in external loop bioreactors as the raiser and downcomer are separated physically, but the power consumed is more than the internal loop bioreactor. Production of betalain, a natural food dye and antioxidant from hairy roots of *Beta vulgaris*, was found to be 2.6 times higher in a bubble column bioreactor than produced in a STR. Additionally the doubling time of the hairy root cultures was also lower in bubble column bioreactor compared to STR (Georgiev et al. 2012). Balloon-type bubble bioreactors are a modification of bubble column bioreactor to reduce foaming and cell wall growth observed in bubble column bioreactors. Unlike bubble column bioreactor (where the diameter of vessel and top of the bioreactor are same), the sparger opens up to a balloon-type vessel (which reduces the foaming and cell wall growth) where the plant cell cultures are grown (Paek et al. 2005). These have been extensively used for large-scale plant micropropagation in a bioreactor (Paek et al. 2005; Cui et al. 2014).

7.3.1.3 Immobilized Bioreactors

Immobilized bed bioreactors (Fig. 7.4) are designed for the use of immobilized plant cells or organ cultures (like hairy roots). They are designed for passage of continuous or intermittent fluid flow which is responsible for transfer of nutrients and oxygen to the cells (Eibl and Eibl 2002). The bed is filled with immobilized particles and the fluid with nutrients and gas flows from the top of the bioreactor. These bioreactors face channeling problem. Channeling is a phenomenon when the fluid does not spend the designed residence time in the bioreactor but escapes through the channel formed between the particles (Shuler et al. 1986). This results in insufficient nutrient transfer and failure of the process. Channeling should be reduced to the minimum by efficient packing or sending the fluid at a very less flow rate to ensure it coats all the particles (Doran 2013).

Trickle bed bioreactors are the most used packed bed bioreactor for plant cells. Headspace of the column is integrated with various nozzles which spray nutrient solution on top of the packed cells, and air is introduced from base for aeration. When the nutrient is sprayed as a mist from the injector or ultrasonic nozzles in the headspace, trickle bed bioreactor gets modified to mist bioreactor. Mist bioreactors were developed to overcome the mass transfer limitations in growing organ cultures in submerged bioreactors (cells suspended in liquid medium and air passed through) (Eibl and Eibl 2008). Submergence increases the hyperhydricity of these cultures

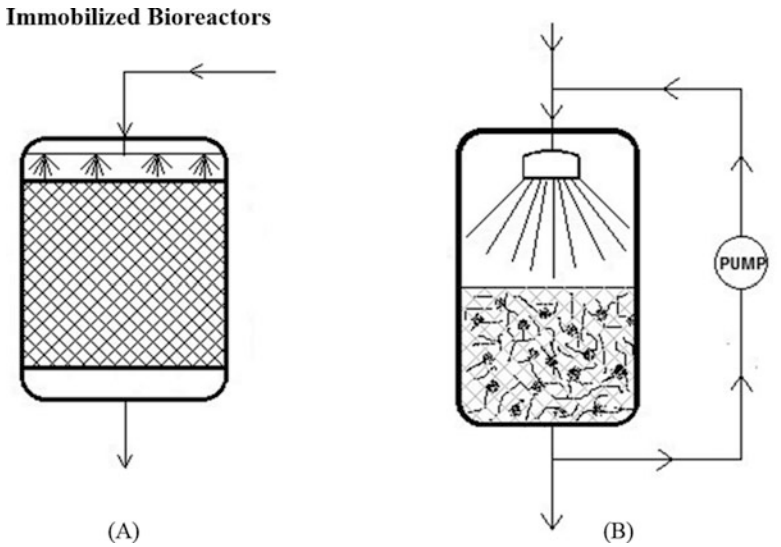

Fig. 7.4 Immobilized bioreactors: (**a**) trickle bed bioreactor, (**b**) mist bioreactor

(hydricity is the amount of moisture stored inside the cell). Rather than supplying oxygen (gas phase) via medium (liquid medium), mist bioreactors expose these organ cultures to continuous gas phase, and the nutrient medium is sprayed as a mist inside the bioreactor. Higher biomass (9.8 g/l) of *Azadirachta indica* hairy roots was obtained in a nutrient mist bioreactor for the production of biopesticide azadirachtin (volumetric productivity of 1.09 mg/l per day) compared to STR (no growth) and nutrient spray bioreactor (4.8 g/l biomass) (Srivastava and Srivastava 2012b).Similarly, a nutrient mist bioreactor was found to be better for production of mouse interleukin-12 (mIL-12) from transgenic tobacco hairy root line (5.3 μg/g fresh weight (FW) mIL-12), which was 49.5% more than the production in airlift bioreactor (Liu et al. 2009).

7.3.1.4 Perfusion Bioreactors

Perfusion bioreactors (Fig. 7.5) are used when there is a need to separate the cells from the medium continuously. It is used for continuous mode of production where the product is extracellular and leaches out in the medium. A porous membrane (pore size <50 μm) is used to segregate the cells from liquid medium. It can also be used for cultivation of immobilized cells which have to be retained inside the bioreactor (pore size is chosen based on the aggregate/organ size). Perfusion bioreactors are used when a certain nutrient or product (which is harmful for the cells) has to be removed continuously from the bioreactor. Membranes are incorporated in the

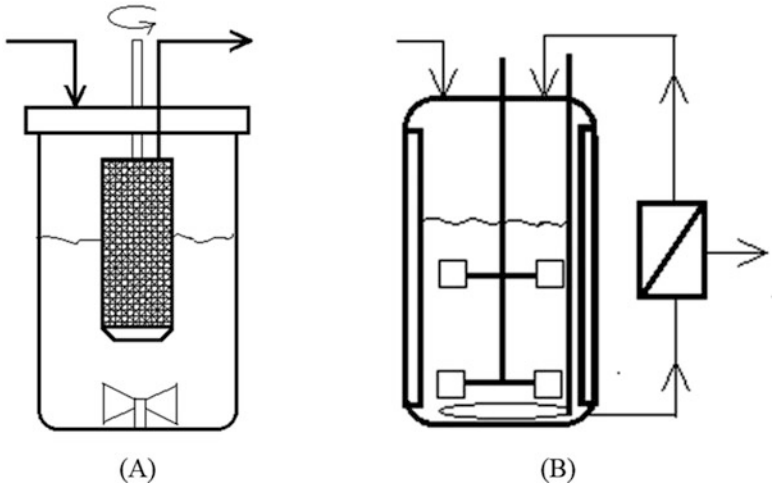

Fig. 7.5 Perfusion bioreactors: (**a**) spin filter bioreactor, (**b**) filtration stirred tank bioreactor

bioreactor where they can be inside the bioreactor, outside, or with medium recycle. The main advantage of using membrane is to prevent the deactivation of the immobilized particle by causing any shear stress and retaining the cells/particles.

Continuous cell/medium separation is a difficulty in perfusion culture. Continuous centrifugation or in situ filtration by membrane or steel mesh can lead to filter clogging when cell density is high (Kawahara et al. 1994). Gravitational sedimentation is considered the most effective way to separate cells from medium in the perfusion culture of plant cells. Su and Arias (2003) obtained complete cell retention and packed cell volume (PCV) of 60% by using a perfusion bioreactor based on cell sedimentation. Su et al. (1996) also reached maximum cell retention efficiency of 100 percent using an airlift bioreactor, which incorporated a cell sedimentation zone delimited by a rectangular baffle in the lower downcomer.

Spin filter bioreactors have a filter separating the medium and cells, which is coupled to a magnet and stirring plate. The filter also acts as an agitator and imparts low shear. The cells can be retained in such bioreactors for longer duration and is best suited for continuous culture of plant cells. However, such bioreactors with built-in cell-settling devices tend to have numerous cells accumulating at the bottom of the reaction tank, causing difficulties with liquid mixing and mass transfer. De Dobbeleer et al. (2006) developed a perfusion STR with four sedimentation columns fixed vertically on the lid of the reaction tank, but failed to find a suitable position for the gas sparger they used. Combining a high perfusion rate with high cell concentration for perfusion bioreactors with built-in cell-settling devices is thus highly challenging. Wang et al. (2010) grew suspension culture of *Glycyrrhiza inflata* in an STR with continuous filtration by gravimetric settling. This was done to remove the spent medium containing certain metabolites which are toxic for cell growth.

7.3.2 Cultivation Strategies

Plant cell systems can be grown based on product accumulation and cell growth. There are three relations for cell growth and product accumulation as follows:

- Growth associated
- Non-growth associated
- Mixed growth associated

When the biomass accumulation is directly proportional to the production of bioactive molecule, the product formation is growth associated. The product accumulates during the exponential growth phase of cells and stops when the cells enter the stationary growth phase (e.g., primary metabolites from microorganisms). In non-growth-associated product formation, the bioactive molecule gets accumulated in the stationary phase of the cell growth. The product of interest is generally a defense molecule produced by the cells, which are produced after cells have reached their maximum growth (e.g., secondary metabolites from microorganisms and plants). When the product is accumulated in the cells during both exponential and stationary phase, it is mixed growth associated (e.g., biomolecules from plant cell/organ cultures in flasks or bioreactor level) (Luedeking and Piret 1959). Based on the relation of cells and product, one of the following cultivation modes can be used.

7.3.2.1 Batch Cultivation

It is a mode of bioreactor operation in which there is no new addition to the system after initial inoculation of cells in the culture medium. It is a closed system where once the fixed volume of medium is inoculated with live cells; it is operated until a certain period of time determined by the shake flask kinetics (Doran 2013). The environment is dynamic with constant change of nutrient consumption and cell growth. The cells follow a sigmoidal pattern of growth. Its best suited for system where there is no substrate or product inhibition. It is suitable for any type of growth-product relation (Eibl and Eibl 2009). Scale-up of bioreactors is easy when the plant cell cultures are cultivated as batch. Batch culture data can be used for modeling the system to further configurations.

7.3.2.2 Fed-Batch Cultivation

It is variation of batch system, where the one or more nutrients are added slowly over a period of time, as high concentration will inhibit either growth or product formation. It is also suited for growth-associated product, where the cells have to be maintained at exponential phase. Production of mono-glucosylated stilbene from

cell suspension cultures of *Vitis vinifera* increased when the medium was replenished (fed) in the 1 l bioreactor after 14 days of growth and harvested after the next 14 days. Stilbene production increased from 0.63 μg/g FW in batch cultivation to 6 μg/g FW in fed-batch cultivation (Ferri et al. 2011). The production of recombinant human alpha-1-antitrypsin (rAAT) in semicontinuous batch mode from transgenic *Nicotiana tabacum* cells in a 2 l STR was 25-fold (603 μg/l) over batch culture (Huang et al. 2001).

7.3.2.3 Continuous Cultivation

It's an open system, where there is continuous exchange of medium. Fresh medium is added continuously, and the same volume is removed from it at the same time. Its main drawback is the maintenance of aseptic conditions in bioreactor for long term (Doran 2013). Contamination may cease every process initiated. Repeated batch (draw and fill) mode can be used for retaining the cells for a longer duration and increasing productivity.

Cultivation mode can be modified to enhance the production. In systems where product is non-growth associated, a two-stage cultivation strategy is used. The cells are grown in one bioreactor and transferred to another bioreactor for product accumulation (Chattopadhyay et al. 2002a, b, c). The medium composition will differ in both, as in the first bioreactor, it will mostly aid rapid growth and the medium in second bioreactor will favor product formation and not much cell growth. Multistage batch culture systems are used for production at large scale.

7.3.3 Bioreactors for Hairy Root Cultivation

Hairy root cultures are the most used organ cultures for development and production of plant-based products. Hairy roots are generated from dicotyledonous plant parts on interaction with gram-negative soil bacterium *Agrobacterium rhizogenes*. They are phenotypically and genotypically very stable. Hairy roots have been found to have stable production of biologically active compounds and have growth rates greater than normal roots. Hairy roots are more sensitive to physical damage (wounding) and shear stress than callus, due to which low-shear impellers and external support (stainless steel plate or styrofoam mesh) are used during cultivation in bioreactors. Excessive branching of hairy roots causes its self-immobilization in the bioreactor and reduces its own biochemical mass transfer of nutrients and oxygen.

Puerarin (an isoflavonoid) production from hairy roots of *Pueraria phaseoloides* was enhanced by 200-fold in a 2.5 l disposable airlift bioreactor with yield of 5570 μg/g DW compared to the yield from shake flask study (Kintzios et al. 2004). The yield of a recombinant protein, human tissue plasminogen activator (t-PA) produced from genetically modified oriental melon (*Cucumis melo*) in 18 l

Table 7.3 Bioreactors used for plant hairy root cultivations

Species	Product	Bioreactor type, volume	Yield	References
Astragalus membranaceus	Astragalosides	Airlift, 2 l	711 mg/l	Ionkava et al. (2010)
Artemisia annua	Artemisinin	Bubble column bioreactor	0.14 μg/g FW	Souret et al. (2003)
		Nutrient mist bioreactor	0.29 μg/g FW	
Panax ginseng	Flavonoids	Airlift balloon-type bioreactor, 5 l	4.8 mg/g FW	Ali et al. (2007)
Silybum marianum	Silymarin	Stirred tank bioreactor, 2.7 l	0.168 mg/g DW	Rahimi et al. (2012)
Stizolobium hassjoo	L-DOPA (3,4-dihydroxyphenylalanine)	Nutrient mist bioreactor, 3 l	0.644 g/l	Huang et al. (2004)
Echinacea purpurea	Cichoric acid	Balloon-type bubble bioreactor, 5 l	26.64 mg/g DW	Jeong et al. (2009)
Salvia sclarea	Diterpenoids	Nutrient sprinkle bioreactor, 10 l	67.5 mg/g DW	Kuźma et al. (2009)
Brugmansia candida	Anisodamine	Stirred tank bioreactor, 1.5 l	10 mg/g DW	Cardillo et al. (2010)
Hypericum perforatum	Hypericin	Balloon-type bubble bioreactor, 3 l	1.4 mg/g DW	Cui et al. (2010)
Harpagophytum procumbens	Iridoid glycosides	Bubble column bioreactor, 3 l		Ludwig-Müller et al. (2008)
Eleutherococcus koreanum	Eleutherosides	Bulb type bubble bioreactor, 3 l	246.41 μg/g DW	Lee and Paek (2012)

bioreactors, was 33 times higher than the production of t-PA in transgenic tobacco plants, suggesting that the mass cultivation of hairy roots in bioreactor is better than production from transgenic plants (Kim et al. 2012). Various bioreactors used for hairy root cultivation are presented in Table 7.3.

7.3.4 Bioreactors for Plant Cell Suspension Cultures

Large-scale bioreactors for production of plant cell-based products have been employed for plant cell suspension cultures (Taxol, shikonin, taliglucerase alfa, etc.). The cells can be homogeneous in suspension for cultivation in a bioreactor with a modified impeller for the hydrodynamic stress. Various configurations of

Table 7.4 Bioreactors used for plant cell suspension cultures

Species	Product	Bioreactor type, volume	Mode of cultivation	Yield	References
Azadirachta indica	Azadirachtin	Stirred tank bioreactor, 3 l	Batch	51 mg/l	Srivastava and Srivastava (2010)
Anchusa officinalis	Acid phosphatase	Perfusion stirred tank bioreactor, 3.3 l	Continuous	300 units/l/day	Su and Arias (2003)
Linum album	Lignan	Stirred tank bioreactor, 5 l	Batch	176.3 mg/l	Baldi et al. (2008a)
Taxus chinensis (cocultivated with *Fusarium mairei*)	Paclitaxel	Stirred tank co-bioreactor, 20 l (divided into two parts of 10 l by membrane)	Batch	25.63 mg/l	Li et al. (2009)
Curcuma zedoaria Roscoe	Essential oil and curcumin	Stirred tank bioreactor, 5 l	Batch	9.69% dry cell weight	Loc et al. (2008)
Commiphora wightii	Guggulsterone	Stirred tank bioreactor, 2 l	Batch	36 µg/l	Mathur and Ramawat (2007)
Nicotiana tabacum	Scopolamine	Stirred tank bioreactor, 5 l	Batch	35.5 mg/l	Moyano et al. (2007)
Pueraria lobata	Puerarin	Stirred tank bioreactor, 5 l	Batch	257 mg/l	Chen and Li (2007)

bioreactors, stirred tank bioreactor (STR), airlift, and bubble column with minor modifications have been successfully used for plant cell suspension cultures. Cell aggregation, foaming, and cell deposition are the common troubles faced with plant cell suspension cultures, which can be overcome with suitable low-shear impeller (less shear but effective in breaking the aggregates) and efficient aeration. Table 7.4 shows different bioreactors used for plant cell suspension cultures.

7.3.5 *Bioreactors for Micropropagation and Embryogenic Suspension Cultures*

Micropropagation is the cloning of a parent plant from any of its tissue to generate large number of progeny in nutrient medium under controlled physical and chemical conditions (Steingroewer et al. 2013). Micropropagation in a bioreactor can generate

Table 7.5 Bioreactors for micropropagation

Species	Bioreactor type, volume	Cultivation period	References
Vaccinium angustifolium	Temporary immersion bioreactor (RITA®)	4 weeks	Debnath (2011)
Vitis vinifera	Airlift, 2 l	6 weeks	Tapia et al. (2009)
Lessertia (Sutherlandia) frutescens	Balloon-type bubble bioreactor, 5 l	6 weeks	Shaik et al. (2010)
Daucus carota	Airlift bioreactor	30 days	Ziv (2010)

Table 7.6 Bioreactors used in embryogenic cultures

Species	Product	Bioreactor type, volume	Yield	References
Eleutherococcus sessiliflorus	Eleutherosides	Balloon-type bubble bioreactor, 3 l	0.1484 mg/g DW	Shohael et al. (2005)
Artemisia judaica	Flavonoids	Bubble column, 0.6 l	60 μg/l	Liu et al. (2004)
		Temporary immersion bioreactor, 0.6 l	100 μg/l	
Eleutherococcus senticosus	Eleutherosides	Balloon-type bubble bioreactor, 3 l	120 μg/g DW	Shohael et al. (2006)
Eleutherococcus senticosus	*E. coli* Entero-toxin B subunit	Airlift bioreactor, 130 l	0.36% TSP	Kang et al. (2006)

up to 10000 progenies with same characteristics in a single batch, which is consistent and efficient than conventional micropropagation, which has led to a less number of progenies in a single batch, and the clones may vary in each batch (Ducos et al. 2009). Some of the biologically active chemicals were found to be produced better in shoot/embryogenic cultivation than in cell suspension/hairy root cultivation. Shikimic acid was produced in a 2 l airlift bioreactor from sandalwood (*Santalum album*) from embryogenic suspension, yielding 0.08% (w/w) shikimic acid in 2–3 weeks (Misra and Dey 2013). Siberian ginseng somatic embryos were produced in a 500 l balloon-type bubble bioreactor (BTBB), where by inoculating 3.5 kg of Siberian ginseng IEDC – induced embryogenic determined cells – 60 kg of mature embryos were harvested after 30 days of culture (Paek et al. 2005). For naturally slow-growing *Stevia rebaudiana* leaf explants, direct shoot bud generation was done in a 1.75 l bubble column bioreactor, and high biomass of about 590 micro cuttings was achieved after 3-week cultivation. The regenerated shoots were then transferred to rooting medium and maintained under controlled conditions (Sreedhar et al. 2008). Sweet pepper (*Capsicum annuum*), a recalcitrant species, was successfully micropropagated in 1 l RITA® airlift bioreactors (Vitropic, France) in 60 days (Grozeva et al. 2009). Various bioreactors used for micropropagation and embryo cultures are in Tables 7.5 and 7.6.

7.4 Application of First Principle-Based Mathematical Modeling for Designing Nutrient Feeding Strategies in Bioreactors

A mathematical model is a real-time representation of the complex bioprocess occurring in the cellular state. The mathematical description of the bioprocess is developed to describe the complex intracellular reactions occurring during the metabolism of the cell which is converting the substrate to products in the fermentation reactions (iitd.vlab.co.in 2013) (Fig. 7.6).

First principle-based mathematical models can be used to simulate different process operating strategies to ensure the major nutrients are at non-limiting and non-inhibitory concentrations in the bioreactor throughout the fermentation process. These optimized cultivation strategies can be implemented in the bioreactor (experimentally) to achieve maximum productivity, thereby reducing the number of experiments required to enhance the efficiency of a particular fermentation process in minimum time without any trial and error fermentation process (Srivastava and Srivastava 2006). Bioprocess kinetic modeling could therefore serve as a biologically logical, yet simple, engineering approach in designing the fresh nutrient feeding strategies in order to obtain high productivity (Kaur et al. 2012).

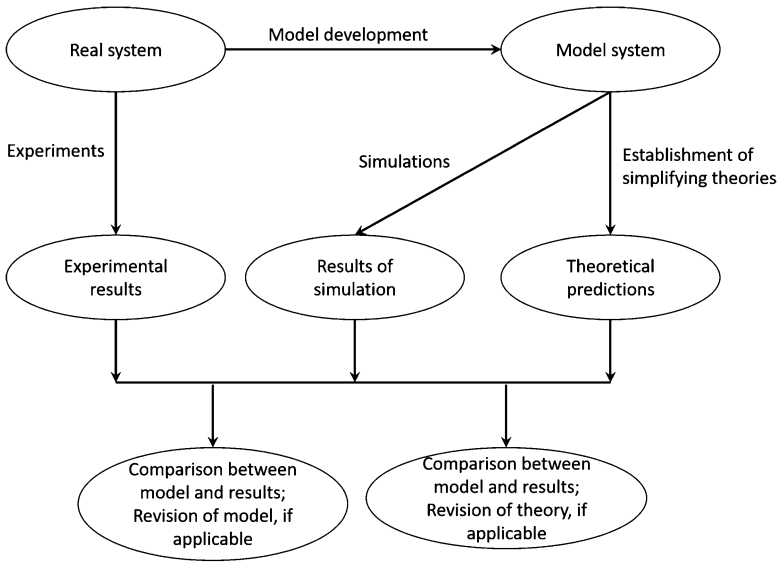

Fig. 7.6 Scheme of mathematical model development and validation (Adapted from Maschke et al. (2015))

Table 7.7 Types of cell kinetic models

Types of cell kinetic models	
Unstructured, distributed	Cells represented by a single component
	Homogeneous system
Unstructured, segregated	Cells represented by a single component
	Heterogeneous system
Structured, distributed	Multiple cell components interact with each other
	Homogeneous system
Structured, segregated	Cells composed of multiple components
	Heterogeneous mixture

Adapted from (Lee 2001)

7.4.1 Types of Models

During the course of growth, the heterogeneous mixture of young and old cells is continuously changing and adapting itself in the medium environment which is also continuously changing physically and chemically. As a result, accurate mathematical modeling of growth kinetics is impossible to achieve. Even with such a realistic model, this approach is usually useless because the model may contain many parameters which are impossible to determine. Therefore, assumptions are made to arrive at simple models which are useful for fermenter design and performance predictions. Various models can be developed based on the assumptions concerning cell components and population as shown in Table 7.7.

The simplest model is the unstructured, distributed model which is based on the following two assumptions:

1. Cells can be represented by a single component, such as biomass during balanced growth (as the biomass doubles, so does other cell components).
2. The population of cellular mass is distributed uniformly throughout the culture. The cell suspension is regarded as a homogeneous solution, and the medium is formulated so that only one component may be limiting the reaction rate. All other components are present at sufficiently high concentrations, so that minor changes do not significantly affect the reaction rate. Bioreactors are also controlled so that environmental parameters such as pH, temperature, and dissolved oxygen concentration are maintained at a constant level (Lee 2001).

For correlating growth rate of cells with substrate concentration in the bioreactor, Monod's model is widely used:

$$\mu = \frac{\mu_m S}{K_S + S}$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate, S is the limiting substrate, and K_S is the Monod's saturation constant based on substrate affinity.

The limiting substrate may also inhibit the cell growth at very high concentration. The effect of inhibition on growth rate can be taken into account by fitting of experimental data into various models demonstrating inhibition kinetics. Few of the growth kinetic models which take into account substrate inhibition are as follows:

$$\mu = \mu_m \left[\frac{K_I}{K_I + S}\right] \quad \text{Prakash and Srivastava (2006)}$$

$$\mu = \mu_m \left[\frac{S}{K_S + S}\right] e^{\frac{-S}{K_I}} \quad \text{Gumel et al. (2014)}$$

$$\mu = \frac{\mu_m}{\left(1 + \frac{K_S}{S}\right)\left(1 + \frac{S}{K_I}\right)} \quad \text{Gumel et al. (2014)}$$

$$\left[\frac{\mu_i}{\mu_m}\right] = \left[1 - \left(\frac{s_i}{s_{mi}}\right)^{ni}\right] \quad \text{Srivastava and Srivastava (2006)}$$

where K_I is the inhibition constant.

Similarly, the product formation can be classified into three types, depending on the relation to the primary metabolism: direct, indirect, or not related (Maschke et al. 2015). In the Luedeking-Piret approach, the product formation rate can be divided into growth and a non-growth-associated component (Luedeking and Piret 1959):

$$\frac{dP}{dt} = \propto \frac{dX}{dt} + \beta X$$

where α and β represent the growth-associated and non-growth-associated product formation constants, respectively (Prakash and Srivastava 2006). Depending on the value of these parameters, product formation kinetics can be demonstrated as growth associated, non-growth associated, or mixed growth associated.

7.4.2 Modeling for Plant Cell and Hairy Root Cultivation

The use of modeling and simulation to study plant growth and developmental processes has increased tremendously over the past few years. By formulating a system of interacting mathematical equations, it becomes feasible for biologists to gain a mechanistic understanding of the complex behavior of biological systems (De Vos et al. 2012).

Mathematical models used for describing hairy root cultivations are highly complex, and yet a lot of potential exists for the identification of more reliable mathematical models (Patra and Srivastava 2015). As it is impossible to determine directly hairy root weight during a run, different techniques have been developed to estimate biomass growth. One of the most used is based on medium conductivity

Table 7.8 Use of mathematical model to enhance product productivity

Species	Product	Culture type	Yield enhancement	References
Azadirachta indica	Azadirachtin	Cell suspension	1.8 fold	Prakash and Srivastava (2006)
Artemisia annua	Artemisinin	Hairy roots	3.7 fold	Patra and Srivastava (2015)
Azadirachta indica	Azadirachtin	Cell suspension	3.8 fold	Prakash and Srivastava (2011)
Catharanthus roseus	Ajmalicine	Hairy roots	2.5 fold	Thakore et al. (2015)
Ajuga reptans	20-hydroxyecdysone	Hairy roots	Threefold	Uozumi et al. (1995)

measurement, which is dependent of the ionic concentrations. As a constant biomass yield from nutrients has been observed, online conductivity measurement has given accurate biomass estimation if there is no nutrient limitation (Mairet et al. 2010)

Few models have been proposed to describe hairy root growth. Different approaches can be discerned (Mairet et al. 2010):

1. Branching model: the increase of biomass is described by several rules concerning the branching kinetics (elongation of branch and formation of new branches). These rules are combined with a population balance approach: the model accounts for the difference between cells in different states.
2. Metabolic model: this approach is based on the metabolic network of the roots. The model uses intracellular nutrients as well as energy shuttles to describe metabolic regulation.
3. Oxygen limited growth kinetic model: this approach is based on fact that oxygen limitation plays a role in growth of hairy roots in shake flasks, considering its branching nature and oxygen limitation observed when scaled up to bioreactor (Palavalli et al. 2012).

These mathematical model-based strategies have been successfully used to enhance the product yield in plant cell and hairy root cultivation (Table 7.8).

7.5 Emerging New Designs of Bioreactors

Bioreactors for plant cell cultures are designed for increasing biomass and productivity. But varying characteristics of plant cells have generated a need to design even better bioreactors. These new bioreactors (Fig. 7.7) are designed to overcome the biochemical limitations, enhance mixing, and reduce the cost and ease of operation.

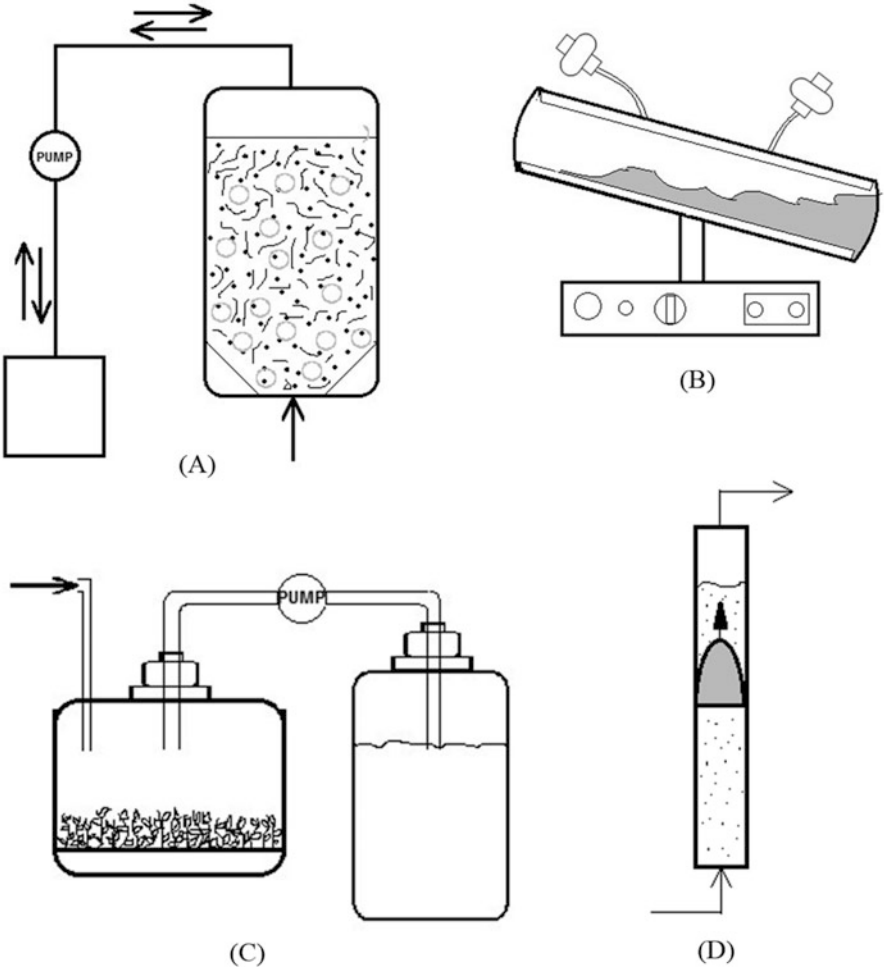

Fig. 7.7 Emerging new design of bioreactors for plant cell and organ cultures: (**a**) Ebb-and-flow regime bioreactor, (**b**) wave and undertow bioreactor, (**c**) temporary immersion bioreactor, (**d**) slug bubble bioreactor

7.5.1 Hydraulically Driven Bioreactors

Hydraulically driven bioreactors use the energy generated by pumping the fluids for mixing and aeration. The pumps ensure circulation of fluid through the loops. Their design is simple and work without moving mechanical parts inside the bioreactor. They cause low shear stress to the cells, and operation is easy. Wave-mixed bioreactor is a hydraulically driven bioreactor designed for shear-sensitive plant cell cultures, which can be grown in a sterile disposable bag made of non-gas permeable plastic. The mixing is provided by the rocking of the bag, to which all

the controllers are attached. They have the advantages of low cost and low shear stress. The mixing, mass, and heat transfer in the wave-mixed bioreactor are characterized by rocking rate, rocking angle, bag type and its geometry, and culture working volume (Huang and McDonald 2012). Oxygen is supplied from the air or gas mixture continuously through headspace aeration. While the wave-mixed bioreactor is rocking, the liquid surface of the medium in the bag is continuously renewed, and bubble-free surface aeration takes place resulting in oxygenation and bulk mixing with less shear stress to cultivated cells (Terrier et al. 2007).

7.5.2 *Immersion Bioreactors*

The temporary immersion bioreactor (TIB), consisting of two vessels (one for holding the plant tissue cultures and another for the liquid medium), was developed to allow cycling of the culture medium by using air pressure or a pump to push the medium from one vessel to the other to immerse the plant tissues and using gravity to withdraw the medium; thus the plant tissues or immobilized plant cells are exposed to the medium intermittently rather than continuously. A separate air or gas mixture is introduced through a sparger to aerate the plant cell or tissue cultures. TIB provides attractive advantages including adequate oxygen transfer and low shear stress to plant tissues (such as hairy root culture) due to the lack of mechanical agitation, although some limitations need to be addressed including vessel size at commercial scale, disposability, and insufficient mixing leading to the accumulation of inhibitory metabolites that can affect cell growth (Ducos et al. 2009). In addition, a modified TIB, consisting of a rigid box placed inside a transparent plastic bag, called a box-in-bag TIB, provides culture headspace between the immersion periods and allows horizontal distribution of biomass for better oxygenation and illumination than that in TIB or other types of immersion bioreactor (Ducos et al. 2009).

7.5.3 *Microbioreactors*

The microbioreactor is designed as a high-throughput platform for cell line selection and evaluation, bioprocess characterization (design space determination), media design and optimization (Betts and Baganz 2006; Diao et al. 2008), and as a scaled-down model to represent the production bioreactor for bioprocess scaling-up purposes (Micheletti et al. 2006). Microbioreactor platforms including microtiter plates (6, 12, 24, 96, with up to 384 wells with a few microliter to milliliter volumes), spin tubes (5–50 ml), shake flasks (25–1000 ml), and parallel miniature stirred and bubble column bioreactor systems (Betts and Baganz 2006) have been implemented for cultivation of many different host cell lines. Feeding, sampling, and harvesting can be automated by using a liquid handling system with an automation control system that can be programmed. Recently the optical sensing systems based on

noninvasive process analytical technology have been used for online measurements of pH, dissolved oxygen, and optical density in a microbioreactor (Zhang et al. 2007). Though microbioreactors are suitable for growing plant cells, there is no literature available on plant cells. Considering its effectiveness on animal cell culture studies, minibioreactor can prove to be successful with plant cells.

7.5.4 Ebb-and-Flow Regime Bioreactor

The ebb-and-flow bioreactor (EFBR) derives its name from the process behavior of its liquid medium which is characterized by its repetitive ebbing and flowing or periodic filling and draining. This bioreactor configuration is a mix between the two bioreactor configurations of the predominantly liquid-phase bioreactor (STR) and the predominantly gas-phase bioreactor (Mist bioreactor). The ebb-and-flow bioreactor has four characteristic operational phases which recur sequentially and intermittently as the liquid medium moves back and forth between the bioreactor vessel and its reservoir. These include the liquid dwell time (LDT), the drain time (DT), the gas dwell time (GDT), and the fill time (FT). The LDT is the phase where the whole reaction volume of the EFBR is completely submerged in liquid and where the bulk of the liquid medium is neither flowing upward nor downward. The GDT is that operational phase where the EFBR reaction volume is predominantly in the gas phase and where mass flow of the bulk liquid medium is not occurring. The operational phases where the bulk flow of the liquid medium takes place are the FT, when the bulk flow direction is upward, and the DT, when the bulk flow direction is downward (Cuello and Yue 2008).

Cuello et al. (2003) were able to successfully cultivate hairy roots of *Hyoscyamus niger* in a 2.5 l ebb-and-flow bioreactor which gave same productivity as in 250 ml Erlenmeyer flasks. It was cultivated in 2.5 l STR and 2.5 l EFBR for scaling up from Erlenmeyer flasks, and EFBR proved to be more efficient. EFBR has been observed to be successful for hairy root cultures, which tend to form clumps and are self-immobilizing.

7.5.5 Slug Bubble Bioreactor

The slug bubble (SB) bioreactor produces artificial slug bubbles and was developed to increase mixing of non-Newtonian fluid in the plant cell bioreactor. Bubble column bioreactors tend to form slug bubbles (based on column diameter and gas velocity) when gas is sparged at high velocity and the bioreactor is filled with fully grown plant cells and highly viscous media. Slug bubble generates significant changes in the hydrodynamic behavior of the system. There exists an onset of upward liquid circulation in the column center and downward liquid circulation

near the column wall. As a result more gas entry takes place in the center, leading to buildup of transverse holdup profile that enhances liquid circulation (Kantarci et al. 2005).

It consists of a vertical flexible plastic cylinder filled with medium up to 80% of its height. Agitation and aeration are achieved through the intermittent generation of large cylindrical single bubbles at the bottom of the system that rise to the top of the cylinder. The bubble size can be controlled by controlling the inlet pressure to form the bubbles. These bubbles are the slug bubbles, and the two-phase flow of gas-liquid formed is known as slug flow (Davies and Taylor 1950; Sousa et al. 2005). Slug bubbles can be described as long bullet-shaped bubbles, which nearly occupy the entire cross section of a pipe. Between the bubble and the pipe walls flows a thin film of liquid; the bubble moves upward at nearly constant speed, while the liquid flows downward as a falling film. The nose of the slug is a very stable region; on the contrary, the rear of the bubble is a region characterized by strong mixing, where all transfer processes are enhanced. Mixing and oxygen transfer are therefore achieved at the same time (Terrier et al. 2007). Terrier et al. (2007) were successful in using 24 l and 64 l of slug bubble bioreactor for production of isoflavones and monoclonal antibodies from suspension cultures of *Glycine max* and *Nicotiana tabacum* BY-2 (bright yellow-2), respectively. Nearly, twofold increase in the isoflavone content (in *G. max* cell line) was observed in slug bubble bioreactor compared to STR with pitched blade turbine impeller.

7.6 Conclusion

Plant cell and hairy root cultivation has proven to be efficient biofactories for production of medicinally/commercially important bioactive metabolites and recombinant protein. Large-scale production of these low-volume high-value compounds has led to modification of the existing bioreactors to suit the requirement of these cultures. Bioreactors have been succesfully developed for commercialization of few plant-based products (Taxol, shikonin, taliglucerase alfa, etc.). The successful use of disposable bag bioreactor vessels as a STR operated at batch mode for production of taliglucerase alfa with recombinant carrot cell suspension cultures is a good example of how far the bioreactor operating process and strategies have come along in the past few years. The overall cost and time required for cleaning of the bioreactor and harvesting of the culture reduces due to the disposable bags, and any chances whatsoever of contamination from the previous batch become negligible. Newer design approaches for better control of the bioreactors and real-time monitoring using artificial intelligence are being used for microbial cultivations. These can be applied to enhance productivity in plant cell bioreactors at a commercial scale. In application of computational tools of modeling and simulation, omic approaches for online monitoring of plant cell constituents also help in evolution of new bioreactors.

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Part II
Progressive Applications

Chapter 8
Hairy Root-Mediated Biotransformation: Recent Advances and Exciting Prospects

Peyman Habibi, Carlos Ricardo Soccol, and Maria Fatima Grossi-de-Sa

Abstract For 35 years, hairy roots have been explored as a promising platform for the production of a variety of compounds in different plant systems. Genetic/biochemical stability, the large-scale production of desired metabolites, low-cost cultural requirements and hormone-independent growth made hairy root as an efficient system for synthesis of new molecules required in pharmaceuticals industry. Moreover, these characteristics make hairy root as an ideal biotransformation system to convert administered organic compounds into useful analogs. Since, the synthesis of many natural products is significantly limited by regioselective and stereospecific properties, which subsequently complicates their chemical synthesis, biotransformation via hairy root systems is an alternative for creation of new therapeutic products because of its ability to perform regioselective and stereospecific reactions. Additionally, the hairy root system contained inherent enzymes, which tackle the occurring of biotransformation reactions, including methylation, oxidation, hydroxylation, glycosylation, reduction, isomerization, and esterification. Hence, the hairy root platform can be considered as an efficient and convenient biotransformation system for the production of new agents with desired physico-chemical properties, sufficient solubility, and low toxicity. The present review recapitulates overall reported progress in hairy root-mediated biotransformation, biotransformation strategies, reaction types involved in hairy root biotransformation, the application of hairy root biotransformation, and strategies involved in end product recovery.

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8.1 Introduction

Plants have evolved a wide range of chemical compounds to protect themselves in response to external invaders such as insect, pests, and microbial pathogens. These valuable compounds can be extracted from different parts of plant such as leaf, shoot, root, and flowers and be investigated for their pharmaceutical properties. In this context, many plant-derived compounds have been used as drugs (codeine, dopamine, paclitaxel, morphine) (Habibi et al. 2017a; Newman and Cragg 2012), food additives and coloring agents (spermidine, anthocyanin, saffron) (Ambati et al. 2014; Delgado-Vargas et al. 2000), pesticides (nicotine, strychnine and azadirachtin) (Maramoroch 1997; Miresmailli and Isman 2014), vitamins, and enzymes. Nearly 300,000 types of secondary metabolites are identified from 1500 plant species (Moon et al. 2015).

Natural products are metabolites that are considered structurally and biologically attractive. Natural compounds constitute more than 2/3 of the antibiotics that are widely used in the pharmaceutical industry (Schmitz et al. 2013). The generation of many natural products is significantly limited by regioselective and stereospecific properties that complicate their chemical synthesis (Wu and Chappell 2008). In this context, biotransformation is an ideal tool to create new therapeutic products because of its ability to perform regioselective and stereospecific reactions (Banerjee et al. 2012). Biotransformation is defined as the specific modification of a given compound to a distinct and structurally similar product by a biological system, which could be microbes, animals, or higher plants. Biotransformation reactions may consist of different events including the generation of stable intermediates that may lack toxic or pharmacological activity. Sometimes, short-lived reactants may also be created. Furthermore, biotransformation reactions can result in chemically stable products with desired pharmacological activities (Fura 2006).

In comparison with semisynthetic routes, which remain costly and have toxic by-products, biotransformation is a well-established technology that provides new compounds with modified molecular structure with high stereo- and regioselectivity and often offers a simpler and easier process, lower costs, and a more eco-friendly result (Liu and Yu 2010). This technology has been used on a wide range of natural products to generate a new library of natural compound analogs. Hence, the emergence of new biotransformed analogs can notably offers new avenues in the area of phytomolecules that are not accessible by chemical semi-synthesis (Srivastava et al. 2013). The capacity of the new generation of modified natural products to improve the toxicity, solubility, and pharmacokinetics of pharmaceutical products could present new opportunities in the pharmaceutical industry. To achieve a successful biotransformation process, some prerequisites should be considered. The system should synthesize the needed enzymes, the substrate or precursors should be

neither toxic to biological system nor useful as a nutrient for system, the compartment cell should take up the substrate easily, and importantly, the rate of product synthesis should be faster than its rate of metabolism (Smetanska 2008). In this context, the system's enzyme biocatalysts may be considered to contribute to the accumulation of totally new pharmaceuticals and may also be applied to transform existing pharmaceuticals by altering their bioactivity spectrum. The biological availability and therapeutic function of drugs, which are demonstrably their most important characteristics, can be enhanced by the establishment of hydrophilic moieties in the substrate and the introduction of protecting groups, respectively. Moreover, the reduction of side effects and boosting the enhancement of drug stability can be achieved by the conversion of parent drug molecules (Pras et al. 1995).

The plant cultured cells have abilities of the regio- and stereoselective hydroxylation, oxido-reduction, hydrogenation, glycosylation, and hydrolysis for various organic compounds as well as microorganisms (Ishihara et al. 2003). However, the generation of natural products in plant systems has encountered some challenges including long doubling time for undifferentiated plant cells as well as the production of a limited range of desired enzymes by plant cells (Giri et al. 2001). These drawbacks can contribute to the complexity and synthesis cost of natural products. Nonetheless, biotransformation using hairy root cultures has tremendous capacity for the synthesis of pharmaceuticals, notwithstanding the disadvantages of plant cells. Scientifically, the application of biotransformation by *Agrobacterium rhizogenes* mediated hairy root is attractive due to its potential to creat new biotransformed compounds in a sustainable manner which unhindered by seasonal variation and pathological restrictions (Veena and Taylor 2007; Banerjee et al. 2012). In hairy root biotransformation system, different exogenous substrates and chemical reactions have been demonstrated. Hence, in this review, we first focus on the substrates and reactions involved in biotransformation, and then summarize biotransformation strategies, as well as its application and finally recapitulate techniques used in boosting the product recovery in biotransformation.

8.2 Hairy Root Biotransformation

Hairy roots are a type of plant tissue that arises at or near wound sites in plants infected by *A. rhizogenes* (Willmitzer et al. 1982). The mechanism of disease begins with the transfer of T-DNA from Ri (i.e., root-inducing) plasmid (200 kb) (White and Nester 1980) into the nuclear genome of the plant. The export of T-DNA from the bacterium to the plant genome activates virulence (*vir*) genes (*rolA*, *rolB*, and *rolC*) that, individually or in combination, induce root formation and the synthesis of secondary metabolites (Habibi et al. 2017a). The functions of *rol* genes were reviewed by Bulgakov (2008) and Bulgakov et al. (2013). In comparison with the whole plant, hairy root culture offers more advantages such as rapid growth, genetic stability, and ease of establishment in petri dishes, Erlenmeyer flasks, or glass

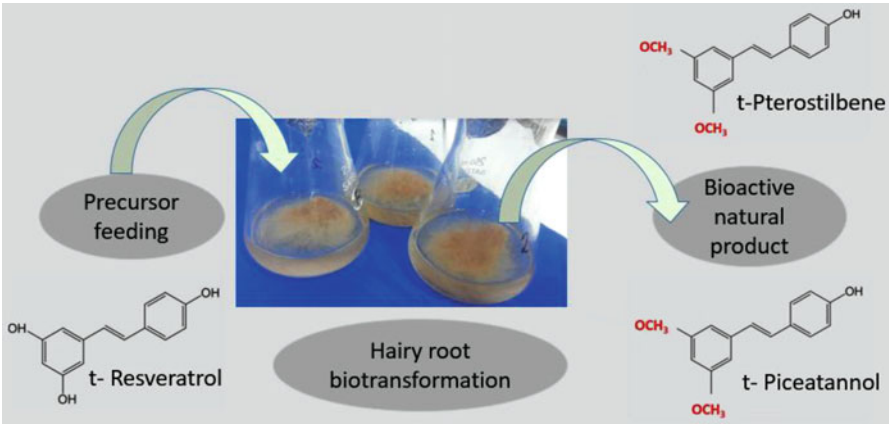

Fig. 8.1 Potential of hairy root biotransformation for the creation of bioactive phytomolecules. In this picture, bioconversion of t-resveratrol into t-piceatannol or t-pterostilbene by hairy root of tobacco (Hidalgo et al. 2017) is demonstrated

bioreactors in growth regulator-free media (Zhao et al. 2013; Habibi et al. 2016). Initially, the use of hairy root as a production platform for secondary metabolites was limited, but over the last 20 years, hairy root system has become a potential biological matrix for various biotechnological applications (Habibi et al. 2017a). An outlook demonstrates that efforts have been given to those strategies that are useful to make this system more practical in term of high productivity at the low cost. However, a sequential analysis of literature indicates that this technique is upgarded to a biotechnology platform where different intra- and interdisciplinary work areas were developed, progressed, and diverged to offer scientific benefits of hairy root-based application such as phytoremediation, molecular farming, biotransformation, etc (Mehrotra et al. 2015).

Moreover, the hairy root system has gained increased attention over other plant cell cultures in terms of biocatalysts due to its genetic and biochemical stability, capacity for multienzyme biosynthesis, and rapid growth (Banerjee et al. 2012). Most of all, various substrate specificities, stereo- and regiospecific modifications, and the continuous secretion of the end product into the culture media for recovery can help to diminish toxicity and feedback inhibition effects, making the hairy root system a potential biotransformation tool for the creation of new and valuable phytomolecules (Fig. 8.1). For instance, the biotransformation of betuligenol (1) into raspberry ketone (2) and betuloside (3) using hairy root culture of *Atropa belladonna* has been reported by Srivastava et al. (2016). Raspberry ketone can be used in flavor and fragrance formulations and in the pharmaceutical and cosmetic sectors and as a dietary supplement, as it can boost the burning of subcutaneous fat. Biotransformation by hairy root cultures has been conceptualized as the structural modification of chemical molecules by the enzymatic activity of root cells (Chandra and Chandra 2011; Banerjee et al. 2012).

8.3 Biotransformation Strategies

The low production of secondary metabolites in plants has led researchers to utilize plant cell and tissue cultures as an alternative system of production. In metabolic investigations, the elucidation of metabolite structures and biosynthetic pathways contributes not only to the improvement of metabolite production but also to the discovery of new secondary metabolites. The hairy root system approach has become a powerful tool to increase the yield of secondray metabolites prodution as well as unravel the biochemical pathways of secondary metabolites. In a biosynthetic pathway, precursors and intermediates are key factors that are formed by enzymatic activation. In some cell suspension cultures, for example, precursors can be a limiting or promoting factor in biosynthetic pathways.

8.3.1 Biotransformation Using Precursor Feeding

Precursor feeding investigation is a suitable strategy to determine limiting factor in secondary metabolite pathways. The feeding of precursors from the terpenoid and tryptophan branches into *Catharanthus roseus* hairy root culture revealed that the terpenoid pathway appears to be rate-limiting (Morgan and Shanks 2000). In this context, Srivastava et al. (2016) demonstrated that the scarcity of near or distant precursors might contribute to the unsatisfactory production of secondary metabolites. The biotransformation of hyoscyamine to scopolamine via hyoscyamine feeding in *Hyoscyamus niger* hairy root revealed that the scopolamine content increased significantly via an enzymatic epoxidation reaction. In this study, differences in the age or the stage of development of hairy root culture were shown to be factors, which could help to increase the yield of scopolamine from hyoscyamine biotransformation. In a precursor feeding investigation, monitoring the degradation of the precursor added to the medium can also help to determine the content of metabolites of interest, as added precursors can be used as nutrients by the hairy roots, resulting in lower levels of the metabolite than expected (Hashimoto and Yamada 1983).

Hairy root biotransformation using precursor feeding can be improved by adding elicitors to culture medium. Feeding experiments in a large-scale culture system with elicitation showed a significant improvement in biotransformation. For instance, the addition of abscisic acid to the medium of *Genista tinctoria* hairy root cultures grown in a basket-bubble bioreactor increased the production of isoliquiritigenin, an isoflavone (Łuczkiewicz and Kokotkiewicz 2005b). *Tropaeolum majus L.* hairy root cultures showed the stimulated biotransformation of two precursor amino acids (phenylalanine and cysteine) and acetylsalicylic acid, leading to increased glucotropaeolin production and suggesting that amino acid precursor availability may limit the stimulation of glucotropaeolin production in *T. majus* hairy root cultures (Wielanek and Urbanek 2006). The results of this study indicated that adding elicitor to the medium could increase the demand for precursors of the defensive

metabolite during elicitation. Another strategy to increase the hairy root biotransformation performance is the overexpression of genes involved in bioconversion of exogenously supplied substrate in other plant systems. In this case, the overexpression of the hyoscyamine-6β-hydroxylase gene in hairy roots of *Nicotiana tabacum* resulted in the bioconversion of exogenously supplied hyoscyamine to the more scopolamine (Häkkinen et al. 2005).

The duration of adding feeding precursor is another important factor in stimulating the biotransformation, and values from 24 h to 25 days have been reported thus far. For example, Wielanek and Urbanek (2006) showed that 24-h treatment of hairy root cultures with Phe and Cys (precursors) and with or without PheP (PAL inhibitor), that is, Phe + Cys or PheP + Phe + Cys, increased the content of glucotropaeolin. This content was further enhanced when the precursors were combined with elicitors, whereupon a stimulating effect on biotransformation was observed on the 3rd day after treatment and maintained until the 6th day of culture. While prolonged exposure to elicitors was reported to be detrimental to hairy root growth, as it decreased biomass and caused blackening or browning (Pitta–Alvarez et al. 2000; Bais et al. 2003), some reports indicated that the combination of precursor feeding and elicitation could be an efficient strategy for avoiding growth inhibition (Qian et al. 2005; Wang et al. 2005; Wielanek and Urbanek 2006).

8.3.2 Biotransformation Using Co-culture Techniques

Co-culture techniques have long been used to investigate cell populations in terms of natural and synthetic interactions. Such systems are of great interest to synthetic biologists for assessing and engineering complex multicellular synthetic systems. Generally, a co-culture is a cultivation setup in which two or more complementary systems (tissues/cells) are cultured together with some degree of contact between them (Goers et al. 2014). The use of co-culture systems to produce valuable secondary metabolites from plant tissue culture has attracted increasing interest, as it is an effective tool for enhancing tissue-specific secondary metabolite production. In a plant co-culture system, the metabolite synthesized by one system translocates to the other to be metabolized as substrate in a further bioconversion process (Subroto et al. 1996).

The culture medium acts as a translocation tool between two systems. The optimization of the culture medium for two systems makes the process complicated, as the medium requirements (such as exogenous hormones) for one system could interfere with the differentiation and metabolism of the other. In this case, the establishment of a transgenic system such as hairy roots can make co-culture feasible, as engineered hairy roots share consistent medium requirements and do not need exogenous regulators. A co-culture of *A. belladonna* shooty teratomas and hairy roots in the same hormone-free medium was established for the conversion of

hyoscyamine to scopolamine (Subroto et al. 1996). The biotransformation results from the root–shoot co-culture studies on *A. belladonna* indicated that in comparison to the individual root and shoot cultures, the co-culture system caused a significant increase in scopolamine production. The reason behind this biotransformation process is that in the root-shoot co-culture technique, the hyoscyamine produced by the hairy roots could be "translocated" via the culture medium along with vascular translocation in the plants, taken up by the shooty teratomas, and finally converted to scopolamine.

Another example of co-culturing shoots and hairy roots has been reported for the production of two secondary metabolites, daidzin and daidzein, in *Genista tinctoria L.* (Łuczkiewicz and Kokotkiewicz 2005a). The result of their investigation showed that in response to exogenous abscisic acid (ABA), hairy roots of *G. tinctoria* released isoliquiritigenin into the medium, from which it was further utilized by shoots to convert this substrate into daidzin and daidzein. Further analysis indicated that using a bioreactor for the separation of tissues with otherwise similar co-culture conditions in shake flask can improve the growth parameters for the large-scale production of secondary metabolites in *G. tinctorial* and also removed difficulties related to the growth measurements and phytochemical analyses in shake flask. In this context, the type of bioreactor has been proved to play a significant role in the level of synthesized compounds (Zobayed et al. 2004). The significant production of podophyllotoxin by the cross-species co-culture of *Linum flavum* hairy roots and *Podophyllum hexandrum* cell suspensions in a dual-bioreactor co-culture system is another relevant example (Lin et al. 2003). Although the total level of podophyllotoxin produced by co-culture in the dual bioreactors was approximately 63% greater than that accumulated by *P. hexandrum* cultured alone, the volumetric efficiency of total podophyllotoxin was not improved by co-culture in the dual-flask and dual-bioreactor systems compared with that in single cultures.

Similar to bioreactor condition as a physical parameter the light condition can also affect biotransformation yield. In this context, Sidwa–Gorycka et al. (2003) investigated the effect of light in a root–shoot system for improving the production of secondary metabolites. Their results strongly supported the idea that umbelliferone synthesized by *Ammi majus* hairy roots was used as a substrate for furanocoumarin metabolism by the *Ruta graveolens* shoots. These data demonstrated the impact of light on secondary metabolite production in co-culture. For example, the production of xanthotoxin was increased under light conditions, while its isomer bergapten showed different results. The co-culture system exhibited a slight increase in the free form of bergapten under light and a decrease in the bound form in darkness. The xanthotoxin:bergapten biotransformation ratio was also affected by light conditions in the co-culture system. This ratio was approximately 1.0 in a cell culture of *R. graveolens* alone and increased to 2.2 and 2.9 in the co-culture system under light and in the dark, respectively, suggesting the effect of light as a physical parameter on the biotransformation rate.

As the production of many secondary metabolites is a defensive reaction in response to microbial contact, the use of growth-promoting microorganisms could be an alternative way to stimulate biotransformation. *Bacillus cereus* is well known as one of the growth-promoting rhizobacterial species that have been shown to boost plant resistance to bacterial and fungal pathogens. As a pioneer in studying a novel and potent tool for improving the accumulation of secondary metabolite from plant hairy root cultures, Wu et al. (2007) proposed a hairy root-*B. cereus* system. Their co-culture system showed an increase in tanshinone synthesis from *Salvia miltiorrhiza* hairy roots. The most probable reason for the increased tanshinone content in the hairy root–bacteria co-culture is the elicitor activity of components released into the medium by *B. cereus*. In this context, compare to chemical elicitors, living components may have different and more complex interactions with the roots, such as protein–protein, gene–gene, and cell–cell interactions. Moreover, it is indicated that the pH drop after bacterial inoculation could be accounted as a stress condition, which might further stimulate secondary metabolite production by hairy roots. Recently, the in vitro co-culture of *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi*, the Columbia root-knot nematode (CRKN), has been reported (Faria et al. 2014). In that study, the co-culture system enhanced the production of volatiles, suggesting that *S. tuberosum* HR/CRKN co-cultures could be considered as a biotechnological tool to investigate the effect of RKN nematotoxic components on the biotransformation capacity of the host plant. However, contamination issue, in which the presence of microbial cells in culture can inhibit the growth of plant tissue or cells, is one of the limiting factors in the use of live microbial cells to stimulate the production of secondary metabolites in plant tissue cultures. Moreover, different medium requirements for live microbial cells could result in the suppression of their growth in plant medium culture demonstrating the need for thoughtful attention regarding their utilization in hairy root culture-based biotransformation. In the latter case, the use of exogenous compounds could produce synergetic effects. For example, (Wu et al. 2007) reported that bacterial growth or survival in hairy root culture relied heavily on casein hydrolysate. Therefore, these systems must be considered more scientifically in terms of media with optimized physical factors for both partners. Additionally, understanding the physiological behavior of the partners and the degree and time of their contact in the medium as well as contact time are further considerations that contribute to improving the biotransformation rate in co-culture systems.

8.3.3 Biotransformation Using Nonspecific/Exogenous Molecules

The substrate specificity and regio-, stereo-, and enantioselectivity make biotransformation systems as a prominent technology for the biosynthesis of secondary metabolites. The regioselective and stereospecific abilities of hairy root cells, the

types of secreted enzymes, and the functional groups in the substrate are among the important keys related to the biotransformation of exogenous substrates. In addition to their characteristically strong and consistent growth/enzymatic backgrounds, stability and low cost, the ability of hairy root cultures to assimilate Ri T-DNA-mediated insertional mutagenesis, as well as adaptability to wide range of substrate, rendered hairy roots as a prominent biotransformation system (Kawauchi et al. 2010; Pandey et al. 2014). The application of hairy root culture for the biotransformation of exogenous substrates into valuable metabolites has been reported (Srivastava et al. 2016). The exogenous substrate for the biotransformation process can be of synthetic or natural origin. Recently, a diversified effort has been focused on generating novel derivatives from nonspecific exogenous artemisinin (Pandey et al. 2015). Accordingly, the modification of the functional groups of the exogenous substrate artemisinin has led to the biosynthesis of novel targets that could not only contribute to combatting drug-resistant malaria but also offer notable tumor necrosis factor (TNF)-lowering ability.

The biotransformation of exogenous substrates has been exploited with and without the transfer of a biosynthetic pathway from one organism to another. Transfer of the human cytochrome P450 hydroxylase 1B1 (*HsCYP1B1*) gene or the *Vitis vinifera* resveratrol O-methyltransferase(*Vv*ROMT) to tobacco hairy roots led to the bioconversion of exogenous *t*-resveratrol into piceatannol or pterostilbene, respectively. These two resveratrol-derived stilbenes are promising metabolites in the treatment of carcinogenic, cardiovascular, and neurodegenerative diseases (Hidalgo et al. 2017). The introduction and expression of lysine decarboxylase (ldc) from the bacterium *Hafnia alvei* to *Nicotiana* hairy roots resulted in a tenfold increase in cadaverine production and a threefold increase in anabasine (Fecker et al. 1993).

Metabolic engineering is under consideration as an alternative for the enhanced production of plant secondary metabolites. The design of expression cassettes harboring suitable and strong promotors for the overexpression of the principle genes involved in a biosynthetic pathway bottleneck has been considered as a prominent strategy to improve the production of secondary metabolites (Habibi et al. 2017b; Capell and Christou 2004). Although there are few reports on the metabolic engineering of hairy roots for the bioconversion of exogenous substrates, Häkkinen et al. (2005) reported scopolamine production by transferring the hyoscyamine-6β-hydroxylase gene from *Hyoscyamus muticus* to tobacco hairy roots. The root cultures did not biosynthesize scopolamine but provided the in vitro capacity to bioconvert exogenous hyoscyamine into its epoxide scopolamine in the culture medium. A further upscale to 5 L was achieved by the development of a turbine-stirred tank reactor for the biotransformation of hyoscyamine into scopolamine by using transgenic hairy roots of tobacco (Moyano et al. 2007). Similarly, the heterologous expression of tryptophan decarboxylase from *C. roseus* in *Peganum harmala* hairy roots resulted in the high production of serotonin from an exogenous substrate (Karuppusamy 2009). Moreover, the expression of the *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) gene from *Pesudomonas fluorescens* by hairy roots of *Beta*

vulgaris led to increase the vanillin level when ferulic acid was added as an exogenous substrate (Singh et al. 2015).

The biotransformation of exogenous substrates with no dependence on metabolic engineering has also been reported for some plant families, such as *Brassicaceae*, *Asteraceae*, *Campanulaceae*, and *Lamiaceae*. The effective bioconversion of butylated hydroxytoluene (BHT) and the consequent production of quinone derivatives such as stilbenequinone by hairy roots of *Asteraceae* and non-*Asteraceae* families clearly showed the unique potential of the enzymatic profile of hairy roots (Banerjee et al. 2012). The hairy roots of *Datura tatula* L. showed striking biotransformation ability toward exogenous p-hydroxybenzyl alcohol, resulting in p-hydroxymethylphenol-β-D-glucoside (gastrodin) production (Peng et al. 2008). Gastrodin is used for its anti-inflammatory, anticonvulsive, analgesic, and antianoxemic properties. It plays a unique role in immunity boosting and blood vessel expansion and is effective as a scavenger of free radicals (Zhou et al. 1979). The production of these molecules by chemical synthesis is very difficult and also expensive and then the production of these compounds by hairy root culture represent the potential of hairy roots to carry out the cost-effective bioconversion of exogenous substrates into valuable products for the pharmaceutical industry.

8.4 Reaction Types

Generally, biotransformation can be categorized into phase I and phase II reactions. Phase I reactions involve the introduction of polar chemical moieties via the creation of polar functional groups or the exchange or modification of existing functional groups by oxidation, hydrolytic, and reduction reactions. This phase can be regulated by enzymes including CYP, FMO, amidase, and esterases. In this context, CYP enzymes are considered key enzymes in drug activation.

8.4.1 Glycosylation

Glycosylation is the most important reaction in the field of glycochemistry and involves one of the most empirically interpreted mechanisms in the science of organic chemistry. Glycosylation is the enzymatic process by which a carbohydrate, i.e., a glycosyl donor, is linked to a hydroxyl or other functional group of another molecule such as a protein, lipid, or other organic molecules. Glycosylation reactions are of interest due to their ability to convert water-insoluble molecules into more water-soluble compounds (Giri et al. 2001). Plant cell cultures or hairy root cultures could be considered as an alternative platform for glycosylation studies since such reactions by microbial transformations or by chemical means are very complicated (Giri et al. 2001). Compared to chemical glycosylation, which requires tedious steps such as the protection and deprotection of the hydroxyl groups of sugar

moieties, one-step enzymatic glycosylation using plant cell cultures is advantageous for glycoside preparation (Shimoda et al. 2002). Moreover, the capability of plant cell cultures, specifically hairy root cultures to glycosylate exogenous substrates and convert it into useful compounds has been successfully reported, as shown in Table 8.1.

Biotransformation involving the glycosylation of gentiobiose and sophorose was performed by ginseng hairy root cultures for the first time in 1990 (Kawaguchi et al. 1990). 3-Epidigitoxigenin beta-D-gentiobioside and digitoxigenin beta-D-sophoroside were obtained as biotransformation products of digitoxigenin by hairy root cultures of ginseng. Digitoxigenin is known as a precursor of cardiac glycosides, which are used as heart ailment remedies to treat congestive heart failure and cardiac arrhythmias by acting on the cellular sodium–potassium ATPase pump (Patel 2016). *P. ginseng* cell and hairy root cultures are being widely investigated as interesting biotransformation tools. The enzymatic biotransformation of phenolics (Furuya et al. 1989; Li et al. 2005; Ushiyama et al. 1989; Ushiyama and Furuya 1989; Yoshikawa et al. 1993), coumarin (Li et al. 2002), and 18-glycyrrhetinic acid (Asada et al. 1993) to their corresponding glycosides by such cultures has been carried out.

8.4.2 Hydroxylation

The regio- and stereoselective hydroxylation of target compounds is an interesting topic regarding the biotransformation of exogenous substrates since it can result in production of valuable substances. Stereospecific hydroxylation is dominant in plant cell culture, as the hydroxylation at C-4 of -terpineol and its acetate provided only trans-isomers (28 and 32, respectively), the hydroxylation of the endocyclic linkage of -terpinyl acetate caused the predominant production of a trans-diol, and the hydroxylation of -terpinyl acetate predominantly resulted in a diol with the hydroxyl group trans to the 1-acetoxyl group. Thus, plant cell cultures have shown the potential to hydroxylate the C–C double bond stereospecifically. Also, regioselective hydroxylation by plant cell cultures has been reported (Suga et al. 1980; Suga et al. 1988; Suga and Hirata 1990). It has been reported that hairy root cultures of *Fragaria x ananassa*, *Lobelia sessilifolia*, *Campanula medium*, and *Lobelia cardinalis* have the ability to hydroxylate trans-cinnamic acid and convert it into p-coumaric acid (Ishimaru et al. 1996).

8.4.3 Reduction

The hairy root culture of several plants have shown an ability to perform biotransformation via reduction reactions. The enantioselective reduction of prochiral ketones into non-racemic chiral secondary alcohols by *Daucus carota* hairy root

Table 8.1 Biotransformation of substrates by different hairy root and plant cell suspension cultures via glycosylation reaction

Plant system	Type of culture	Substrate	Product	References
Ajuga reptans L.	Cell suspension culture	Emodin Aloe–emodin	6-O-β-malonyl galactoside 11-O-β-malonyl galactoside	Nadia et al. (2005)
Brassica napus	Hairy root	Natural ketone 1-(5-acetyl-2-hydroxyphenyl)-3-methylbutan-1-one	4-acetyl-2-(3-methylbutanol)-phenyl-O-β-D-glucopyranoside	Orden et al. (2006)
C. roseus	Cell suspension culture	Cinobufagin	Desacetylcinobufagin 16-O-β-D-glucoside 3-epi-desacetylcinobufagin 16-O-β-D-glucoside 3-oxo-desacetylcinobufagin 16-O-β-D-glucoside Cinobufagin 3-O-β-D-glucoside	Ye et al. (2002)
C. roseus	Cell suspension culture	Capsaicin 8-Ordihydrocapsaicin	Capsaicin 4-O-(6-O-β-D-xylopyranosyl)-β-D-glucopyranoside Capsaicin 4-O-(6-O-α-L-arabinopyranosyl)-β-D-glucopyranoside 8-Nordihydrocapsaicin 4-O-(6-O-β-D-xylopyranosyl)-β-D-glucopyranoside 8-Nordihydrocapsaicin 4-O-(6-O-α-L-arabinopyranosyl)-β-D-glucopyranoside	Shimoda et al. (2007)
C. roseus	Callus suspension culture	2-Hydroxybenzyl alcohols 3-Hydroxybenzyl alcohol	Primeveroside vicianosides	Shimoda et al. (2002)
C. roseus	Callus suspension culture	(RS)-1-phenylethanol	Vicianoside [α-L-arabinopyranosyl-(1–6)-β-D-glucopyranoside], primeveroside [β-D-xylopyranosyl-(1–6)-β-D-glucopyranoside], and gentiobioside [β-D-glucopyranosyl-(1–6)-β-D-glucopyranoside]	Hirata et al. (2001)
Coleus forskohlii	Hairy root	Methanol Ethanol 2-Propanol	Methyl β-D-glucopyranosides, methyl β-D-ribo-hex-3-ulopyranosides Ethyl β-D-glucopyranosides Ethyl β-D-ribo-hex-3-ulopyranosides 2-Propylβ-D-glucopyranosides	Li et al. (2003)
Eucalyptus perriniana	Cell suspension culture	Bisphenol	2,2-bis(4-β-d-Glucopyranosyloxyphenyl)propane	Hamada et al. (2002)

Levisticum officinale	Hairy root	Geraniol	Geraniol glycosides	Nunes et al. (2009)
L. sessilifolia	Hairy root	(−)- Epicatechin Protocatechuic acid	(−)-Epiafzelechin 7-O-β-D-glucopyranoside Rotocatechuic acid 3-O-β-D-glucopyranoside	Yamanaka et al. (1995)
N. tabacum	Cell suspension culture	3-Hydroxycoumarin 4-Hydroxycoumarin 7-Hydroxycoumarin 6,7-Dihydroxycoumarin	3-O-β-D-glucopyranosylcoumarin 4-O-β-D-glucopyranosylcoumarin 7- O-β-D-glucopyranosylcoumarin 6-O-β-D-glucopyranosyl-7-hydroxycoumarin	Hirata et al. (2000)
Panax ginseng	Root and cell cultures	Paeonol	2 -O-β-D-glucopyranoside 2-O-β-D-xylopyranoside 2-O-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside 2-O-β-D-xylopyranosyl(1 → 6)-β-D-glucopyranoside	Li et al. (2005)
P. ginseng	Hairy root	18-β-Glycyrrhetinic acid	30-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl] 18 β-Glycyrrhetinic acid 30-O-(6-O-malonyl-β-D-glucopyranosyl) 18-β-glycyrrhetinic acid 3-O-[6-O-malonyl-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl] 18-β-glycyrrhetinic acid	Asada et al. (1993)
P. ginseng	Hairy root	Digitoxigenin	3-Epidigitoxigenin β-D-gentiobioside Digitoxigenin β-D-sophoroside 3-Epidigitoxigenin β-D-glucoside digitoxigenin β-D-glucoside, periplogenin β-D-glucoside	Kawaguchi et al. (1990)
P. ginseng	Hairy root	(RS)-2-Phenylpropionic acid	(RS)-2-Phenylpropionyl β-D-glycopyranoside (2RS)-2–0-(2-phenylpropionyl)D-glucose (2RS)-(2-phenylpropionyl)6–0-β-D-xylopyranosyl β-D-glycopyranoside	Yoshikawa et al. (1993)
Polygonum multiflorum Thunb	Hairy root	Furannoligularenone	3-Oxo-eremophila 1,7(11)-dien-12,8-olide 3-Oxo-8-hydroxy-eremophila1,7(11)-dien-12,8-olide	Yan et al. (2008)
Rhodiola sachalinensis	Cell suspension culture	Tyrosol	Salidroside	Xu et al. (1998)

cultures has been reported (Caron et al. 2005). Because chiral alcohols are important compounds for the formation of pharmaceuticals, flavoring agents, pesticides, pheromones, fragrances, and advanced materials such as liquid crystals, the stereoselective reduction of prochiral ketones into non-racemic chiral secondary alcohols is essential for the synthesis of many products (Caron et al. 2005). In other research, the biotransformation of benzaldehyde- and acetophenone-type derivatives by hairy roots of *Pharbitis nil* was investigated (Kanho et al. 2005). In the case of 3,4-dihydroxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, and salicylaldehyde, the formyl groups were reduced. Moreover, *P. nil* hairy root cultures exhibited other biotransformative capabilities such as the glucosylation of phenolic and benzylic hydroxyl groups and phenol dimerization by oxidation.

In a similar way, the biotransformation of four 4-hydroxybenzene derivatives by a hairy root culture of *P. multiflorum* was reported. The results showed that *P. multiflorum* hairy roots not only were able to stereoselectively and regioselectively glucosylate phenolic hydroxyl groups of compounds but also reduce 4-hydroxybenzaldehyde to its corresponding alcohol. Additionally, the conversion of prochiral diketones into the corresponding (S)-1 -hydroxy compound via stereo- and regioselective bioreduction by hairy roots of *B. napus* has been demonstrated (Orden et al. 2006). In addition to the enantioselective bioreduction of natural prochiral diketones, *B. napus* hairy roots were able to glycosylate and hydrolyze the acetate derivative 4-(acetyl-2-(3-methylbut-2-enoyl)-phenylacetate). The natural acetophenone derivatives showed anti-inflammatory activity (Favier et al. 1998). Likewise, hairy root cultures of *Raphanus sativa* (Orden et al. 2009), *Cyanotis arachnoidea* (Zhou et al. 1998), and *Rheum palmatum* (Lixin et al. 2002) indicated the potential to carry out bioreduction reactions in biotransformation processes.

8.4.4 Oxidation

The oxidation reactions in biotransformation processes are mainly restricted to aldehyde and alcohol substrates. Previously, an oxidation process using the enzymatic systems of 15 different whole plants has been reported (Andrade et al. 2006). In this study, the authors evaluated the catalytic potential by performing the alcohol oxidation of a racemic mixture to produce ketones or to achieve the enantiomeric enrichment of the alcohol. In comparison to microbe biotransformation, aldehyde oxidation is a rare process in hairy root biotransformation. However, the oxidation of the formyl group of 3,4,5-trimethoxy benzaldehydeto produce 3,4,5-trimethoxy benzoic acid has been reported in *A.belladonna* hairy roots. Moreover, the biotransformation of 4-(p-hydroxyphenyl) butan-2-ol or betuligenol into 4-(p-hydroxyphenyl)-2-butanone or raspberry ketone by oxidative processing hairy root cultures of *A. belladonna*, *C. roseus*, and *N. tabacum* has been reported (Häkkinen et al. 2015; Srivastava et al. 2013). Additionally, hairy root cultures of *Anisodus tanguticus* were used in a biotransformation process to modify the structure of dehydroepiandrosterone (DHEA) via an oxidative reaction into five DHEA-related compounds (Liu et al. 2004).

Oxidative biotransformation reactions of phenol-like compounds also have been performed by hairy root culture. Phenols, as aromatic compounds, are considered a major class of hazardous pollutants because of their carcinogenicity, recalcitrance to degradation, high toxicity, and presence in industrial wastewaters. 2,4-Dichlorophenol has been found to cause lethargy, tremors, and convulsions in mice (Borzelleca et al. 1985). 2,4-dichlorophenol is highly toxic because it is readily captured by the skin, resulting in poisoning (causing hepatic and renal failure, pulmonary edema, and hemolytic anemia) or in teratogenic and carcinogenic effects.

Hairy root cultures of *B. napus* have demonstrated the capability to remove 2,4-dichlorophenol (Agostini et al. 2003). The data indicated that hairy root cultures were able to eliminate 2,4-DCP from aqueous solutions in the presence of H_2O_2 via oxidation catalyzed by the peroxidases of the hairy roots. Recently, a report on whether hairy root cultures of tobacco are useful and efficient for the removal of the halogenated derivative2,4-dichlorophenol was published by (Talano et al. 2010). This study showed the remarkable efficiency of tobacco hairy roots for eliminating high concentrations of 2,4-dichlorophenol by an oxidative process in the presence of the lignin-type products formed during 2,4-dichlorophenol transformation and its compartmentalization in hairy root cell walls.

Similarly, the transformation of phenol and chlorophenols by hairy root cultures of *D. carota*, *Ipomoea batatas*, and *Solanum aviculare* via oxidative processes (de Araujo et al. 2006) indicated the significant role of biotransformation in the process of phytoremediation by hairy root systems. In this context, hairy root approaches could be considered as an alternative way to enhance the removal process as well as to protect the enzymes involved, thereby reducing the costs and improving the process at an industrial scale (Habibi et al. 2017a).

8.4.5 Other Reaction Types

Hairy root cultures have also shown the ability to perform other kinds of biotransformation reactions, such as esterification and acetylation. Biotransformations including the esterification of stearic acid, palmitic acid, myristic acid, and lauric acid with digitoxigenin have been reported (Kawaguchi et al. 1990).

Acetylation is another kind of biotransformation reaction that has been demonstrated in hairy root cultures of *Anethum graveolens* (Faria et al. 2009). In this context, to investigate the influence of the biotransformation capacity on growth and on the production of volatile compounds, two oxygen-containing monoterpene substrates, geraniol and menthol, were added to hairy root cultures of *A. graveolens*. The results showed that the added geraniol was converted into ten new products including the alcohols linalool, citronellol, and a-terpineol; the aldehydes neral and geranial; the esters citronellyl, neryl, and geranyl acetate; and linalool and nerol oxides, while a substantial amount of the added menthol was transformed into menthyl acetate. In the same way, the biotransformation ability of hairy roots of *L. officinale* was investigated by the addition of menthol or geraniol as

a substrate (Nunes et al. 2009). In contrast to the hairy root cultures of *A. graveolens*, no new volatiles were elucidated after the addition of menthol to the hairy roots of *L. officinale*; however, the addition of geraniol resulted in the production of geranyl acetate, α-terpineol, nerol/citronellol/neral, and linalool.

8.5 Biotransformation for Phytoremediation Applications

Phytoremediation is known as an eco-friendly approach for the remediation of contaminated soil and water by using plant systems. Plants are autotrophic systems that exploit sunlight and carbon dioxide as sources of energy and carbon. A successful microbe-based phytoremediation system must meet various conditions, including the ability of microbes with the desired metabolic activity to survive in an environment containing the bioavailable chemical as well as inducers to activate the expression of necessary enzymes. These requirements make this system inappropriate and costly. In terms of bioremediation, plant cell cultures are an alternative system that can be employed for phytoremediation to abolish or decrease the concentration of toxic organic and inorganic pollutants in soil, air, wastewater, groundwater, and biowaste (Habibi et al. 2017a). The process of phytoremediation consists of a range of detoxification phases including transformation (phase I), conjugation (phase II), and compartmentation (phase III). Each phase involves its own reactions and processes to detoxify contaminants. In this context, the biotransformation phase can be considered a critical step in the detoxification process.

However, unlike bacteria and mammals, plant-based systems involved in phytoremediation research are usually impoverished in the catabolic enzymes necessary to perform the full metabolism of recalcitrant organic compounds, potentially resulting in slow and incomplete treatment (Eapen et al. 2007). The incomplete transformation of contaminants in plants causes the release of toxic compounds from plant tissues, potentially leading to the presence of contamination in the food chain (Yoon et al. 2006).

The use of genetically engineered systems with enhanced degradative capabilities, such as hairy root cultures, has made phytoremediation the most promising, cost-effective, and resource-conserving tool for environmental remediation (Oller et al. 2005). Hairy root cultures have been considered for use as a superior model system to investigate phytoremediation processes such as the rhizofiltration, phytostabilization, and phytoextraction of organic and inorganic pollutants because of their biochemical and genetic stability (Majumder and Jha 2012) and their easy maintenance (De Araujo et al. 2002). Physiologically, they follow a prolific root growth template like that of a real root, which is a prerequisite for increasing the effectiveness of phytoremediation processes, and therefore provide reliable and stable biomass throughout the whole year without environmental effects (Doran 2009).

In this context, the phytoremediation of 2,4-dichlorophenol by *B. napus* hairy root cultures in an oxidative reaction has been reported (Agostini et al. 2003).

2,4-Dichlorophenol (2,4-DCP) is a commercially produced substituted phenol used in the manufacturing of germicides, herbicides (especially 2,4-dichlorophenoxyacetic acid), pesticides, germicides, resins, and antiseptics (Buchanan and Nicell 1997). Many efforts have been developed to establish strong methods to eliminate this compound because of its toxicity not only to humans but also to aquatic life, since it has been found to be as much as 50 times more toxic than phenol to some organisms (Edwards and Santillo 1996). Significant removal of 2,4-DCP was accomplished in short reaction times, within the first 5–15 min of incubation with *B. napus* hairy roots. The main mechanism involved in the elimination of this phenolic compound was the peroxidase-catalyzed oxidative coupling of phenolic compounds. One of the interesting advantages of the hairy root system in phytoremediation is the possibility of re-using the radical biomass for large-scale applications, as hairy root cultures have been successfully re-used six times, according to the determination of their peroxidase activity in phytoremediation processes (Agostini et al. 2003).

The phytoremediation of explosive compounds, leading to the amelioration of great environmental risks, is another area of remarkable interest. The phytoremediation of trinitrotoluene (TNT) by plant systems has attracted attention to research on the potential of plants for the biotransformation of this compound. In this regard, knowledge of the plant metabolism of nitroaromatic compounds is one of the most significant topics for understanding natural attenuation processes and phytoremediation applications. Hence, hairy root cultures have contributed to the understanding of the transformation pathways of trinitrotoluene. Based on the structure of trinitrotoluene, two initial transformation processes occur: (1) the reduction of one or more nitro groups, yielding hydroxylamino or amino groups, and (2) the oxidation of either the methyl group or the ring itself. In this context, hairy roots act as a "green-liver" model to provide complementary information about the conjugation of trinitrotoluene monoamine derivatives during the plant metabolism of trinitrotoluene and allow us to determine the contributions of both plants and microbes to the removal of TNT (Bhadra et al. 1999; Wayment et al. 1999). With recent advances in gene and pathway identification, hairy root cultures have provided information on the TNT transformation pathway and identified specific enzymes that are responsible for oxidative transformations. Cytochrome P-450 is one of the most plausible enzyme candidates for the oxidative metabolism of TNT. Banerjee et al. (2002) showed that hairy root cultures of A. *belladonna* can produce cytochrome P-450 and illuminate the basic mechanisms involved in trinitrotoluene degradation by this enzyme. These findings not only introduce the possibility of new genetic and biochemical approaches to study TNT transformation pathways but also provide good information on the toxicity of the final products and their effects on the ecosystem. In the analysis of toxicity, most TNT studies with hairy root cultures have been performed on stationary phase cultures (cultures that are metabolically active but not undergoing growth) (Lauritzen III 1998).

8.6 Product Recovery

Triggering plant tissue culture to produce the highest possible level of secondary metabolites is an advantage for large-scale production. However, efficient methods of product recovery are important for the commercial success of such processes. In this context, enhancing product release or exudation from plant cell tissues into the culture medium could be of substantial interest in terms of recovery and cost. For instance, the accessibility of metabolic enzymes in the biotransformation process as well as the facility of membrane transport could be influenced by targeted transport of the formed product into a second phase, which is introduced into the aqueous medium or two-phase system. This process enables the rapid removal of the product from the plant cells, thereby inhibiting its further interaction with cellular and medium components. This process is beneficial for the production of secondary metabolites that are unstable, toxic, or thermodynamically unfavorable (Woodley et al. 2008). Santamaria et al. (2011) demonstrated that rather than a lack of key biosynthetic enzymes involved in product degradation, the volatility of the substances synthesized as well as enzymatic and nonenzymatic degradation can boost the low production of secondary metabolites. The creation of an artificial compartment for the product can substantially affect the biosynthetic pathways in plant cell culture. In this case, the introduction of a second liquid or solid phase into the aqueous medium could enhance product recovery. In this context, Lee and Shuler (2000) showed that the presence of Amberlite XAD-7 resin increased the yields of ajmalicine and serpentine synthesized by *C. roseus*. Therefore, the removal and sequestering of product in a non-biological location may improve total production and recovery. Avoiding any type of feedback inhibition, initiating the release of secondary metabolites stored in the cells, and inhibition of metabolite degradation by excreted catabolic enzymes and acids are considered advantages of two-stage systems (Smetanska 2008). Immobilization, elicitors, and membrane permeabilization are other suitable techniques that have been reported for exudation processes (Cai et al. 2012).

Immobilization techniques may help to overcome some barriers and limitations related to plant cell suspensions, including low and variable product yields, high susceptibility to shear and slow growth rates (Wilson and Roberts 2012). Immobilization is a fruitful technique in the exudation process because it allows high cell concentrations as well as continuing product recovery (Brodelius 1985). Immobilization is a tool that confines a catalytically active enzyme or cell and inhibits its entry into the mobile phase, which contains the substrate and product (Knorr et al. 1985). Immobilized plant cells are very interesting for use in single- and multi-step biotransformations of precursors to target compounds as well as for the de novo production of secondary metabolites.

Immobilized plant cells can be used as biocatalysts for use in biotransformation. In comparison with the use of freely suspended cells, immobilized plant cells could act as reusable catalysts and allow the easy separation of the formed product from the biomass (Smetanska 2008). Immobilization can exert a dramatic effect on cellular

physiology and secondary product synthesis. Various immobilization techniques have been established, including adsorption, covalent coupling, and entrapment. The most widely used method is the entrapment of cells in a gel or combination of gels that can polymerize surrounding them. The immobilization of plant cells in biotransformation processes by applicable matrixes such as alginate (*Digitalis lanata*) (Alfermann and Petersen 1995), agarose (*C. roseus*) (Asada and Shuler 1989), and the polyurethane foam (*Papaver somniferum*) (DiCosmo and Misawa 1995) has been previously reported to increase production of the secondary metabolites ajmalicine, digoxin, and codeine, respectively, from their substrates or precursors. Simplicity and the maintenance of cell viability are two important characteristics for matrix selection.

Products synthesized by plants are stored in the vacuole compartments. Improving the transfer of compounds from vacuoles to the culture medium could be advantageous in terms of cost and product recovery. Cell permeabilization is based on the formation of pores in plant cell membranes and has been reported to facilitate the transport of substrates into the cell and the secretion of formed compounds from the cell (Brodelius and Pedersen 1993). Basically, maintaining cell viability and promoting the transfer of high amounts of substrates and metabolites into and out of the plant cells are two prominent advantages of cell permeabilization. There are various techniques for cell permeabilization to initiate the release of products from plant cell cultures, including both chemical treatments (e.g., high ionic solution, external pH changes, dimethyl sulfoxide (DMSO), Tween 20, chitosan) and physical treatments (e.g., ultrasonics, ultra-high pressure, high electric field pulses) (Dornenburg and Knorr 1997; Knorr et al. 1985). In the case of chemical treatment, the permeabilization agent must not inhibit cell growth and should be able to increase the pore size of the cell reversibly (Prakash and Srivastava 2011). The application of physical and chemical treatments to cell permeabilization was well reviewed by Cai et al. (2012).

The separation of target molecules from multiple other substances, which is considered as a downstream process, is an integral part of the biosynthesis of a desired product, and the final cost of the produced compound significantly depends on the cost of downstream processing during product recovery. More broadly, the formulation and packing of the desired product are considered as downstream processes (de Carvalho et al. 2017). The improvement of overall productivity has been demonstrated to be a significant goal for established biotechnology. Bioseparation is a critical step in the production of highly valuable biomolecules, as it ensures the standardization of the product. The bioseparation process should achieve the recovery of desired molecules with high purity. Therefore, the establishment of improved separation processes as early as possible is essential for economic success (Basaran and Rodriguez-Cerezo 2008). In a biotransformation process that results in a mixture of the precursor, product, and by-products, the bioseparation of the target molecule should result in high purity of the desired molecule. The method used for separation depends on the nature of the molecule and affects its purity, yield, and most importantly the characterization of its activity. The separation of several compounds has been carried out in small-scale processes

including column salt/solvent-induced precipitation, chromatography, and electrophoresis techniques, while the development of large-scale separation processes involving these units has encountered some problems in terms of low product recovery and high operation costs.

8.7 Conclusion

In comparison with semisynthetic routes, which remain costly and result in toxic by-products, biotransformation is a well-established technology that provides new compounds with modified molecular structures via high stereo- and regioselectivity and often also offers a simpler, easier, and more eco-friendly process with lower costs. In recent years, hairy root systems have gained increasing attention over other plant cell cultures in terms of biocatalysts due to its genetic and biochemical stability, capacity for multienzyme biosynthesis, and rapid growth. Most of all, various substrate specificities, stereo- and regiospecific modifications, and the continuous secretion and recovery of the end product into the culture media can help to diminish toxicity and inhibitory feedback effects, making hairy root systems a potential biotransformation tool for the creation of valuable new phytomolecules. Biotransformation by hairy root cultures has been conceptualized as the structural modification of chemical molecules by the enzymatic activity of root cells. The process of biotransformation by hairy root systems involves diverse strategies such as precursor feeding, co-culture systems, and exogenous molecule supplementation. Due to the ease of using this system as a transgenic system, and given the great progress in functional genomics and epigenetic studies, it is necessary to establish new hairy root lines with the ability to more efficiently express and accumulate enzymes involved in bioconversion as well as in the biotransformation of contaminants in phytoremediation under in vitro or field conditions or in the environment. Moreover, wide ranges of substrates can be subjected to hairy root-based biotransformation to create valuable new compounds for the pharmaceutical industry. Additionally, the optimization of internal factors (upstream elements involved in gene expression) and external factors (growth medium, elicitors, bioreactor design, product recovery, selection of hairy root lines with strong enzymatic pools) could greatly influence hairy root-based biotransformation. This hairy system platform will continue to advance and help scientists to decipher many interesting phenomena in various fields of plant science - biotechnology and their applications.

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Chapter 9
Hairy Roots as Bioreactors for the Production of Biopharmaceuticals

Marcello Donini and Carla Marusic

Abstract Production of heterologous proteins in plant systems has become a valuable biotechnological approach as demonstrated by the recent approval of the first plant-made pharmaceutical for the treatment of the rare genetic disorder known as Gaucher's disease in carrot cell cultures. The main advantages of plants compared to classical expression systems such as bacteria, yeasts or animal cell cultures are the intrinsic safety (e.g. absence of human pathogens), cost-effectiveness and the possibility to manipulate protein post-translational modifications such as glycosylation. Among plant-based production systems, hairy root cultures offer further advantages represented by the possibility of using defined cultivation conditions under contained environment which are attractive for an industrial scale production, the maintenance of product homogeneity and the ease of purification and recovery of the biopharmaceutical product secreted in the culture medium. Several biopharmaceutical products have been successfully produced in hairy root cultures such as vaccine components, enzymes and monoclonal antibodies. In this context, we recently described the production of a tumour-targeting monoclonal antibody with a human-compatible glycosylation pattern in glyco-engineered hairy root cultures. In this chapter we will describe the recent advances in the generation of hairy root cultures expressing heterologous proteins and the strategies adopted to produce biopharmaceuticals, with particular focus on antibodies, and to increase their stability and secretion into the culture medium. Overall, hairy root cultures represent an innovative and promising biotechnological system for the production of plant-made biopharmaceuticals.

Keywords Hairy roots · Molecular farming · N-glycosylation · Antibodies · Vaccines

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9.1 Introduction

Plant tissues and plant cell-based platforms represent a valid system for the production of secondary metabolites but also heterologous proteins (Doran 2000). Since 20 years, the use of plant cells or tissues as production platforms of heterologous proteins is growing thanks to several advantages such as the possibility of producing the molecules in sterile contained conditions and the final quality of recombinant products. In addition, plant cells and tissues allow post-translational modifications, have low production costs and are safer due to the lower risk of contamination by viruses, pathogens and toxins dangerous to humans. Furthermore, the ability of plant cells to propagate indefinitely without the need for sexual reproduction offers a solution to other problems relating to gene segregation and long-term transgene stability in agricultural crops. Alongside, the protein of interest can be secreted into the culture medium easing the downstream product recovery and purification process (Doran 2013). A successful example of the use of plant cells to produce human biopharmaceuticals is represented by the drug ELELYSO® for the treatment of the rare metabolic disorder called Gaucher's disease commercialized by Pfizer, which is composed of the recombinant enzyme α-glucocerebrosidase produced in carrot cell suspensions (Grabowski et al. 2014). Among cultured plant tissues used for the production of heterologous proteins, hairy roots (HRs) have been widely used and will be described in detail in the next paragraphs. Hairy roots are neoplastic tissues resulting upon infection of monocot and eudicot plants with *Agrobacterium rhizogenes* (recently revised as *Rhizobium rhizogenes*) a gram-negative soil bacterium of the family Rhizobiaceae. In nature, the pathogen enters into the plant cell after a wound, introducing into the genome of the infected plant, a T-DNA segment from its root-inducing (Ri) plasmid (White et al. 1985).This T-DNA carries a set of oncogenes that act by disturbing the phytohormone auxin control inducing a new hormonal balance allowing the formation of proliferating roots, called HRs, which emerge at the wounded site. An important feature of these root systems is that they can be easily maintained in culture indefinitely. Hairy roots have been used for a variety of purposes over the last 30 years, ranging from metabolic engineering of bioactive substances to the production of recombinant proteins (Ono and Tian 2011; Srivastava et al. 2016). HR-based production systems offer several advantages such as the possibility to be cultured under contained conditions, fast biomass accumulation, their genetic stability and their ease and rapidity of production (Hu and Du 2006; Mehrotra et al. 2015). Another important peculiarity of HR cultures is the possibility to secrete the heterologous proteins in the culture medium greatly facilitating and decreasing the costs of downstream processes. Among the disadvantages there is the low yield of heterologous protein generally secreted in the culture medium and the challenges of setting up large-scale production in bioreactors. Major advantages and challenges of HRs are reported in Table 9.1. For these reasons, a wide range of different heterologous proteins have been produced in HRs ranging from vaccines to antibodies.

Table 9.1 Advantages/challenges of the production of heterologous proteins in hairy roots

Advantages
The final product can be secreted in the medium and is more homogeneous than a product extracted from plant tissues
Efficient downstream processing when the protein is secreted in the culture medium
Absence of human pathogens
Production in sterile contained bioreactors
Long-term genetic stability compared to cell suspension cultures
Fast growth and large biomass accumulation
Challenges
Low protein yield in the culture medium
Culture scale-up
Plant-type glycosylation of proteins

9.2 Production of Hairy Root Cultures Expressing Heterologous Proteins

Transgenic HR cultures can be generated using two different approaches: (i) by infecting transgenic plants expressing the protein of interest with wild-type *A. rhizogenes* which represents the most 'classical' approach and (ii) by infecting wild-type plants with *A. rhizogenes* transformed with a plant expression vector carrying the gene(s) of interest.

(i) In the case of HRs generated from transgenic plants already expressing the protein of interest, plant leaf discs, after sterilization, are inoculated with a culture of non-transformed *A. rhizogenes.* The leaf discs are then incubated in plates containing antibiotics such as kanamycin for the selection of the transgene and cefotaxime generally used to eradicate any residual contamination by *A. rhizogenes* in the emerging roots. Approximately after 20 days, the first hairy roots emerge each representing a different clonal line, and these are screened for heterologous protein expression. The single clones expressing the protein of interest can be cultured on plates with solid medium or grown in shake flasks in liquid medium. An example of HRs derived from *Nicotiana benthamiana* (a close relative of tobacco), expressing the red fluorescent protein (RFP) obtained in our laboratory, is shown in Fig. 9.1. A disadvantage of this strategy is represented by the fact that it is time-consuming since it requires the generation of transgenic lines expressing the protein of interest. Moreover, the maximum expression levels that can be obtained are generally those of the original transgenic plant.

(ii) An alternative strategy for the generation of HRs is represented by the co-infection of wild-type plant tissues with a mix of recombinant *A. rhizogenes* strains bearing one or more genes of interest. These genes are typically cloned in T-DNA cassettes of standard plant expression binary vectors, using strong constitutive promoters such as the cauliflower mosaic virus 35S promoter (Odell et al. 1985) and viral translational enhancers such as the *omega* (Ω) 5′ leader sequence of tobacco mosaic virus (TMV) (Gallie and Kado 1989). This approach usually

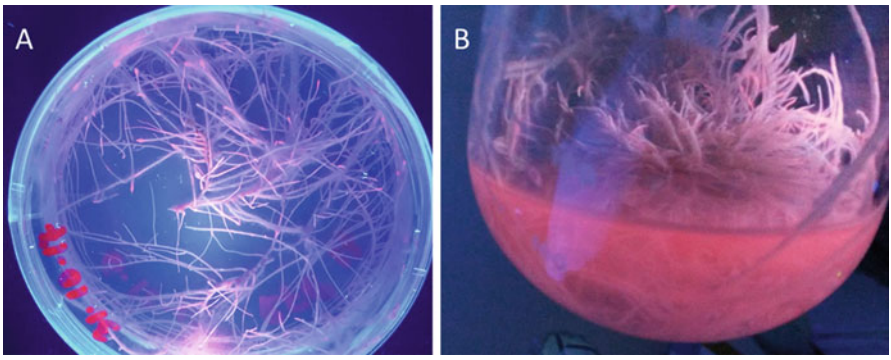

Fig. 9.1 Hairy roots of *N. benthamiana* expressing the red fluorescent protein (RFP). RFP was visualized using a mercury vapour lamp. (**a**) Hairy roots on solid medium; (**b**) hairy roots grown in a shake flask with liquid medium

generates a lower percentage of clones expressing the recombinant protein compared to the 'classical' transformation strategy but is very rapid and suitable for the expression of complex multimeric proteins such as monoclonal antibodies (mAbs) which require the assembly of two heavy chains (HC) and two light chains (LC) to form the functional molecule. For instance, Lonoce and colleagues showed the successful expression of a tumour-targeting mAb in *N. benthamiana* HRs, even if the expression efficiency obtained using the co-transformation strategy was lower compared to that observed in HRs derived from transgenic *N. tabacum* (only 20% of the emerging roots expressed the protein compared to the 60% in *N. tabacum*), although it must be noted that the overall expression levels between the two systems were equivalent (30–40 μg/g of fresh weight) (Lonoce et al. 2016). This result demonstrated that co-infection of leaf discs with a mix of *A. rhizogenes* carrying antibody heavy chain (HC) and light chain (LC) coding sequences can be used for the rapid establishment (approx. 2 month) of stable HR clones expressing functional mAbs. Moreover, a previous study showed that the same multiple gene co-transformation strategies successfully led to the co-expression of β-glucuronidase (GUS) and green fluorescent protein (GFP) in *N. tabacum* HRs (Huang et al. 2013). Several studies demonstrated that transgene expression in HRs is strongly influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA) affecting not only protein expression but also hairy root growth and morphology (Kim and Veena 2007; Huang et al. 2013). This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic HRs expressing heterologous proteins in different plant species (Wongsamuth and Doran 1997; Häkkinen et al. 2014; Lonoce et al. 2016). For this reason, the screening of a large number of clones

generally permits the selection of hairy root lines with high accumulation levels of the heterologous protein.

In addition to the stable integration into the plant cells, the gene of interest can be transiently expressed in HRs using recombinant plant viral vectors. Transient or epichromosomal transformation differs from stable transformation as the exogenous sequence does not integrate into the genome of the plant/tissue and the transgene is not inherited by the progeny. Viral transient expression systems are based on the ability of plant viruses to infect plant tissues and spread from cell to cell. An important advantage is the speed of production, but the main limitations are represented by the possible loss of the foreign gene during viral replication and the size constraints of the gene that can be accommodated by the viral genome. The different strategies published in literature exploit the ability of plant viruses, such as tobacco mosaic virus (TMV), to infect root tissues and spread from cell to cell. For example, a work aimed at determining the feasibility of using transient viral infection of HR cultures for propagating wild-type and recombinant plant viruses was based on TMV (Shadwick and Doran 2007a). Hairy root cultures obtained by infecting seedlings of *Nicotiana tabacum* and *N. benthamiana* with *A. tumefaciens* were first infected with wild-type TMV to evaluate the kinetics of root growth and virus accumulation and the correlation between HR biomass and viral accumulation levels. The results revealed that virus infection did not affect the HR proliferation and biomass increase. Then HRs were infected with TMV-GFP expression vector (TMV-30B). The infectivity of virus was high initially but declined as the culture progressed, and no GFP expression was revealed, probably due to the loss of GFP gene during viral replication as already observed in leaf infection (Shadwick and Doran 2007b). A different strategy was used by Skarjinskaia and colleagues to express GFP and human growth hormone (hGH) in HRs (Skarjinskaia et al. 2008). In this work HR cultures were generated from *N. benthamiana* leaves systemically infected with two TMV 30-derived expression vectors, carrying GFP- or hGH-encoding sequences. The results showed that about 70% of HR clones expressed the heterologous proteins and that the GFP and hGH accumulation levels were maintained stable through 3 years of HR propagation (Skarjinskaia et al. 2008).

To address the potential instability of the plant viral expression vector that may limit the range of their application in HR cultures, a new strategy based on the pBID4 hybrid vector also known as 'launch vector' was described (Musiychuk et al. 2007). The hybrid vector was derived from an *Agrobacterium* binary vector (pBI121) and contained the TMV genome expression cassette, in which the CP gene was replaced with the gene of interest. *N. benthamiana* HR cultures, obtained by infection with recombinant *A. rhizogenes* carrying different launch vectors for several vaccine targets such as the human papilloma virus (HPV) E7 oncogene and a fusion protein containing the *Bacillus anthracis* protective antigen toxin (PA), showed the feasibility of long-term foreign protein production in HRs using this system (Skarjinskaia et al. 2013).

9.3 Enhancing the Production of Heterologous Proteins in Hairy Roots

One of the main advantages of using the HR expression system is the possibility to secrete correctly folded and functional recombinant proteins into the culture medium easing the downstream purification process. Several approaches have been applied to trigger the secretion of the heterologous proteins in the medium such as the use of hormones (auxins) that have been demonstrated to increase heterologous protein accumulation (Häkkinen et al. 2014). Indeed, it was shown that auxins play a role in root development by increasing lateral and adventitious root formation that may provide increased surface area for the production and secretion of recombinant antibodies (Drake et al. 2009). Another interesting observation was made in roots of tobacco plants grown in hydroponic culture medium induced with auxins. Authors identified mRNAs encoding several cell wall remodelling genes, including a subtilisin-like protease and expansins that indicate a possible role of auxins in cell wall remodelling (Madeira et al. 2016). Recently, several studies demonstrated that the addition of the synthetic auxin 1-naphthaleneacetic acid (NAA) into the culture medium greatly increased the accumulation of recombinant antibodies (Lonoce et al. 2016). In addition to the use of auxins, some strategies were aimed to increase protein synthesis by adding potassium nitrate (KNO_3) as a nitrogen source. It was shown that the use of KNO_3 could enhance the accumulation of the mAb M12 antibody in tobacco HRs by almost two times (Häkkinen et al. 2014). Once secreted, heterologous proteins could be attacked by proteases; the addition of protein stabilizers was shown to be a valid strategy to protract the half-life of secreted proteins. The most widely used are polyvinylpyrrolidone (PVP) and bovine gelatin which in some cases are demonstrated to significantly increase the accumulation of monoclonal antibodies in HR medium (Wongsamuth and Doran 1997; Häkkinen et al. 2014). Another approach to enhance heterologous protein expression was the fusion of the E7 oncoprotein of HPV with stabilizing molecules such as the LickM carrier from *Clostridium thermocellum*. This fusion protein antigen was transiently expressed in roots of *N. benthamiana* using the launch vector and successfully evaluated as prophylactic and therapeutic vaccine in mouse models (Massa et al. 2007).

HRs expressing recombinant proteins have been established from numerous species of dicotyledonous plants such as *Brassica rapa*, *Solanum lycopersicum*, *Lotus corniculatus*, *N. tabacum* and *N. benthamiana* showing consistency in target gene expression over an extended period of time (Georgiev et al. 2012). Among the successfully produced heterologous proteins, we can find antibodies (Lonoce et al. 2016), vaccine antigens (Skarjinskaia et al. 2013), the human enzyme alkaline phosphatase (Gaume et al. 2003), the tissue plasminogen activator (t-PA) (Kim et al. 2012) and the murine cytokine interleukin-12 (Liu et al. 2009). In particular, most of them were secreted in the culture medium easing the downstream purification processes. Among these, the highest yields were obtained in *N. tabacum* hairy

Table 9.2 Different approaches used to increase the yield of antibody molecules in hairy root cultures

Medium additive	Expression host	Antibody	Yield of secreted antibody	Culture type	References
NAA 19 mg/L PVP 1.5 g/L KNO_3 14 g/L	Tobacco	Anti-vitronectin tumour-targeting mAb M12	5.9 mg/L	Shake flask/aeration/mixing Medicel bioreactor	Häkkinen et al. (2014)
NAA 19 mg/L PVP 1.5 g/L KNO_3 14 g/L	Tobacco and *N. benthamiana*	Anti-tenascin C tumour-targeting mAb H10	2.70 mg/L and 2.24 mg/L	Shake flask	Lonoce et al. (2016)
PVP 1.5 g/L	Tobacco	Anti-*Streptococcus mutans* mAb Guy's 13	1.4 mg/L	Shake flask	Sharp and Doran (2001a, b)
KNO_3 0.1% PVP 1.0 g/L Gelatin 9.0 g/L	Tobacco	Anti-*Streptococcus mutans* mAb Guy's 13	10.8 mg/L	Shake flask/air-lift bioreactor	Wongsamuth and Doran (1997)
PVP 1.0 g/L Gelatin 5.0 g/L	Tobacco	Catalytic mAb 14D9	625 μg/L	Shake flask	Martínez et al. (2005)

PVP polyvinylpyrrolidone, *NAA* 1-naphtaleneacetic acid, *KNO_3* potassium nitrate

roots expressing the murine mAb Guy's 13 against the cariogenic dental pathogen *Streptococcus mutans* (Wongsamuth and Doran 1997) and the tumour-targeting human IgG M12 (5.9 mg/L) (Häkkinen et al. 2014) (Table 9.2).

9.4 Production of Antibodies in HR Cultures

Monoclonal antibodies (mAbs) are useful tools in medicine, biology and biochemistry because of their binding specificity and stability both in vitro and in vivo. Antibodies generated by hybridoma technology or from phage display libraries are widely used in medical research and disease diagnosis (Geyer et al. 2012), while mammalian cell cultures are still the favoured systems for the production of commercial mAbs, even if the increasing demand and the high costs are encouraging the development of alternative expression platforms. Among alternative expression systems, plants represent ideal bioreactors for the production of antibodies. In fact, plants offer a cost-effective expression platform that can assemble such complex multimeric molecules with high quality (Yusibov et al. 2016). Another advantage of the production of antibodies in plants is represented by the possibility to direct the

protein expression in specific cell compartments or to specific cell storage compartments such as seeds. Both transgenic plants and transient expression systems based on whole plants have proved to be valuable platforms for the production of recombinant immunoglobulins. Nevertheless, expression of antibodies in plants still presents several drawbacks related to the extraction procedures, purification steps, quality of the final product and regulatory issues (Sabalza et al. 2014). Extraction procedures represent an important step, since these can influence the quality of the final product even after purification (Platis et al. 2008). In fact, recombinant protein extraction is often conducted in the presence of unwanted plant proteins and compounds such as proteolytic enzymes, pigments, alkaloids, phenolics, polysaccharides and DNA that can reduce the efficiency of protein extraction and the final quality. Moreover, strict parameters are imposed by regulatory bodies that control the introduction of plant-derived immunoglobulins in the market (Ma et al. 2015). For all these reasons, HRs have several advantages over whole plants, mainly due to the possibility of producing antibodies in sterile contained conditions and secreting the recombinant proteins in the culture medium which greatly facilitates the downstream processes. The first example of a protein of pharmaceutical interest in HRs was reported by Wongsamuth and colleagues in 1997, expressing the anti-*Streptococcus mutans* mAb Guy's 13 (Wongsamuth and Doran 1997). Since then there have been several examples of mAbs expressed in HRs (Table 9.2) and in particular two tumour-targeting antibodies, the anti-tenascin C mAb H10 and the anti-vitronectin mAb M12 (Häkkinen et al. 2014; Lonoce et al. 2016). In the latter work, authors optimized an induction protocol for the cultivation of tobacco HRs secreting the mAb M12 in the culture medium. The addition of a nitrate source (KNO_3), a synthetic auxin (NAA) and a stabilizing agent (PVP) enhanced mAb M12 yield by 30-fold, and about 57% of the antibody produced was secreted in the medium. Characterization of the purified antibody showed that it possessed a typical plant glycosylation pattern, which still represents a major issue for plant-derived antibodies to be used in human therapy. In another approach, the expression of the catalytic murine IgG1 14D9 in *N. tabacum* HR cultures was studied. Antibody 14D9 catalyzes the stereo-selective transformation of achiral enol ethers having a potential practical application in organic synthesis. Authors demonstrated that the addition of PVP (at 1.5 g/L) and gelatin (at 5.0 g/L) increased the total 14D9 amounts in the culture medium reaching levels of about 625 μg/L (Martínez et al. 2005). Antibody purification from HR culture medium demonstrated to be particularly facilitated requiring a simple two-step procedure constituted by, first, clarification of the medium by centrifugation or filtration and, second, affinity chromatography step using protein A resin. In the case of the mAb H10 antibody, Lonoce and colleagues reported a 60% recovery of the total antibody from the culture medium compared to the 30% obtained from the root extracts, with final yields of 1.5 mg/L of purified antibody (Lonoce et al. 2016).

9.4.1 *Production of Glyco-optimized Antibodies in Plants and HR Cultures*

Post-translational modifications occurring in antibodies have been extensively studied, and it has been demonstrated that glycosylation is crucial for many fundamental biological processes, including antibody-dependent cellular cytotoxicity (ADCC), complement activation and Fcγ receptor-binding ability (Gomord et al. 2005). These modifications are often essential for the stability and biological activity of a protein especially in the case of antibodies (Gomord et al. 2010). In fact, it has been evaluated that in these molecules, different glycosylation patterns could affect protein half-life in blood and, in some cases, the capability to interact with the components responsible for the stimulation of the immune system (Jefferis 2009). In eukaryotes, oligosaccharides are added on specific sequences of secreted proteins in the endoplasmic reticulum (ER) in a process called N-glycosylation. Biosynthesis of N-glycans can be divided into two steps, which occur sequentially in the ER and downstream in the Golgi. In the ER, glycosylation between mammals and plants is highly similar showing minimal differences in N-glycans found on mature glycoproteins (Gomord et al. 2010) (Fig. 9.2). The major differences are found in the Golgi apparatus where plant-specific complex-type glycans are added by the enzymes β-1,2-xylosyltransferase (XylT) and α-1,3-fucosyltransferase (FucT) absent in mammalian cells (Lerouge et al. 2000) (Fig. 9.2). Furthermore, plant glycoproteins differ from their mammalian counterpart for the lack of β-1,4-galactose, sialic acid complex-type glycans, core α-1,6-fucose and the

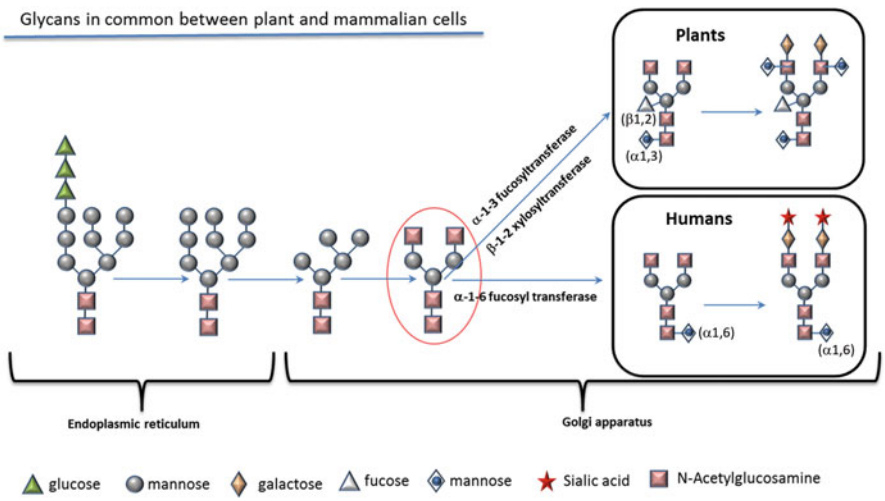

Fig. 9.2 Different glycosylation patterns among plants and mammals. Silencing of the plant endogenous α-1,3 fucosyltransferase (FucT) and β-1,2 xylosyltransferase (XylT) genes was used to obtain heterologous proteins with human-compatible glycans. The main glycan structure found in these recombinant proteins is circled

homologous of the mammalian N-acetylglucosaminyltransferases involved in further branching of the bi-antennary N-glycans. As described in literature, proteins harbouring plant sugars could induce adverse side effects when used in human therapy. In fact, the immunogenicity of these N-glycan epitopes is well documented, and their role in allergy has not yet been clarified (van Ree et al. 2000; Bardor et al. 2003; Altmann 2007). Based on this concern, new strategies have been adopted to engineer antibody glycosylation profiles in order to reduce problems related to the presence of typical plant sugars. Modulation of the N-glycosylation profile of plant-produced antibodies is largely described in literature and known as 'glyco-engineering'. Several strategies have been exploited until now to modulate the glycan structure such as retention of immunoglobulins in the ER using C-terminal KDEL tags (Sainsbury et al. 2010; Loos et al. 2011a, b; Lombardi et al. 2012), production of aglycosylated antibodies through mutation of the HC-specific N-glycosylation site (Lombardi et al. 2012), co-expression of antibodies with mammalian glyco-enzymes (Vézina et al. 2009) and expression of antibodies in plants in which genes coding for glycosyltransferases are inactivated or silenced by expression of siRNA (Strasser et al. 2008). In these ΔXTFT *N. benthamiana* plants, RNA interference strategy was used to obtain a targeted downregulation of the expression of endogenous α-1,3 fucosyltransferase (FucT) and β-1,2 xylosyltransferase (XylT) genes. The absence of β-1,2-xylose and α-1,3-fucose in the final purified antibodies was confirmed by mass spectrometry (MS) analysis and immunoblotting, demonstrating the possibility to use these engineered plants as an efficient expression platform for the production of human mAbs without detectable plant-specific N-glycan residues (Strasser et al. 2014). This strategy showed the possibility to obtain plant-derived immunoglobulins harbouring a human-compatible N-glycosylation pattern representing an ideal production platform of immunotherapeutic proteins. In a recent work, Lonoce and colleague generated HRs starting from glyco-engineered ΔXTFT *N. benthamiana* plants (Lonoce et al. 2016). The tumour-targeting monoclonal antibody mAb H10 was expressed by co-infecting leaf discs of the glyco-engineered plant with recombinant *A. rhizogenes* carrying mAb H10 heavy and light chain cDNAs. Selected HR clones accumulated mAb H10 in the culture medium with yields of 2–3 mg/L. N-Glycosylation profiles of antibodies purified from HR supernatant revealed glycan structures lacking xylose and fucose residues. This represents a step forward towards the exploitation of root cultures for the production of human therapeutic antibodies, demonstrating that the co-infection of glyco-engineered plants with recombinant *A. rhizogenes* is an efficient strategy for the generation of HRs expressing mAbs with a human-compatible glycan profile.

9.5 Conclusions

We have shown that HR cultures are an advantageous system for the production of different recombinant proteins that span from vaccine antigens and enzymes to therapeutic monoclonal antibodies. Although HRs offer several advantages, there are still many challenges to be faced in order to compete with traditional production

systems such as yeasts and animal cell cultures. Among these the most important one is related to the low accumulation yield of recombinant protein that is generally achieved in the culture medium. In the case of mAbs, the highest accumulation levels reported were in the range of 20 mg/L, a value which is still far from those (several grams per litre) obtained in Chinese hamster ovary (CHO) cell cultures. For this reason current and future efforts should be focused on enhancing the secretion of recombinant proteins from HRs as well as in minimizing the proteolytic degradation occurring in the culture medium. Several studies suggest that the optimization of expression constructs, the use of different inducers of secretion, such as synthetic auxins, as well as novel stabilizing agents could effectively increase the accumulation levels of recombinant proteins making HRs a more competitive commercial production platform. Another aspect that could increase the impact of HRs is also the possibility of producing therapeutic proteins with tailored glyco-modified profiles. Indeed, glycosylation was demonstrated to exert a profound impact in the biological function of several proteins and particularly in the case of antibodies. Monoclonal antibodies lacking fucose residues are currently evaluated in clinical studies for their enhanced therapeutic efficacy and are considered as novel drugs showing improved characteristics over the original molecules ('biobetters'). In conclusion, HRs show great potential as a novel production platform of biopharmaceuticals endowed with enhanced functionality.

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Chapter 10
Phytoremediation of Persistent Organic Pollutants (POPs) Utilizing Transgenic Hairy Root Cultures: Past and Future Perspectives

Yoshihiko Nanasato and Yutaka Tabei

Abstract Persistent organic pollutants (POPs) are halogenated compounds that were once widely used. Common properties of POPs are persistence in the environment, high toxicity to s and wildlife, bioaccumulation in biological tissues, biomagnification through food chains, and long-range transport. Although they are now banned or restricted in many countries owing to their persistence in the environment, POPs are of particular concern for continuing potentially adverse effects on human health and the environment. Therefore, urgent action is required to address the global elimination, remediation, cleanup, and safe disposal of POPs. Phytoremediation has received attention for mitigating POPs pollution and is appropriate for in situ degradation of pollutants over a large area, which contributes to its cost-effectiveness. However, ordinary plant species are unable to take up POPs from soil. Furthermore, there is no specific enzyme that degrades or detoxifies POPs in plants. Some plant species, for example, *Cucurbita* species, possess the unique ability for uptake of significant amounts of POPs and are considered hyperaccumulators of POPs. Genes encoding POPs-degrading enzymes have been isolated from POPs-degrading microorganisms. Generating transgenic hyperaccumulator plants expressing POPs-degrading enzymes might have promise as a practical means of phytoremediation of POPs. However, production of transgenic plants is laborious and time-consuming. The hairy root culture system has several advantages compared to other tissue culture systems. In addition, we discuss the utility of "composite plants," chimeras containing wild-type shoots with transgenic hairy roots, from the perspective of biosafety and rapid evaluation of phytoremediation ability in a POPs-contaminated field.

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10.1 Introduction

A number of man-made halogenated organic compounds have been manufactured and used for many purposes such as pesticides, herbicides, refrigerants, fire retardants, electrical insulation oil, paints, and solvents. Of great concern, some of these compounds are highly detrimental to the health of organisms through bioaccumulation in their tissues and biomagnification in food chains. These compounds are highly resistant to natural degradation and are capable of long-range transport even to regions where these compounds have never been used. These compounds are now called persistent organic pollutants (POPs) (the Stockholm Convention, UNEP, http://chm.pops.int). Under the Stockholm Convention, POPs are categorized as those subject to elimination, restriction, and unintentional production, and there has been an increase in the number of compounds listed as POPs (Table 10.1). Despite the restriction and banning of these compounds, contamination of breast milk, water, and soil by POPs has been reported over all regions in the world. Recently, a research group reported detection of some POPs in fauna from the deepest ocean trenches (>10,000 meters) (Jamieson et al. 2017). General information on POPs is available on the website of the Stockholm Convention. This review focuses on previous research and future perspective on phytoremediation of POPs using hairy root cultures.

1. Phytoremediation of POPs
2. Transgenic approach for degradation of POPs
3. Test system for phytoremediation of POPs using hairy root cultures
4. New application of composite plants for safer genetically modified organisms
5. Conclusions and future perspective

10.2 Phytoremediation of POPs

"Phytoremediation" was coined in the 1990s to describe the use of plants for removal of toxic metals (Raskin et al. 1994; Salt et al. 1995), and this technology has been used to remove various organic and inorganic pollutants (Pilon-Smits 2005). Since phytoremediation is ultimately driven by solar energy, it is more economical than methods such as excavating, washing, and burning soil or pump-and-treat systems (Schnoor 1997). Another advantage of phytoremediation is that the original soil remains after treatment because there is no need to remove the soil. Compared to other chemical treatments, phytoremediation is appropriate for in situ

Table 10.1 All POPs listed in the Stockholm Convention[a]

		New POPs added to the Stockholm Convention				
	The initial 12 POPs	In COP4 (2009)	In COP5 (2011)	In COP6 (2013)	In COP7 (2015)	In COP8 (2017)[b]
Annex A (elimination)	Aldrin	α-Hexachlorocyclohexane	Technical endosulfan and related isomers	Hexabromocyclododecane (HBCDD)	Polychlorinated naphthalenes	Decabromodiphenyl ether (c-decaBDE)
	Chlordane	β-Hexachlorocyclohexane			Hexachlorobutadiene (HCBD)	Short-chain chlorinated paraffins
	Dieldrin	Lindane (γ-hexachlorocyclohexane)				
	Endrin	Chlordecone				
	Heptachlor	Hexabromobiphenyl				
	Hexachlorobenzene (HBC)	Hexabromodiphenyl ether and heptabromodiphenyl ether (octaBDE)				
	Mirex	Pentachlorobenzene (PeCB)				
	Toxaphene	Tetrabromodiphenyl ether and pentabromodiphenyl ether				
	Polychlorinated biphenyls (PCB)					
Annex B (restriction)	DDT	Perfluorooctane sulfonic acid (PFOS), its salts, and perfluorooctanesulfonyl fluoride (PFOS-F)				

(continued)

Table 10.1 (continued)

		New POPs added to the Stockholm Convention				
	The initial 12 POPs	In COP4 (2009)	In COP5 (2011)	In COP6 (2013)	In COP7 (2015)	In COP8 (2017)[b]
Annex C (unintentional production)	Polychlorinated dibenzo-*p*-dioxins (PCDD)	Pentachlorobenzene (PeCB)			Polychlorinated naphthalenes	Hexachlorobutadiene (HCBD)
	Polychlorinated dibenzofurans (PCDF)				Pentachlorophenol and its salts and esters	
	Hexachlorobenzene (HBC)					
	Polychlorinated biphenyls (PCB)					

[a]Adapted from the Stockholm Convention web page (http://chm.pops.int/TheConvention/ThePOPs/AllPOPs/tabid/2509/Default.aspx)
[b]http://chm.pops.int/TheConvention/ConferenceoftheParties/Meetings/COP8/tabid/5309/Default.aspx

degradation of pollutants in a large area, which contributes to its cost-effectiveness. Mechanisms for transport of contaminants to land plants are divided into sorption of contaminants by the roots and distribution within the shoots through xylem and phloem transport. The flow of organic pollutants from the soil to plants is expressed by the root concentration factor and the transpiration stream concentration factor (Briggs et al. 1982).

Obviously, bioavailability of a pollutant is important for its remediation. The bioavailability depends on the chemical properties of the pollutant, soil properties, environmental conditions, and biological activity. One important chemical property of a pollutant that affects its movement in soils is its hydrophobicity. Hydrophobicity is usually expressed as the octanol-water partition coefficient, or log K_{ow}. A high log K_{ow}value corresponds to high hydrophobicity. POPs are extremely hydrophobic molecules (log $K_{ow} > 3$) that are tightly bound to soil organic matter and do not dissolve in soil pore water (Briggs et al. 1982). This lack of bioavailability limits their ability to be phytoremediated, leading to their classification as recalcitrant pollutants. Volatility of pollutants, expressed as their Henry's law constant (H_i), is another important chemical property (Davis et al. 2002). POPs are often recognized as semi-volatile compounds.

Phytoremediation is categorized into types reflecting the specific mechanism involved in the removal of the pollutant: (i) phytoextraction, removing pollutants from soils by concentrating them in aerial parts of plants; (ii) phytovolatilization, releasing pollutants into the atmosphere through plant transpiration; (iii) phytostabilization, reduction of the mobility of pollutants in soil; (iv) phytostimulation, enhancing microbial activity for degradation of pollutants in the rhizosphere by plants; and (v) phytodegradation, breakdown of pollutants within plant tissues. These technologies are not mutually exclusive because accumulation, stabilization, and volatilization can occur simultaneously.

For efficient removal of a pollutant in the soil, the ability to take up large concentrations of pollutants is essential. Plant species that accumulate high levels of inorganic elements are so-called hyperaccumulators (Brooks et al. 1977; Peer et al. 2005). For heavy metals, many hyperaccumulator or metallophyte species have been investigated such as *Elsholtzia splendens*, *Silene vulgaris*, *Thlaspi caerulescens*, *Alyssum* species, *Arabidopsis halleri*, *Sedum alfredii*, *Brassica juncea*, and *Pteris vittata* for Cu, Zn, Ni, Cd, Pb, Se, and As (Ma et al. 2001; Song et al. 2004; Weng et al. 2005; Liu et al. 2007; Singer et al. 2007; Milner and Kochian 2008; Ueno et al. 2008). Some plant species have been investigated for remediation of toxic organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and explosives (Hannink et al. 2001; Harms et al. 2002; Tang and Mo 2007). As well as ordinary organic pollutants, there are reports on uptake of or tolerance to POPs such as DDT, PCBs, and HCHs in various plant species (Table 10.2) (Calvelo Pereira et al. 2006; Mo et al. 2008; Mikes et al. 2009; Abhilash and Singh 2010; Abhilash et al. 2013; Dubey et al. 2014; Rissato et al. 2015).

One major factor making phytoremediation of POPs difficult is the poor uptake of POPs by plants. Because of their extremely hydrophobic properties (log $K_{ow} > 3$), they are recalcitrant to uptake by plant species from soil. Although there is no clear benchmark for a hyperaccumulator of organic pollutants, *Cucurbita* species are generally considered as hyperaccumulators of POPs. The species has been extensively studied for uptake and translocation of POPs, such as DDT, PCBs, dieldrin,

Table 10.2 Examples of studies on phytoremediation of POPs

Species	POPs	References
Withania somnifera Dunal	Lindane (γ-hexachlorocyclohexane)	Abhilash and Singh (2010)
Jatropha curcas L.	Lindane (γ-hexachlorocyclohexane)	Abhilash et al. (2013)
Spinacia oleracea L.	Lindane (γ-hexachlorocyclohexane)	Dubey et al. (2014)
Raphanus sativus	HCHs, DDT, HCB, and PCBs	Mikes et al. (2009)
Zea mays	DDT	Mo et al. (2008)
Ricinus communis L.	HCHs, chlordane, DDT, aldrin, dieldrin, endrin, heptachlor, and other organochloride pesticides	Rissato et al. (2015)
Avena sativa L. *Chenopodium* spp. *Solanum nigrum* L. *Cytisus striatus* (Hill) Roth. *Vicia sativa* L.	HCHs	Calvelo Pereira et al. (2006)
2 cultivars of *Cucurbita pepo* L. *Cucumis sativus* L. cv.	PCDD/PCDF	Hülster et al. (1994)
2 cultivars of *Cucurbita pepo* L. *Spinacia oleracea* cv.	*p,p'*-DDE (a metabolite of DDT)	White (2001)
2 cultivars of *Cucurbita pepo* L. *Festuca arundinacea* cv. *Lolium multiflorum* cv. *Medicago sativa* cv.	DDT	Lunney et al. (2004)
Cucurbita pepo L. cv. *Cucurbita pepo* × *C. texana* cv. *Cucumis sativus* L. cv.	DDTs, chlordane	Mattina et al. (2006)
32 plant species 34 cultivars of *Cucurbita* sp.	Dieldrin, endrin	Otani et al. (2007)
3 cultivars of *Cucurbita pepo* L.	PCDD/PCDF, PCBs	Inui et al. (2008)
Cucurbita pepo L. *Cucurbita maxima* Duch. *Cucurbita moschata* Duch. *Cucurbita sativus* L. *Brassica oleracea* var. *Glycine max* Merrill	HCHs, dieldrin, endrin, heptachlor, and DDT	Namiki et al. (2013)

(continued)

Table 10.2 (continued)

Species	POPs	References
Solanum lycopersicum Mill. *Zea mays* L.		
10 cultivars of *Cucurbita maxima* Duch.	Heptachlor	Sugiyama et al. (2013)

endrin, and dioxins (Table 10.2) (Hülster et al. 1994; White 2001; Lunney et al. 2004; Mattina et al. 2006; Otani et al. 2007; Inui et al. 2008; Namiki et al. 2013; Sugiyama et al. 2013). Although the molecular mechanisms for uptake of extremely hydrophobic organic pollutants are not fully understood, candidate key factors have been reported for uptake and solubilization of POPs (Murano et al. 2010; Inui et al. 2013, 2015).

10.3 Transgenic Approach for Degradation of POPs

Another factor making it difficult for phytoremediation of POPs is the difficulty in breaking them down into nontoxic compounds. Moreover, some of them are toxic to plant cells. In other words, rapid degradation of POPs after uptake is required for efficient phytoremediation. Some POPs can be degraded in plant cells via endogenous enzymatic activities. Wilken et al. (1995) determined the metabolism of 10 PCB congeners in cell cultures from 13 plant species. However, metabolites can be as persistent and toxic as the parent POPs (Machala et al. 2004; Grimm et al. 2015). One effective solution is utilization of a set of catabolic enzymes isolated from POPs-degrading bacterial strains (Chakraborty and Das 2016). There are several examples of candidate genes. The genes encoding the terminal oxygenase components (*dbfA1A2*) of dibenzofuran 4,4a-dioxygenase (DFDO) isolated from *Terrabacter* sp. and the genes encoding carbazole 1,9a-dioxygenase (CARDO) isolated from *Pseudomonas* sp., respectively, degrade polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) (Habe et al. 2001). The genes encoding biphenyl dioxygenase (*bph* genes) isolated from *Rhodococcus* sp. can degrade PCBs (Masai et al. 1995). The *bph* genes are also involved in degradation of DDE, a primary decomposition product of DDT (Nguyen et al. 2013). The *lin* genes encoding dehalogenases isolated from *Sphingobium* sp. are involved in degradation of γ-HCH (Nagata et al. 2007) and β-HCH (Ito et al. 2007). Extensive studies have identified and characterized the degradation pathway and genes involved in these bacterial strains. Therefore, utilization of the entire set of genes, rather than a single gene, is desired for complete detoxification. Direct application of these bacteria into contaminated soil, often called bioaugmentation (Singer et al. 2005), is also an attractive approach. But many factors such as temperature,

moisture, pH, and organic matter content affect the viability of bacteria introduced into soil, and it has been observed that the number of exogenous microorganisms decreases shortly after soil inoculation (Mrozik and Piotrowska-Seget 2010). We believe a strategy using transgenic plants expressing one or more POPs-degrading enzymes offers a shortcut to produce plants for phytoremediation of POPs. Uptake and detoxification of organic pollutants, for example, explosives and herbicides, have been reported using transgenic plants (Kawahigashi et al. 2005; Uchida et al. 2005; Abhilash et al. 2009; Shimazu et al. 2011; Vail et al. 2014).

10.4 Test System for Phytoremediation of POPs Using Hairy Root Cultures

From the view point of practical applications, it is crucial to use hyperaccumulator plants as hosts for introduction of POPs-degrading enzymes. However, to our knowledge, there are only reports with non-hyperaccumulator plants such as *Arabidopsis thaliana* or *Nicotiana tabacum*. One of the major bottlenecks is the lack of tissue culture and transformation systems for POPs hyperaccumulators. Although transformation systems have been reported for *Cucurbita* species (Tricoli et al. 1995; Shah et al. 2008; Nanasato et al. 2011, 2013), it is still laborious and time-consuming to produce transgenic *Cucurbita* compared with model plant species. It is also difficult to identify other candidate plant species for development of tissue culture and transformation systems. Moreover, it takes a long time to evaluate whether an introduced heterologous enzyme will be stable and work well in plant cells. Characterization of metabolites of POPs generated by introduced transgenes in the plants is also required. From this perspective, in vitro plant cell culture systems such as callus and cell suspension cultures are suitable biotechnological tools for rapid analysis of transgenic plant cells and fundamental studies of the interactions between plants and pollutants. In particular, hairy root cultures produced by infection with *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) are an ideal tool with several specific advantages: (i) genetic and biochemical stability, (ii) rapid growth in standard media without phytohormones, (iii) low costs of implementation and easy maintenance of a lot of transgenic lines, and (iv) possibility of development in industrial bioreactor models (Veena and Taylor 2007; Agostini et al. 2013; Adrián et al. 2017). Moreover, hairy root cultures have an organized structure and secondary cell walls, so they can bridge the gap between in vitro culture systems and in vivo whole plants. In addition, plant roots are in direct contact with soil or water, and hence the pollutants present therein, so a hairy root culture system is much more suitable for phytoremediation studies than other cell culture systems. More than 450 species of plants are susceptible to infection by *A. rhizogenes* (Porter 1991). There are reports on development of hairy root cultures for *Cucurbita* species (Katavic et al. 1991; Ramírez-Ortega et al. 2015) and other candidates for hyperaccumulating or tolerant plant species such as *Jatropha curcas* and *Withania*

Table 10.3 Hairy root cultures for phytoremediation of POPs

Species	POPs	Introduced genes	Reference
Cichorium intybus *Brassica juncea*	DDT	–	Suresh et al. (2005)
Solanum nigrum	PCBs	–	Kučerová et al. (2000)
		–	Rezek et al. (2007)
Cucurbita moschata Duch.	Lindane (γ-hexachlorocyclohexane)	*linA*	Nanasato et al. (2016)

somnifera (Kajikawa et al. 2012; Thilip et al. 2015). Accumulation of and tolerance to heavy metals such as Cu, Cd, Ni, U, and Zn have been investigated using hairy root cultures of hyperaccumulators (Nedelkoska and Doran 2000a, b; Eapen et al. 2003; Subroto et al. 2007; Vinterhalter et al. 2008). In an example for organic pollutants, Wevar Oller et al. (2005) demonstrated the utility of transgenic hairy root cultures for degradation of phenol. In contrast, only a few reports have described phytoremediation of POPs using hairy root cultures (Table 10.3). Suresh et al. (2005) reported uptake and degradation of DDT by hairy root cultures of *Cichorium intybus* and *Brassica juncea.* Metabolites of degradation (DDD, DDE, DDMU, and some unidentified products) ranged from 10% to 20% of the applied DDT in the hairy root cells after 10 days of incubation. A hairy root culture of *Solanum nigrum* was used to determine metabolites of various PCB congeners (Kučerová et al. 2000; Rezek et al. 2007). Transgenic hairy root cultures were generated to evaluate the function of *linA*, a gene encoding γ-HCH dehydrochlorinase isolated from a γ-HCH-degrading microorganism (Imai et al. 1991) in *Cucurbita moschata* (Nanasato et al. 2016). The LinA protein, targeted to the apoplast, accumulated in the hairy root cultures, which degraded more than 90% of the applied γ-HCH (1 ppm) in overnight incubation. These results indicate that *linA* has high potential for phytoremediation of environmental γ-HCH.

10.5 New Application of Composite Plants for Safer Genetically Modified Organisms

As described above, a transgenic approach is critical for phytoremediation of POPs in practice. However, it introduces another problem: gene flow from transgenic plants, especially through transgenic pollen. Several techniques to produce plants that do not produce pollen have been reported, such as genetically engineered male sterility (Zhan et al. 1996; Beals and Goldberg 1997; Konagaya et al. 2008) and plastid transformation (Ruf et al. 2007; Svab and Maliga 2007). These techniques have been applied to *A. thaliana*, *N. tabacum*, and some crop plants. It is however unclear whether these techniques are efficient for other plant species. A composite

plant system is an alternative to tissue culture-derived transgenic studies. A composite plant is a chimera containing wild-type shoots with transgenic roots established by *A. rhizogenes* transformation, first reported using *Lotus corniculatus* (Hansen et al. 1989). Collier et al. (2005) applied the system to make composite plants with roots containing transgenes. The system has been applied in various plant species such as *Brassica oleracea*, *Coffea arabica*, *Nicotiana benthamiana*, and *Cucurbita pepo* (Collier et al. 2005; Alpizar et al. 2006; Li et al. 2010; Ilina et al. 2012). Because composite plants have wild-type shoots, they could be used in field trials for phytoremediation of POPs even though they have transgenic roots. PCR using primers to amplify *rol*, *vir*, and *HrcA* genes can confirm that the roots have been transformed and the hairy root-inducing bacteria have been eliminated (Nanasato et al. 2016). One of the important advantages of the system is that with composite plants, there is no need for a tissue culture step. In addition, the plants can be grown in soil. A flaw of hairy root culture systems is that they can be cultured only in liquid media. A composite plant system can overcome this flaw. Composite hyperaccumulators with transgenic roots containing POPs-degrading enzymes may help to screen plants and transgenes for production of POPs-phytoremediating plants.

10.6 Conclusions and Future Perspective

POPs have been widely manufactured and released into the environment. After the 1962 publication of *Silent Spring* by Rachel Carson, unwitting use of POPs was reconsidered, and later their manufacture and use were restricted by the Stockholm Convention. Nevertheless, environmental pollution by POPs remains, and their spread has been global. Phytoremediation is a promising technology for in situ remediation of many areas contaminated with POPs, but problems remain for practical application. First, the underlying aspects of plant physiology including the molecular mechanisms for uptake and translocation of POPs bound to the soil into plant shoots are not fully understood. This study could help enhance the accumulation of POPs in plants. Not only understanding the plant physiology but also the rhizosphere biology is likely required, because synergetic action of phytoremediation and plant-assisted bioremediation would be an attractive option for enhancement of remediation in the environment. Second, there is a need for exploring genes encoding POPs-degrading or POPs-detoxifying enzymes. Development of metagenomics and whole-genome sequencing technology may help to discover novel genes encoding POPs-degrading enzymes. In any event, evaluation of the genes in POPs-hyperaccumulator plant cells is required. Hairy root culture systems are ideal for rapid evaluation. We previously demonstrated the potential of a transgenic hairy root culture for evaluation of a phytoremediation system (Nanasato et al. 2016). Further exploration using transgenic composite plants containing other POPs-degrading enzymes could help phytoremediation of POPs become more widely applicable.

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Chapter 11
Use of Hairy Root System to Study Signaling Pathways During Nodule Formation

Swarup Roy Choudhury and Sona Pandey

Abstract Nodule formation by a specialized group of plants is one of the most beneficial plant-environment interactions, where atmospheric nitrogen is biologically fixed into ammonia, which is subsequently converted to nitrates and amino acids. The molecular basis of nodule formation has been studied in detail, and work done in the past few years has led to seminal discoveries, connecting the initial signal perception by the root hairs to the downstream signaling events and finally to cellular and developmental changes that result in organogenesis and nodule formation. Although the physiology of biological nitrogen fixation has been well known for many years, the exquisite molecular details of nodule formation have been made possible, mostly, by using the transgenic hairy roots on composite plants. Development of hairy roots by *Agrobacterium rhizogenes* (*A. rhizogenes*) infection provides an excellent experimental system to rapidly and efficiently evaluate the effect of changes in the expression of specific genes or gene families on a range of root phenotypes. By using this system, the Nod factor receptor-mediated signaling has been linked to the infection thread formation and nodule organogenesis, two critical events of nodulation. The use of hairy root system has made it possible to uncover the details of signaling and developmental events using molecular genetics, genomics, proteomics, and cell biological approaches, making the nodulation signaling pathway one of the best understood in leguminous plants. This article provides an overview of multiple rhizobium-legume interaction studies that utilized the hairy root system to uncover the signaling pathways and offers perspectives on its future uses in the context of the development of novel gene-editing capabilities in plants.

Keywords Biological nitrogen fixation · Hairy roots · Legumes · Nodulation · Rhizobia · Symbiosis

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11.1 Biological Nitrogen Fixation

Nitrogen is an essential element for plant survival. It is a key constituent of amino acids, the building blocks of proteins, as well as of chlorophyll, a pigment required for photosynthesis. Increasing nitrogen content biologically in the soil is an effective strategy to produce higher crop yields while reducing chemical nitrogen fertilization and, subsequently, environmental pollution. Among plants, legumes (family Fabaceae) and few nonlegumes (some member of Cannabaceae) are able to fix atmospheric nitrogen in soil through symbiotic association with rhizobia, by a process known as biological nitrogen fixation (BNF). In agricultural systems, BNF is an environmentally sound alternative to chemical fertilizers and economically beneficial for crop production.

Only a subset of bacteria can convert atmospheric nitrogen to ammonia for BNF in the host plants, due to the catalytic activity of nitrogenase. These important nitrogen-fixing bacteria are called diazotrophs which include gram-negative *Rhizobia* sp. and gram-positive *Frankia* sp. Rhizobia are responsible for the most efficient nitrogen fixation processes by the formation of root nodules on legumes and few nonlegumes (Oldroyd and Downie 2008; Desbrosses and Stougaard 2011). Different genera of rhizobia including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* are capable to form a symbiotic association with different legumes depending on their genetic compatibility. Symbiotic interaction between diazotrophs and legumes is responsible for the majority of BNF, although minor contribution from certain actinomycete members such as *Frankia* sp. which can form symbiotic relationship either by root hair infection or intercellularly with a broad spectrum of plant families belonging to Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Elaeagnaceae, Rhamnaceae, Datiscaceae, and Coriariaceae also exists. In addition, some diazotrophs including *Azospirillum* sp. and *Azoarcus* sp. form endophytic relationships with a wide variety of cereal roots. Finally, certain cyanobacteria, mainly *Nostoc* sp., can fix atmospheric nitrogen to colonize different plant organs (Santi et al. 2013; Pawlowski and Bisseling 1996).

11.2 Nodule Formation in Legumes

In this chapter, our main focus is on the underlying signaling mechanisms of root nodule symbiosis in legumes via rhizobia and how the use of hairy roots has helped uncover the exquisite details of these pathways (Fig. 11.1). The legume family is the third largest family of flowering plants and includes plants varying from annual herbs to large trees with many agronomically and economically important crops. Research on legumes is driven, to a large extent, by their biological nitrogen-fixing capacity. The formation of nodules leading to nitrogen fixation is quite complex and tightly regulated but also inadequately understood at the molecular level. However, recent advances in genome sequencing and development of hairy root

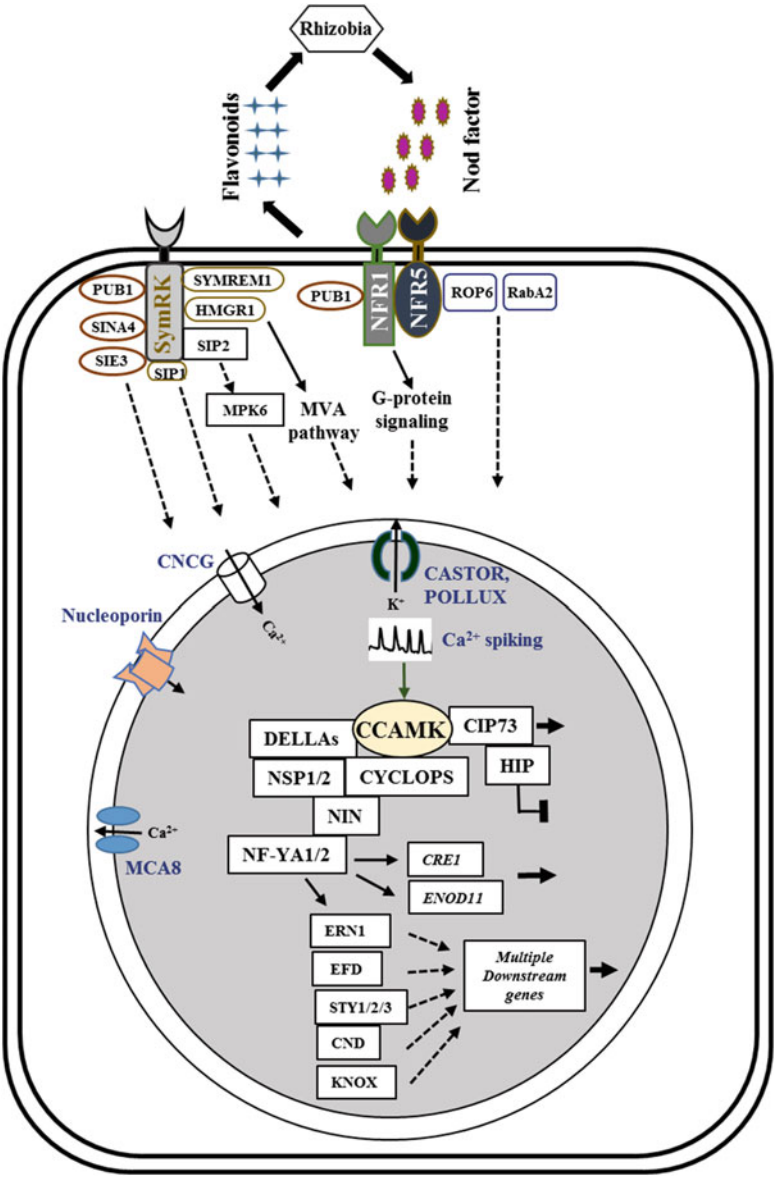

Fig. 11.1 Schematics of nodulation signaling as established using hairy root transformation system. Infection starts by secretion of flavonoids from roots, which trigger the production of bacterial Nod factors (NF). NF perception involves plant receptor-like kinases comprising lysin motifs NFR1 and NFR5 and a leucine-rich repeat SYMRK receptor. Receptors propagate signal from the plasma membrane to cytosol via heterotrimeric and monomeric (ROP6, RabA2) G-proteins, MAP kinase pathway (SIP2), HMGR1-dependent mevalonate (MVA) pathway, ubiquitination (PUB1, SINA4, SIE3), and other proteins (SYMREM1, SIP1) to generate unidentified secondary messengers, which are essential for calcium flux and calcium spiking in

transformation methods of different legumes have helped tremendously our understanding of the signaling mechanisms during nodulation.

Generally, two major types of nodules are formed on legumes: (a) determinate nodules that are characterized by a spherical shape and the lack of a persistent nodule meristem, producing ureide products, e.g., in *Lotus japonicus* and *Glycine max*, and (b) indeterminate nodules that are characterized by a cylindrical shape and the presence of a persistent nodule meristem, producing amide products, e.g., in *Medicago truncatula* and *Pisum sativum* (Sprent 2007). While determinate nodule initials arise from the outer or mid-cortical cells of the root, indeterminate nodule initials arise from the inner cortical cells. Two essential steps are needed for both types of nodule development: one an early infection phase and two a late developmental phase or organogenesis. The infection phase is started by the secretion of flavonoids from the legume roots, which trigger the production of lipochitin oligosaccharide known as Nod factors (NFs) from bacteria. NFs are sensed by Nod factor receptors (NFRs) present in the roots, and a number of morphological, biochemical, and cellular changes begin which allow the rhizobia to invade the host root cells. The most common entry strategy for rhizobia is by epidermal root hair curling and infection thread (IT) formation, observed in *L. japonicus*, *M. truncatula*, *G. max*, and *Phaseolus vulgaris* (Oldroyd and Downie 2008). Another nonclassical rhizobial invasion strategy is known as crack invasion. In this case, the rhizobia access the cortical cells through epidermal cracks, a point of epidermal damage, which is generally caused by the emergence of lateral roots. This is commonly observed in the Aeschynomeneae tribe of legumes, e.g., *Arachis hypogea* and *Sesbania rostrata* (Sprent 2007; Oldroyd and Downie 2008). After access to the host cell, most rhizobia invade the cytoplasmic space of the host cells via an endocytosis-like process and stimulate the root cells to proliferate by cortical cell division. Subsequently, the rhizobia in the infected plant cells are enclosed within membrane-bounded structures that develop into symbiosomes, where they differentiate into a nitrogen-fixing form called bacteroids. This symbiosome membrane maintains the exchange of substrate and signal molecules between host plant cell and the bacteroids (Verma and Hong 1996). Finally, the cortical cells of symbiosomes continue to divide and fuse together to form the nodule (Oldroyd and Downie 2008; Desbrosses and Stougaard 2011).

The progress in understanding the molecular details of nodulation signaling has been greatly improved by the use of two model plant species, *M. truncatula* and

Fig. 11.1 (continued) the nucleus. Several potassium and calcium channels, calcium pump, and nucleoporins modulate the calcium flux at the nuclear membrane that may activate the calcium/calmodulin-dependent protein kinase (CCaMK) by triggering the calcium spiking inside the nucleus. CCaMK, the master regulator, interacts with other proteins and transcription factors to activate transcriptional programs, leading to stimulation of multiple downstream genes. Dotted arrows indicate proposed signaling routes, whereas solid arrows indicate established signaling pathway. Inside the nucleus arrowheads and blunt heads represent positive and negative regulators, respectively

L. japonicus, although the studies in soybean, peas, peanuts, and few other leguminous species have also been helpful. Both these model species are diploid, have sequenced genome with modest genome sizes, are important representatives of indeterminate and determinate nodules, respectively, and are amenable to genetic manipulation by hairy root transformation as well as by tissue culture-based transgenic plant development (Boisson-Dernier et al. 2001; Limpens et al. 2004; Stiller et al. 1997). Besides, the availability of genetic variants of these two plants from different resources makes it easier for further studies (https://medicago-mutant.noble.org/mutant) (Sandal et al. 2006; Cheng et al. 2014). In addition, the genome-wide synteny between these two plants and other legumes suggests that the study of these model legumes will provide important insight into the important biological questions related to nodulation in other plants as well.

11.3 Hairy Root Transformation: A Strategy for Functional Analysis of Genes

Efficient plant transformation by *Agrobacterium tumefaciens* has been described only in few model legumes (Iantcheva et al. 2013). This procedure is plagued by lengthy shoot regeneration period to analyze the transgenic constructs, and the transformation frequency is relatively poor. To avoid these complications encountered with *A. tumefaciens* transformation techniques, it was important to find a convenient way to allow more rapid evaluation of gene function in the model and other legumes. To address this problem, *Agrobacterium rhizogenes*-mediated hairy root transformation technique was developed. It is a versatile and adaptable model system for a wide variety of plants. Recently, hairy root transformation system has been extensively used to rapidly generate transgenic roots for genetic and molecular analysis.

Hairy roots originate from plants due to the *A. rhizogenes*-mediated transformation. The genetic determinant of hairy root infection is a *rol* gene cluster located on the *A. rhizogenes* root-inducing Ri plasmid (White et al. 1985). This powerful and simple transformation tool facilitates the integration of novel genes into the infected host plant. During this transformation process, the infection occurs within a host plant with a compatible *A. rhizogenes* strain which results in the formation of chimeric transgenic plants, consisting of untransformed shoots with multiple transgenic hairy roots (Lin et al. 2011). In addition to its speed and efficiency, this system offers multiple advantages: (i) the transgenic roots are stably transformed in contrast to transient transformations achieved by biolistic methods, so the results obtained from such studies are more physiologically relevant; (ii) the vectors typically have a GFP marker that allows easy identification of transgenic roots; (iii) the roots remain responsive to various biological treatments, so the effects of transgenes on root biology and physiology can be easily evaluated in an approximately natural environment; (iv) the system provides an efficient way of evaluating multiple constructs

for expression and functionality in a relevant genetic background; (v) the constructs used with *A. rhizogenes* can be used with *A. tumefaciens* without the need for any alteration, so the same constructs can be used for the generation of stable transgenic plants. The system therefore becomes useful for the evaluation of plant-microbe interactions, plant-fungus interactions, plant-nematode interaction, secondary metabolite production, host-parasitic plant interaction, etc. (Boisson-Dernier et al. 2001; Limpens et al. 2004; Chandra and Chandra 2011).

The root nodule (RN) symbiosis has been actively studied for the last three decades using hairy root transformation. The first hairy root transformation by *A. rhizogenes* was reported for *Lotus corniculatus* (Jensen et al. 1986). Subsequently it has been extended to other legumes for nodulation studies (Table 11.1), for example, *Trifolium repens* (Diaz et al. 1989), *Vigna aconitifolia* (Lee et al. 1993), *G. max* (Cheon et al. 1993), *Vicia hirsuta* (Quandt et al. 1993), *L. japonicus* (Stiller et al. 1997; Kumagai and Kouchi 2003), *Trifolium pratense* (Diaz et al. 2000), *M. truncatula* (Boisson-Dernier et al. 2001), *P. sativum* (Clemow et al. 2011), *A. hypogea* (Sinharoy et al. 2009), *S. rostrata*, and *Phaseolus* spp. (Estrada-Navarrete et al. 2007). In the following sections, we will the signaling circuit of nodulation, which has been uncovered based on research using hairy root transformation.

11.4 Role of Hairy Roots in Establishing Flavonoids as a Host-Derived Early Signal for Activation of Bacterial Nod Factors

Flavonoids are one of the largest classes of phenylpropanoid-derived plant secondary metabolites with different functions in plants. More than 10,000 different flavonoids have been identified and are classified into two major groups: 2-phenylchromans (flavonoids) and 3-phenylchromans (isoflavonoids). These secondary metabolites are involved in multiple physiological processes including plant structural integrity, protection against ultraviolet (UV) radiation and phytopathogens, auxin transport, coloration of flowers, and importantly nodulation signaling process (Ferreyra et al. 2012). During nodulation, legume roots release specific flavonoids into the surrounding soil to attract the rhizobia. Flavonoids also act as auxin transport inhibitors inside the plant roots to change its direction and accumulate auxin at specific sites to allow cortical cell division for nodule formation. To assess the functional role of flavonoids during nodulation genetically, hairy root transformation was used in *M. truncatula*. RNAi-mediated knockdown of *chalcone synthase* gene, which catalyzes the first committed step in the flavonoid biosynthesis pathway, significantly inhibited flavonoid production in transgenic hairy roots. These flavonoid-deficient transgenic roots were unable to initiate nodules, although the auxin transport remains unaffected in this root (Wasson et al. 2006). To investigate further details of the role of individual flavonoids, different biosynthetic enzymes of the flavonoid pathway including *isoflavone synthase*, *chalcone*

Table 11.1 Examples of successful hairy root transformation in different legumes

Plant common name	Scientific name	Family	*Agrobacterium rhizogenes* strain	*Rhizobia species*	Protocol
Barrel clover	*Medicago truncatula*	Fabaceae	*Agrobacterium rhizogenes* ARqua1	*Sinorhizobium meliloti* strain RCR2011	Boisson-Dernier et al. (2001)
Bird's-foot trefoil	*Lotus japonicus*	Fabaceae	*Agrobacterium rhizogenes* LBA1334	*Mesorhizobium loti* Tono	Kumagai and Kouchi (2003)
Soybean	*Glycine max*	Fabaceae	*Agrobacterium rhizogenes* K599	*Bradyrhizobium japonicum* (61A76)	Cheon et al. (1993)
Common bean	*Phaseolus vulgaris*	Fabaceae	*Agrobacterium rhizogenes* K599	*Rhizobium tropici* strain CIAT899	Estrada-Navarrete et al. (2007)
Pea	*Pisum sativum*	Fabaceae	*Agrobacterium rhizogenes* AR12 and AR1193	*Rhizobium leguminosarum* bv. viciae 128C53K	Clemow et al. (2011)
Peanut	*Arachis hypogea*	Fabaceae	*Agrobacterium rhizogenes* R1000	*Bradyrhizobium* sp. (Arachis) NC92	Sinharoy et al. (2009)
Moth bean or Turkish gram	*Vigna aconitifolia*	Fabaceae	*Agrobacterium rhizogenes* A4	*Bradyrhizobium* sp. *cowpea strain 3456*	Lee et al. (1993)
Hairy tare or tiny vetch	*Vicia hirsuta*	Fabaceae	*Agrobacterium rhizogenes* 15834, C58C1, AR12, R1000, ARqua1, ARqua2	*Rhizobium leguminosarum* bv. viciae	Quandt et al. (1993)
White clover	*Trifolium repens*	Fabaceae	*Agrobacterium rhizogenes* LBA9402	*Rhizobium leguminosarum* bv. viciae	Diaz et al. (1989)
Red clover	*Trifolium pratense*	Fabaceae	*Agrobacterium rhizogenes* LBA 1334	*Rhizobium leguminosarum* bv. trifolii ANU843, *Rhizobium leguminosarum* bv. viciae 248, *Mesorhizobium loti* E1R, M. loti E1R, *Sinorhizobium meliloti* 2011 pMP604	Diaz et al. (2000)

reductase, *flavone synthase*, and *chalcone synthase* were silenced by hairy root transformation in *M. truncatula*. These experiments revealed that the activation of rhizobial Nod operon and sustained induction of NF biosynthesis during infection thread development were indeed facilitated by flavone, whereas auxin transport was modulated by flavonols. Conversely, no significant role was assigned to isoflavonoids during nodulation signaling (Zhang et al. 2009). Overall, these data confirmed that legumes use different flavonoid compounds to activate the rhizobial nod operon and auxin transport modulation in roots during nodulation, underpinning a link between RN symbioses and auxin signaling through flavonoids.

11.5 The Plasma Membrane-Localized Components of Nodulation Signaling and Role of Hairy Roots in Their Discovery

Flavonoids stimulate the transcription of bacterial nodulation genes leading to the production of a lipochitin oligosaccharide signal, the Nod factors (NFs). NFs are perceived by a pair of membrane-bound LysM receptors which lead to multiple cellular responses including deformation and curling of root hairs for eventual invasion of rhizobia and cortical and pericycle cell divisions (Oldroyd and Downie 2008; Oldroyd et al. 2011; Wais et al. 2002). LjNFR1 and LjNFR5 in *L. japonicus*, MtLYK3 and MtNFP in *M. truncatula*, and GmNFR1α and GmNFR1β and GmNFR5α and GmNFR5β in *G. max* are the LysM-RLKs (LysM-receptor-like kinases) crucial for NF perception and activation of symbiotic signaling (Oldroyd et al. 2011). Both LysM receptors (NFR1 and NFR5) interact with each other to form a heterodimer, which can initiate downstream signaling. NFR1 contains an active kinase domain, whereas NFR5 lacks several conserved kinase subdomains and acts as a co-receptor. Mutants of these genes show complete impairment of nodule formation due to the lack of NF perception (Radutoiu et al. 2003; Limpens et al. 2003; Madsen et al. 2003;Smit et al. 2007). Functional roles of these receptors were established by the use of transgenic hairy roots. For example, overexpression or complementation of *NFR1* in *nfr1* mutant (*nod49*) or *NFR5* in *nfr5* mutant (*nod133*) background by strong constitutive or native promoter results in nodule formation after rhizobial infection in soybean (Indrasumunar et al. 2011; Indrasumunar et al. 2010; Lin et al. 2011; Roy Choudhury and Pandey 2015). Similarly, functional complementation of *nfr* mutants using the *A. rhizogenes* hairy root transformation revealed that NFR1 kinase activity is essential for the in vivo function of NFR1, and NFR1 can activate the NFR5 by phosphorylation (Madsen et al. 2011). These data led to an important question: How does the signal perception by membrane-bound NFRs connect with the downstream signaling in the nucleus? Again, hairy root transformation-based research helped elucidate many nuclear and cytoplasmic components of this signaling pathway.

In addition to the LysM receptors, another leucine-rich repeat receptor-like kinase (LRR-RLK) proteins play a significant role in nodulation by infection initiation as well as for the internalization of bacteria in cortex cells during symbiosome formation (Endre et al. 2002). This LRR-RLK commonly known as symbiosis receptor kinase (SymRK) in *L. japonicus*, DOES NOT MAKE INFECTIONS 2 (DMI2) in *M. truncatula*, NORK (nodulation receptor kinase) in *G. max*, and SYM19 (Symbiosis 19) in *P. sativum* contains three LRR domains, a transmembrane domain, and an intracellular kinase domain. Several studies revealed that SymRK interacts with and functions downstream of the NFR1/LYK3 and NFR5/NFP receptors (Endre et al. 2002; Stracke et al. 2002; Markmann et al. 2008; Oldroyd and Downie 2008). Although the activation mechanisms of SymRK are unclear, it is proposed that these receptor-like proteins form a complex with NFR proteins. It is also possible that SymRK accepts yet unknown extracellular signals by its LRR domain (Stracke et al. 2002). Recently the autophosphorylation of a tyrosine residue of SymRK was determined to be important for regulating its symbiotic activity (Saha et al. 2016). *RNAi*-mediated knockdown of *SymRK* by hairy root transformation in *M. truncatula* and *S. rostrata* established that the protein is crucial not only for early infection stage but also for symbiosome formation during nodule development (Capoen et al. 2005; Limpens et al. 2005).

11.6 The Cytosolic Components of Nodulation Signaling and Role of Transgenic Hairy Roots in Their Identification and Characterization

Relatively few cytoplasmic components involved in regulation of nodule formation immediately following the NF perception are known to date. To identify potential interacting proteins of the receptors, a yeast two-hybrid-based cDNA library screen was performed using LYK3 of *M. truncatula* (Andriankaja et al. 2007). This study identified PUB1, a UND-PUB-ARM protein or U-box (PUB) E3 ubiquitin ligase protein, as an interactor of LYK3. PUB1 is strongly induced by NFs, specifically in the roots during nodulation. Additionally, PUB1 is phosphorylated by LYK3 in vitro. To address the question of the physiological role of PUB1, both knockdown and overexpression approaches were used in hairy root transformation system. A strong increase in the number of nodules was observed by suppressing *PUB1* levels, whereas its overexpression caused a delay in nodulation. This study established that a possible receptor-mediated, phosphorylation-based mechanism modulates PUB1 (or E3 ubiquitin ligases in general) in controlling plant-rhizobial interactions by functioning as a negative regulator of LYK3 signaling pathway (Mbengue et al. 2010).

A similar yeast-based library screening was performed by using kinase domain of NFR5 in *L. japonicus*. This screen identified Rho-like GTPase (ROP6) protein, which interacts with NFR5 in a GTP-binding-dependent manner. Again, to establish

the role of ROP6 in regulating nodulation, transgenic hairy roots were generated by RNAi-mediated silencing of *ROP6*. A detailed study of transgenic hairy roots at different developmental stages confirmed that rhizobium entry was not influenced by ROP6, but ROP6 is most likely responsible for the establishment of infection thread (IT) growth through the root cortex. Suppression of *ROP6* resulted in fewer nodules, whereas its overexpression or expression of a constitutively active version of ROP6 (ROP6-CA) using hairy root transformation exhibited extensive root hair deformation after rhizobium (*Mesorhizobium loti*) infection, resulting in an increase of infection threads and nodule number (Yuan et al. 2012). Further study on ROP6 has led to a model of clathrin-mediated endocytosis by clathrin triskelion (CHC1), as an interactor of ROP6. The potential role of CHC1 was also ascertained by reduction of nodule number in transgenic hairy roots after overexpression of inactive domain of CHC1 or silencing of CHC1 during hairy root transformation (Wang et al. 2015). Taken together these data suggest a possibility of endocytosis of NFRs by the potential link between NFR5 and clathrin via ROP6 GTPase during nodulation.

A suppressive subtractive approach in response to infection with *Rhizobium etli* strains in *P. vulgaris* found a GTPases of the Rab subfamily, RabA2, which is responsible for the polar growth of root hair. Interestingly, nodulation was impaired in *RabA2* RNAi-silenced hairy roots indicating nodulation in *RabA2* RNAi plants is most likely the consequence of a compromised vesicle trafficking, which is required for deposition of cell wall material for the infection thread formation (Blanco et al. 2009). These data suggest the involvement of GTPases in signaling during nodulation.

One of the most well-defined membrane-bound signaling systems present in all eukaryotes is the heterotrimeric G-protein complex, consisting of Gα, Gβ, and Gγ proteins. Earlier pharmacological evidences provided the evidence for the involvement of heterotrimeric G-proteins in atmospheric nitrogen-fixing nodulation process in leguminous plants (Kelly and Irving 2003; Sun et al. 2007). Different downstream components of the heterotrimeric G-protein signaling, including phospholipase C and D, phosphatidic acid, diacylglycerol pyrophosphate, monomeric G-proteins, and MAP kinases, have been proved to involve in the regulation of nodulation (Pingret et al. 1998; Sun et al. 2007; Kelly and Irving 2003; Peleg-Grossman et al. 2007; Oldroyd et al. 2011; Tirichine et al. 2006). To directly establish the involvement of heterotrimeric G-protein during nodulation signaling, specific subunits of this signaling complex were knocked down or overexpressed in soybean hairy root system. Detailed analyses of the transgenic root phenotypes revealed that the Gβ and Gγ subunits act as positive regulators of nodule development, whereas the Gα subunits act as a negative regulator (Roy Choudhury and Pandey 2013). To establish the direct role of G-protein signaling per se in regulation of nodulation process in soybean, additional members of the G-protein complex were evaluated. A regulator of G-protein signaling, a GTPase activity-accelerating protein (GAP), which deactivates the G-protein cycle, positively regulated nodule development as revealed by gene silencing and overexpression approaches using hairy root transformation (Roy Choudhury and Pandey 2015). To probe if the heterotrimeric G-proteins are directly interacting with the NFRs, an interaction screen was performed. Both the Gα

proteins and RGS proteins interacted with the NFR1 protein of soybean. Furthermore, NFR1 was able to phosphorylate the RGS proteins, and the phosphorylation led to an increase in its activity. This model suggested that at least one of the functions of the NFRs after activation is to phosphorylate the RGS proteins, which deactivates the Gα protein. Because the Gα protein is a negative regulator of nodule formation, its deactivation led to successful nodulation. To further validate this model, it was hypothesized that if one of the roles of the NFRs is to phosphorylate RGS proteins, then introduction of a phosphomimic mutant of RGS protein in a mutant lacking the receptor should be able to restore nodulation, at least partially. To confirm this hypothesis, a phosphomimic version of RGS protein was introduced in the *nod49* (NFR1) mutant of soybean by hairy root transformation. Partial restoration of nodule formation was observed, validating the hypothesis that the heterotrimeric G-protein cycle is acting directly downstream of the NFRs to control nodule formation in plants (Roy Choudhury and Pandey 2015, 2016).

The proteins functioning downstream of the SymRK complex and the signaling pathways that follow are also beginning to be explored (Stracke et al. 2002). In search of the potential interacting partner of SymRK, a yeast-based library screening was performed by using intracellular kinase domain of *L. japonicus* SymRK as bait. An AT-rich interaction domain (ARID) containing SymRK-interacting protein 1 (SIP1) was identified as an interacting partner of SymRK (Zhu et al. 2008). Silencing or overexpression of *SIP1* using transgenic hairy roots led to reduced or increased nodule numbers, respectively, suggesting a positive role of SIP1 during nodulation (Wang et al. 2013). Yeast-based library screening also identified SymRK-interacting protein 2 (SIP2) as another potential interacting partner of SymRK in *L. japonicus*. SIP2 belongs to the plant MAPKK family, and in vitro analysis revealed that SymRK has a specific inhibitory effect on the kinase activity of SIP2 toward its substrate MPK6 (Chen et al. 2012). To establish the functional role of SIP2, hairy root transformation was used to knock down its expression. Suppression of *SIP2* reduced infection thread formation and nodule organogenesis, indicating a positive role of SIP2 in nodulation similar to SIP1. Overall, these data suggest that the route of signal transmission from SymRK to downstream components is likely via the MAPK-based signaling module.

Several E3 ubiquitin ligases were also identified as potential interactors of SymRK in yeast-based screening. Similar to NFR1, SymRK can interact with and phosphorylate PUB1, an E3 ubiquitin ligase in *M. truncatula* (Vernie et al. 2016; Mbengue et al. 2010). Additional genetic analysis revealed that PUB1, via its ubiquitination activity, is essential for rhizobial infection and nodulation. Another E3 ubiquitin ligase, SEVEN IN ABSENTIA (SINA4), interacts with the kinase domain of SymRK in *L. japonicus*. Ectopic expression of *SINA4* negatively influenced SymRK protein levels for its ubiquitination activity resulting in the impairment of infection thread formation and a strong reduction in bacteroid abundance. Additionally, promoter analysis of *SymRK* and *SINA4* after hairy root transformation has shown partially overlapping expression patterns of these genes during rhizobial infection and early nodule development (Den Herder et al. 2012). Furthermore, another SymRK-interacting E3 ubiquitin ligase, SIE3, has been shown to bind

with and use SymRK as a substrate for ubiquitination in *L. japonicus*. Silencing of *SIE3* transcripts via RNAi in hairy roots inhibited infection thread development and nodule organogenesis, whereas overexpression resulted in increased nodule numbers (Yuan et al. 2012). Overall, these data imply that the modulation of protein turnover of membrane-bound receptors like NFR and SymRK by ubiquitination is a key regulatory strategy during RN symbiosis.

In addition to the ubiquitin ligases, yeast-based library screening also identified 3-hydroxy-3-methylglutaryl-CoA reductase1 (HMGR1), a key enzyme regulating the mevalonate (MVA) pathway, as a specific interactor of the SymRK or DMI2 kinase domain in *M. truncatula* (Kevei et al. 2007). The N-terminal of HMGR1 catalytic region is sufficient and specific for binding to DMI2. RNAi silencing of *HMGR1* by hairy root transformation indicated a requirement of HMGR1 activity in the infection process during nodulation. It was also predicted that the active DMI2-HMGR1 complex at early root hair infection induced an invagination of the plasma membrane to initiate infection thread growth when rhizobia were entrapped in a root hair curls. These data also revealed that the Nod factor signaling recruits specific isoprenoid biosynthesis pathways via DMI2-HMGR1 for the production of cytokinins and steroids to modulate the cell division, which is essential for nodule organogenesis. In addition, an analysis of epidermal cells of *HMGR1-RNAi* transgenic hairy roots after application of NFs exhibits altered Ca^{2+} spiking and *ENOD11* (a key transcription factor of nodulation) expression in *M. truncatula*, indicating a role for the mevalonate pathway in early RN symbiotic signaling (Venkateshwaran et al. 2015).

Another example of a potential interactor in *M. truncatula* is the symbiotic remorin 1 (SYMREM1) protein, which is usually required for plant-bacteria interactions. This SYMREM1 can specifically interact with the symbiotic RLKs including LYK3/NFR1, NFR5/NFP, and DMI2/SymRK. The study of hairy roots in transgenic RNAi lines suggested that SYMREM1 functions as a scaffolding protein, and it might be required at the preinfection stage through the regulation of receptor proteins for the perception of bacterial signaling molecules (Lefebvre et al. 2010).

11.7 The Nuclear Components of Nod Factor Signaling and Their Identification via Hairy Root Transformation

11.7.1 Ion Channels and Nucleoporins

Calcium ions are key secondary intracellular messengers for a multitude of processes, relaying precise information by their ability to produce a wide variety of molecular signatures in both animal and plant cell signaling. Calcium signals are generated by a number of channels and pumps. In response to NFs, two different

calcium responses have been observed in legume roots, calcium flux and calcium oscillations. Calcium influx arises rapidly after receiving bacterial NFs, and subsequently a wave of calcium influx begins at the root hair tips and moves along the length of the root hair cell toward the nuclear membrane for membrane depolarization. Calcium oscillations or calcium spiking is observed approximately 10 minutes after the initial signal within the nucleus (Wais et al. 2002).

The mechanisms underlying calcium spiking during RN symbioses in the nucleus of root cells, which function downstream of the receptor and the cytosolic signaling, were enigmatic. A major breakthrough was attained by the characterization of genetic mutants, providing crucial information for understanding the nodulation signaling pathways. The two mutants of *L. japonicus*, *castor* and *pollux* genes, retained Ca^{2+} influx at the root hair tip but were impaired in the perinuclear Ca^{2+} spiking, which was required for establishing symbiotic relationships. The electrophysiological, yeast complementation and localization studies suggested that CASTOR and POLLUX are potassium-permeable cation channels. Homologs of these genes were identified in *M. truncatula* where DMI1 (DOES NOT MAKE INFECTIONS1) was characterized as a putative ortholog of POLLUX and in *P. sativum* where SYM8 (SYMBIOSIS8) was characterized as a putative paralogs of CASTOR (Edwards et al. 2007; Matzke et al. 2009). CASTOR and POLLUX share similarity with the NAD-binding TrkA domain of bacterial K^+ channels (Ane et al. 2004; Imaizumi-Anraku et al. 2005; Chen et al. 2009). Although CASTOR and POLLUX were reported to be localized in plastids, later investigation unveiled that DMI1 (POLLUX ortholog in *M. truncatula*) is restricted to the nucleus periphery and has a direct role in conducting ions in the nuclear compartment (Riely et al. 2007). In order to test the biological function of CASTOR and POLLUX, hairy root transformation was performed by complementing two mutants, namely, *castor-12* and *pollux-5*, respectively, with native genes expressed with a constitutive promoter. The results confirmed a positive role of CASTOR and POLLUX in mediating perinuclear Ca^{2+} spiking by the release of calcium from the nuclear envelope to modulate the nodulation signaling (Charpentier et al. 2008). A series of cross-species complementation experiments by transgenic hairy root transformation revealed that both DMI1 in *M. truncatula* and SYM8 in pea also have the capacity to compensate for the loss of CASTOR and POLLUX in *L. japonicus*, uncovering an unexpected twist in the evolution of ancestral and essential symbiotic proteins. An additional complementation assays using hairy roots revealed that a single amino acid change in DMI1 (serine to alanine substitution in the filter) is responsible for the improvement of DMI1 by enhancing the Ca^{2+}-induced Ca^{2+} release and reducing potassium conductance (Venkateshwaran et al. 2012). These data provide novel insights into the mechanism of DMI1 or CASTOR and POLLUX as calcium ion channels and underline its importance during rhizobial infection.

Considering that calcium changes also occur in the cytoplasm, an additional component, preferably a calcium pump, would be required at the nuclear membrane for efficient reuptake of the nuclear calcium. In an attempt to elucidate such components, MCA8, a nuclear-localized SERCA-type calcium ATPase, was

identified in *M. truncatula*. MCA8 localization was confirmed in transgenic hairy root cells by immunogold labeling. Unlike DMI1, MCA8 is present on both inner and outer nuclear membranes and at the ER to modulate the nuclear calcium oscillations by capturing the released calcium into the nuclear-associated cytoplasm. Furthermore, silencing of *MCA8* by hairy root transformation diminished NF-induced calcium oscillations, confirming its role as a calcium pump (Capoen et al. 2011).

Recently, for the identification of additional calcium channel proteins, hairy root transformation-based gene silencing approaches were used to assess the roles of different members of the cyclic nucleotide-gated channel (CNGC) gene family. RNAi-mediated silencing of *CNGC15a*, *CNGC15b*, and/or *CNGC15c* correlated with the defects in symbiotic associations. Localization studies confirmed that CNGC15 proteins are present in the nuclear envelope and are permeable to Ca^{2+}. Moreover, hairy root transformation revealed that variants of CNGC15 members complemented their respective mutant phenotypes establishing their roles in nuclear Ca^{2+} oscillations and in the symbiotic signaling pathway (Charpentier et al. 2016).

Characterization of another nodulation-deficient mutant *nup133* in *L. japonicus* by genetic and physical mapping, followed by sequencing of the mutant alleles, identified nucleoporins as regulators of nodulation signaling, especially by working at the junction of nuclear and intracellular plastid organelle membranes. To ascertain functionality of NUP133, *in planta* complementation of mutant alleles was performed using the hairy root transformation. *NUP133* gene successfully restored the mutant phenotype confirming its role in a rapid nuclear-cytoplasmic communication after host-plant recognition of symbiotic microbes (Kanamori et al. 2006). Later, another putative nucleoporin gene, *NUP85*, was identified through positional cloning and phenotypic analysis of a mutant from *L. japonicus*. Complementation of the *nup85* mutant with the putative nucleoporin-like gene demonstrated that *NUP85* is a prerequisite for nodule formation (Saito et al. 2007). Overall, multiple biochemical and genetic results suggest that nucleoporins such as NUP133 and NUP85 likely modulate the permeability of the nuclear pores to calcium ions, thereby facilitating the calcium spiking. Alternatively, nucleoporins might facilitate transport of POLLUX or CASTOR through the nuclear pore to the inner nuclear membrane (Matzke et al. 2009).

Major components of the nodulation signaling pathway including DMI2/SYMRK/Sym19, DMI1/POLLUX, NUP85, NUP133, and CASTOR are responsible for the establishment of both nodulation and mycorrhization. All these proteins are directly or indirectly involved to facilitate the calcium spiking for following a common symbiotic pathway. A genetic screen of a mutant related to arbuscular mycorrhizal (AM) symbiosis identified a WD40 repeat protein related to the nucleoporins, known as NENA. To test the functionality and localization of NENA during nodulation, hairy root transformation-based assays were performed. Complementation of *nena-1* mutant with the corresponding gene under native or constitutive promoter restored the nodule formation capacity. Interestingly NENA is localized at the nuclear rim by its interaction with NUP85 (Groth et al. 2010), implying an additional role of a nucleoporin in the control of symbiotic associations.

11.7.2 The CCaMK Complex

The LysM receptor kinase mutants (*nfr1* and *nfr5*) lacked both calcium influx and calcium spiking, whereas five other mutants including *SymRK* (LRR receptor kinase), *castor*, *pollux* (cation channels), *nup133*, and *nup85* (nucleoporins) were defective for calcium spiking but retained a calcium flux suggesting that these two steps can be delineated (Shaw and Long 2003; Miwa et al. 2006).

In *M. truncatula*, *dmi1* (*pollux*) and *dmi2* (*SymRK*) mutants were defective for calcium spiking, whereas *dmi3* mutants showed steady calcium spiking in response to NFs, suggesting that calcium spiking acts downstream of both DMI1 and DMI2 but upstream of DMI3. *DMI3* codes for CCaMK, a calcium/calmodulin-dependent serine-threonine protein kinase. *dmi3* mutants form no nodules, but this phenotype can be fully complemented by the introduction of the wild-type genomic sequence of *DMI3* gene by hairy root transformation (Levy et al. 2004). Furthermore, hairy root transformation of *snf1* (spontaneous nodule formation) mutant of *L. japonicus* with a candidate *CCaMK* gene resulted in the formation of spontaneous nodules, independent of the NFs, suggesting a central regulatory position of CCaMK upstream of all components required for cell cycle activation (Tirichine et al. 2006). Hairy root-based complementation analysis of another *CCaMK* mutant in *L. japonicus* (*ccamk-3*) by wild-type and gain-of-function variant of CCaMK ($CCaMK^{T265D}$) revealed that the protein is specific for nodule development (Shimoda et al. 2012) and works downstream of the common symbiotic pathway which is shared by nodulation and mycorrhization. This is different from DMI1 and DMI2 proteins, which are a part of the common symbiotic pathway.

Given the central importance of CCaMK, several methodologies have been used to identify its interacting partners in the last few years. A yeast-based approach identified a novel protein named IPD3 (interacting protein of DMI3) from *M. truncatula* as an interacting partner of CCaMK. Localization studies and promoter analysis by hairy root transformation revealed that IPD3 expresses primarily in the root vasculature and co-localizes with DMI3 to the nucleus (Messinese et al. 2007). Further characterization of *ipd3* mutants in *M. truncatula* confirmed that IPD3 function is partially redundant, i.e., nodulation (and mycorrhization) was initiated but then aborted (Horvath et al. 2011). PsSYM33 is an ortholog of IPD3 in *P. sativum* (Ovchinnikova et al. 2011), which also has a role in nodule development in pea. The IPD3 homolog in *L. japonicus* turned out to be the CYCLOPS gene. CYCLOPS is a phosphorylation target of CCaMK. The involvement CYCLOPS in rhizobial infection during symbiotic signaling was further confirmed after hairy root transformation-based complementation of *cyclops* mutant by the corresponding gene. Since *cyclops* mutants retained the ability to initiate cortical cell division during nodule organogenesis, it suggests that CYCLOPS is dispensable for nodule organogenesis (Yano et al. 2008; Limpens and Bisseling 2014). Moreover,

cyclops mutants cannot be complemented by either CCaMK gain-of-function mutant ($CCaMK^{T265D}$) or wild-type CCaMK indicating that CYCLOPS is positioned downstream of CCaMK in the symbiotic pathway (Hayashi et al. 2010). Later, hairy root transformations in different mutants of *L. japonicus* confirmed that CYCLOPS, a DNA-binding transcriptional activator, activates the *NODULE INCEPTION (NIN)* genes in a phosphorylation-dependent manner to regulate the symbiotic signaling (Singh et al. 2014).

Similar to CYCLOPS, CIP73, which belongs to the large ubiquitin superfamily, has been found to be a direct interacting partner and phosphorylation target of CCaMK. RNAi-mediated silencing of *CIP73* in *L. japonicus* hairy roots resulted in fewer nodules, suggesting that CIP73 is a positive regulator of nodulation (Kang et al. 2011). Further yeast-based experimental analysis recognized a cochaperone protein, HIP (HSC/HSP70 interacting protein), as an interacting partner of CIP73. Unlike CIP73, the suppression of HIP expression in hairy roots led to increased nodule numbers, indicating that HIP is a negative regulator of nodulation (Kang et al. 2015).

CCaMK-CYCLOPS complex initiates nodule organogenesis following calcium oscillations in the host nucleus. Further investigation of downstream signaling identified DELLA protein in *M. truncatula*, which are the central regulators of gibberellic acid (GA) signaling. These DELLAs increased the phosphorylation of CYCLOPS by forming a complex with CCaMK-CYCLOPS. To assess whether DELLA proteins have a role during symbiosis, hairy root transformation was used to decrease the expression of *DELLAs* by RNAi approaches. Knocking down *DELLAs* caused a decrease in nodule number in the hairy roots demonstrating their positive regulatory roles in RN symbiosis (Jin et al. 2016). Furthermore, DELLAs can form a protein complex with transcription factors NSP1-NSP2 (nodulation signaling pathway 1 and 2) and are able to form a connection between CYCLOPS and NSP2 (Jin et al. 2016) indicating their role in GA-mediated RN symbiosis.

11.7.3 Transcription Factors Involved in RN Symbiosis

Both *NSP1* and *NSP2* of *M. truncatula* encode genes with similarity to members of the GRAS family of putative transcriptional regulators or transcription factors. *SYM7* of *P. sativum* is a possible ortholog of *NSP2*. In addition to the classical genetic analysis, hairy root transformation was used to decipher the function of NSP1 and NSP2 by complementation and subcellular localization. Complementation of *nsp1 and nsp2* mutants by native genes and subcellular localization using native promoter-driven *NSP1/2-GFP* established that both these proteins co-localize with CCaMK in the nucleus, and CCaMK acts directly upstream of NSP1 and NSP2 (Catoira et al. 2000; Kalo et al. 2005; Smit et al. 2005). NSP1-NSP2 heteropolymerization is essential for nodulation signaling (Hirsch et al. 2009). *NSP1* binds to the promoter of the NF-inducible genes, namely, *ENOD11*, *ERN1*,

and *NIN*. To assess the functional role of NSPs, *nsp2* mutants were complemented with the wild-type gene as well as the domain-swapped *NSP2* or variant *NSP2* which can no longer dimerize with NSP1. Termination of nodule formation in domain-swapped NSP2 and a decreased nitrogen fixation activity in variant *NSP2* transgenic hairy roots confirmed their role as functional heterodimers.

To identify additional regulatory components of nodulation, a transposon-tagged *L. japonicus* mutant *nin* (nodule inception) was identified, which produces no nodules (Schauser et al. 1999). NIN is an essential transcription factor responsible for initiating nodulation-specific symbiotic processes, and it acts downstream of the *NSP* genes. *Sym35* gene required for root nodule development in *P. sativum* is an ortholog of *NIN* (Borisov et al. 2003). Hairy root transformation confirmed the functional complementation of *nin-1* mutants by *NIN1*. Additionally, the overexpression of *NIN* in *M. truncatula* induced cortical cell divisions leading to spontaneous nodule development in the transgenic roots in the absence of rhizobia, suggesting that NIN functions downstream of CCaMK (Soyano et al. 2013; Marsh et al. 2007). Transcriptional profiling and promoter analysis revealed that *NIN* restricts the *ENOD11* expression through competitive inhibition of *ERN1* (Vernie et al. 2015).

To investigate the downstream targets of NIN, two transcriptional targets, namely, *NF-YA1* and *NF-YB1*, were identified by a screen for suppressors of the *L. japonicus har1-1* hypernodulation phenotype. These NF-Ys (A, B, C subunits) are heterotrimeric CCAAT box-binding transcription factors. HAP2 and HAP3 in *M. truncatula* are the possible orthologs of LjNF-YA1 and LjNF-YB1. Interestingly, *RNAi*-mediated silencing of *NF-YA1* in *L. japonicus* hairy roots did not inhibit the epidermal responses and led infection thread formation and growth but prevented cortical cell division required for the development of nodules. Similar response was exhibited by the loss of function of *NIN*. Conversely, overexpression of *NIN* and *NF-Y* genes in *L. japonicus* enhanced cell division during nodule formation, implying that NIN is a key player in root nodule organogenesis and NF-Y subunits function downstream of NIN (Soyano et al. 2013; Combier et al. 2006). Additionally, gene expression analysis in the hairy roots of *NF-YA* RNAi lines suggested that *NF-YA1/2* acts upstream of *ERN1* and *ENOD11* in the nodulation signaling pathway (Laloum et al. 2014). Recently, three more members of SHI/STY (SHORT INTERNODES/STYLISH) transcription factor gene families, namely, STY1, STY2, and STY3, have been identified as direct targets of *NF-YA1* (Hossain et al. 2016). A cytokinin receptor CRE1 (cytokinin response element 1) is essential for nodule organogenesis (Plet et al. 2011; Gonzalez-Rizzo et al. 2006), and *CRE1* promoter-driven expression of GUS in *M. truncatula* hairy roots was significantly reduced in the *nin-1* mutant compared to the wild type. This suggests that NIN binds to the *CRE1* promoter and activates *CRE1* expression in the root cortex (Vernie et al. 2015; Soyano et al. 2014).

A genetic screen in a population of fast neutron-mutagenized *M. truncatula* plants identified a gene, *BIT1* (*branching infection threads 1*), necessary for the infection thread formation. Overexpression of auto-activated CCaMK in *bit1-1* mutants by hairy root transformation did not produce any spontaneous nodules, demonstrating

that *BIT1* functions downstream of CCaMK for the activation of nodule organogenesis. Overexpression of *ENOD11-GUS* in *bit1-1* mutants showed severely reduced *ENOD11-GUS* induction after NFs application, confirming BIT1's role in nodulation pathway. Furthermore, an ethylene response factor (ERF) required for nodulation, ERN (ERF required for nodulation), complemented the *bit1-1* mutant phenotype and confirmed that ERN is necessary for NF signaling and functions by activation of *ENOD11* (Middleton et al. 2007; Andriankaja et al. 2007). Silencing and overexpression of *EFD* (ethylene response factor required for nodule differentiation), another ERF transcription factor, by hairy root transformation affected the nodule development by regulation of the cytokinin pathway genes (Vernie et al. 2008). These data provide a new connection between ethylene and cytokinin pathway transcription factors during nodulation signaling.

RNAi-mediated silencing and overexpression studies in hairy root system also demonstrated the role of KNOX transcription factors in nodule development in *M. truncatula* (Di Giacomo et al. 2017). Similarly, gene silencing also revealed that a Myb transcription factor, *control of nodule development* (*CND*), is also involved in regulation of soybean nodulation (Libault et al. 2009).

11.7.4 Downstream Regulatory Genes Involved in Nodule Development

Genetic studies and transcriptome analysis have identified a number of downstream genes essential in NF signaling. To understand the molecular mechanisms of these genes, hairy root transformation became a suitable tool to assess them by complementation analysis, *RNAi*-mediated gene silencing, overexpression, and promoter analysis. Table 11.2 lists a number of genes, which were identified as potential regulators of nodule formation by using hairy root approaches. Further targeted analysis will pinpoint how these genes are connected to the established modules of nodulation signaling.

11.8 Long-Distance Control of Nodulation

Nodulation is an extremely energy-requiring process, and plants control both the timing and number of nodules formed by a shoot-derived protein which encodes a putative leucine-rich, serine-threonine receptor kinase with homology to *Arabidopsis* CLAVATA1 (CLV1). This receptor-like kinase is activated from root-derived CLE peptides. The lack of CLV1 protein due to gene disruption causes hyper- or supernodulation in legume roots due to a defect in the systemic negative feedback mechanism called autoregulation of nodulation (AON). AON is initiated during nodule development by the synthesis of a root-derived signal named "Q" or

Table 11.2 Examples of downstream genes responsible for nodule formation as confirmed by hairy root transformation

Gene name	Scientific name (Plant)	Gene silencing (RNAi)	Gene silencing (Micro RNA)	Over-expression	References
ENOD40 (*ENOD40-1* and *ENOD40-2*)	*Medicago truncatula*	+	–	–	Kumagai et al. (2006), Wan et al. (2007)
CDC16 (*CELL DIVISION CYCLE16*)	*Medicago truncatula*	+	–	–	Kuppusamy et al. (2009)
RbohA (*NADPH oxidase*)	*Medicago truncatula*	+	–	–	Marino et al. (2011)
nsRING (an RING-H2 finger domain protein)	*Lotus japonicus*	+	–	–	Shimomura et al. (2006)
GS52, an *ecto-apyrases*	*Glycine max*	+	–	–	Govindarajulu et al. (2009)
FWL1 (*FW2-2-like1*)	*Glycine max*	+	–	–	Libault et al. (2010)
EXPB2, an *expansin* gene	*Glycine max*	+	–	+	Li et al. (2015)
SGF14c/SGF14l, an G-box factor	*Glycine max*	+	–	–	Radwan et al. (2012)
PT5, a phosphate transporter	*Glycine max*	+	–	+	Qin et al. (2012)
UPS1 (*ureide permease 1*)	*Glycine max*	+	–	–	Collier and Tegeder (2012)
ACP, an acyl carrier protein	*Glycine max*	+	–	–	Wang et al. (2014)
S6 kinase 1	*Glycine max*	+	–	–	Um et al. (2013)
GH3, *GRETCHEN HAGEN 3*	*Glycine max*	–	+	–	Damodaran et al. (2017)
NMHC5, a sucrose regulatory MADS-box transcription factor	*Glycine max*	–	–	+	Liu et al. (2015)
Early nodulin 93 (*ENOD93*)	*Glycine max*	–	+	–	Yan et al. (2015)
Mannosyl-oligosaccharide 1, *2-alpha-mannosidase* (*MNS*)	*Glycine max*	–	+	–	Yan et al. (2016)
Rhizobium-induced peroxidase 1(*RIP1*)	*Glycine max*	–	+	–	Yan et al. (2016)
Rbohb (NADPH oxidase)	*Phaseolus vulgaris*	+	–	–	Montiel et al. (2012)
IFR1, an isoflavone reductase gene family	*Phaseolus vulgaris*	+	–	–	Ripodas et al. (2013)

(continued)

Table 11.2 (continued)

Gene name	Scientific name (Plant)	Gene silencing (RNAi)	Gene silencing (Micro RNA)	Over-expression	References
RACK1, a receptor for activated C kinase	*Phaseolus vulgaris*	+	–	–	Islas-Flores et al. (2011)
TOR, a protein kinase gene, rapamycin	*Phaseolus vulgaris*	+	–	–	Nanjareddy et al. (2016)
TPS9, a class II trehalose-6-phosphate synthase	*Phaseolus vulgaris*	+	–	–	Barraza et al. (2016)
HK1, a cytokinin receptor histidine kinase	*Arachis hypogaea*	+	–	–	Kundu and DasGupta (2017)

CLE peptide. CLE peptides move from the roots to shoots through xylem after inoculation with rhizobia and are perceived by CLV1. In *L. japonicus*, CLE Root Signal 1 (CLE-RS1) and CLE Root Signal 2 (CLE-RS2) are representative members of CLV3-like peptides and are strong candidates for the root-derived signal, which modulate nodulation by following CLV signaling pathway. *CLE12* and *CLE13* are two representative CLE peptide genes in *M. truncatula*, which potentially bind to CLV1. A hairy root transformation study in *M. truncatula* showed that overexpressing both these peptide genes inhibited nodulation systemically, and knockdown of *CLE12* and *CLE13* resulted in an increase in nodule number (Okamoto et al. 2009; Mortier et al. 2012). Additionally, the *CLE-RS1/2* of *L. japonicus* can directly bind to CLV1 or HAR1, and the suppression of nodule numbers due to the overexpression of *CLE-RS1/2* depends on CLV1/HAR1 (Okamoto et al. 2013). Similarly, three candidates of CLE peptide-encoding genes, *RIC1*, *RIC2*, and *NIC1*, have been identified in soybean. Overexpression of these peptides in wild-type plants inhibits nodulation, whereas their overexpression in *clv1* or *nark* mutants had no effect on the nodule number, confirming that nodule number inhibition by CLE peptide is CLV1/NARK1 (nodulation autoregulation receptor kinase) dependent (Reid et al. 2011; Lim et al. 2011).

A screen for supernodulating mutants, defective in AON, identified loss-of-function alleles of several genes. For example, the *rdn1* mutant of *M. truncatula* and a *nod3* mutant of pea exhibit increased nodulation and reduced root growth. In *M. truncatula*, this mutant phenotype was rescued by expressing *RDN1* (*ROOT DETERMINED NODULATION1*) by hairy root transformation (Schnabel et al. 2011) suggesting that it may have a role in the production or transport of CLE peptides (Li et al. 2009).

These CLE peptides activated CLAVATA1 leucine-rich serine-threonine receptor kinase protein which is essential for shoot-controlled regulation of root growth, nodule number, and nitrate sensitivity of symbiotic development. The supernodulation phenotype is caused due to a mutation in a *CLV1*gene known as

SUNN1 (super numeric nodules) in *M. truncatula* and *HAR1* in *L. japonicus* (Nishimura et al. 2002; Schnabel et al. 2005). In pea and soybean, the orthologs of this gene are named *SYM29* and *NARK*, respectively (Krusell et al. 2002; Searle et al. 2003). Additional proteins, corresponding to CLAVATA2, which is known to work together with CLV1, have also been identified in *L. japonicus* (*CLV2*) and *P. sativum* (*sym28*) (Krusell et al. 2011). Another LRR-RLK kinase, KLAVIER (KLV), identified from *L. japonicus* is also involved in shoot regulation of nodulation (Miyazawa et al. 2010). Some of the phenotypes of *klavier* mutants are similar to the *clv1/har1* mutant phenotype suggesting that KLAVIER is likely involved in the CLV signaling pathway.

Two kinase-associated protein phosphatases (KAPP1 and KAPP2) interact with the phosphorylated kinase domains of NARK or CLV1. Both KAPP1 and KAPP2 are transphosphorylated by NARK, and, in turn, the PP2C domain of the KAPP1 and KAPP2 dephosphorylates NARK receptor to relay the signal generated by the formation of shoot-derived inhibitor (SDI). The SDIs enter the phloem and move down to the roots to prevent further nodule development (Miyahara et al. 2008; Lin et al. 2010).

TML (TOO MUCH LOVE) encodes a kelch repeat-containing F-box protein, which has a role in AON signaling. Gene silencing and overexpression approaches by hairy root transformation revealed that TML, HAR1, and CLE-RS1/RS2 negatively regulate nodule organogenesis in the same genetic pathway. Furthermore, TML might suppress the nodulation signaling downstream of the HAR1 and CLE peptides and might function in the long-distance regulation of the legume-rhizobium symbiosis (Takahara et al. 2013).

11.9 Conclusions and Future Work

As is evident from the examples listed in the previous sections, the use of hairy roots has been transformative in studying and deciphering almost every aspect of nodulation signaling. It was especially useful as early on, most legumes were considered recalcitrant to tissue culture-based transformation and regeneration. Recent advances in the genome-editing technologies are going to make it even more useful, as constructs can be evaluated using the hairy root system before investing in stable transformation and genetic manipulation of important leguminous crops. There are already studies demonstrating its feasibility (Wang et al. 2017). This could be especially useful in case of polyploid legumes where multiple genes can be edited simultaneously to achieve desired phenotypes and potentially improved nitrogen use efficiency in crops.

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Chapter 12
Hairy Roots as a Tool for the Functional Analysis of Plant Genes

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Abstract With its root-inducing (Ri) plasmid, *Agrobacterium rhizogenes* is a valuable alternative to transfer gene constructs into the genome of plant species which are difficult to stably transform with disarmed strains of *Agrobacterium tumefaciens*. Composite plants consisting of transformed hairy roots induced on a non-transgenic shoot have been reported in an increasing number of legume and nonlegume plant species. They were first used in the model legumes *Medicago truncatula* and *Lotus japonicus* to study the symbiotic interaction with rhizobia. Since then, composite plants have been shown to be effective to investigate the function of genes involved in mycorrhizal symbiosis, root-nematode and root-pathogen interactions, resistance response of plant roots to parasitic weeds, root development and branching, and the formation of wood. The different methodologies developed to generate composite plants and the applications of co-transformed hairy roots for studying gene function are discussed in this chapter, together with recent opportunities offered by genome editing technologies in hairy roots.

Keywords *Agrobacterium rhizogenes* · Composite plant · Gene functional analysis · Genome editing · Hairy root

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12.1 Introduction

Once a gene has been isolated, the exploration of its function begins with DNA sequence analysis together with a search of public databases for characterized genes with similar sequences. However, such a comparison involves certain risks since similarity at the nucleotide level does not always mean the gene product will have a similar structure and function. Additional information can be obtained by analyzing the spatiotemporal expression of the studied gene and its response to several biotic and abiotic factors. Nevertheless, one of the most significant steps in the functional analysis usually involves the study of transgenic plants in which the gene has been knocked out by mutagenesis, overexpressed, or downregulated (Rhee and Mutwil 2014). With the alterations observed in the plant phenotype, important conclusions can be drawn concerning the function of the corresponding gene.

Agrobacterium tumefaciens-mediated transformation is the most popular technique to generate transgenic plants. However, a major problem linked to the use of this bacterium is the need for efficient organ regeneration and transformation in plants (Anami et al. 2013). To study genes expressed in plant roots, *Agrobacterium rhizogenes* offers a valuable alternative to disarmed strains of *A. tumefaciens*. This gram-negative soil bacterium is responsible for the development of hairy root disease in many dicotyledonous plants as well as in some gymnosperms and monocotyledonous plants (Tepfer 1990). In a process similar to that described for *A. tumefaciens*, *A. rhizogenes* transfer into the genome of the infected host plant a T-DNA fragment from the bacterial root-inducing (Ri) plasmid carrying oncogenes that encode enzymes which control auxin and cytokinin biosynthesis (Koplow et al. 1984; Britton et al. 2008). The resulting modifications in the hormonal balance induce the formation of roots at the wounding site which are morphologically different from normal roots. The so-called hairy roots are characterized by rapid hormone-independent growth, are much more branched, have numerous root hairs, and exhibit plagiotropic root development. *A. rhizogenes* has proven to be a valuable tool for generating transgenic roots which are easy to grow and can be used for a range of biological applications including metabolic engineering and phytoremediation, as well as for the production of valuable secondary metabolites and recombinant proteins (Guillon et al. 2006; Talano et al. 2012; Mehrotra et al. 2015).

A. rhizogenes hairy roots have other valuable applications in many areas of basic plant research. This pathogenic bacterium can be used to generate composite plants consisting of transformed hairy roots induced on a non-transgenic shoot (Beach and Gresshoff 1988; Hansen et al. 1989; Collier et al. 2005). Binary vectors carrying appropriate gene constructs can be introduced into oncogenic strains of *A. rhizogenes*; the resulting bacteria can then be used to obtain co-transformed hairy roots which integrate both the T-DNA from the Ri plasmid and the T-DNA from the genetically engineered binary vector. The co-transformation procedure enables more rapid analysis of transformed roots than the methods used to generate plants which are stably transformed by disarmed *A. tumefaciens* or by direct gene

Table 12.1 Composite plant-inducible families and species

Family	Genus/species
Apiaceae	*Daucus carota*
Brassicaceae	*Arabidopsis thaliana* *Brassica oleracea*
Casuarinaceae	*Allocasuarina verticillata* *Casuarina glauca*
Chenopodiaceae	*Beta vulgaris*
Convolvulaceae	*Ipomoea batatas*
Cucurbitaceae	*Cucurbita pepo*
Datiscaceae	*Datisca glomerata*
Fabaceae	*Aeschynomene indica* *Arachis hypogaea* *Glycine max* *Lotus corniculatus* *Lotus japonicus* *Lupinus albus* *Medicago truncatula* *Phaseolus vulgaris* *Pisum sativum* *Sesbania rostrata* *Trifolium pratense* *Trifolium rubens* *Vicia hirsuta* *Vigna aconitifolia* *Vigna unguiculata*
Lauraceae	*Persea americana*
Malvaceae	*Hibiscus esculenta*
Myrtaceae	*Eucalyptus camaldulensis* *Eucalyptus grandis*
Poaceae	*Zea mays*
Rhamnaceae	*Discaria trinervis*
Rosaceae	*Prunus* spp.
Rubiaceae	*Coffea arabica*
Salicaceae	*Populus* sp.
Solanaceae	*Lycopersicon esculentum* *Nicotiana benthamiana* *Nicotiana tabacum* *Petunia x hybrida* *Solanum tuberosum*
Theaceae	*Camellia sinensis*

Adapted from Collier et al. (2005) and completed with recent references

transfer techniques, such as biolistic or protoplast electroporation. Composite plants have now been reported in at least 18 plant families including about 40 species (Table 12.1), and the utility of the co-transformed hairy roots for investigating the function of genes involved in different aspects of root development and biotic interactions is now well established.

Different methods to generate composite plants using *A. rhizogenes* are described in this chapter, and their contribution to the functional analysis of candidate genes involved in different physiological processes is illustrated. In addition to promoter studies and downregulation of gene expression resulting from RNA interference (RNAi) experiments, the recent development of genomic mutations induced by the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system in composite plants is presented.

12.2 *Agrobacterium Rhizogenes*-Mediated Transformation: Technical Aspects

12.2.1 Factors Involved in a Successful T-DNA Transfer by A. rhizogenes

A successful transfer of a T-DNA into a plant species involves many factors that need to be optimized, especially when the host is poorly susceptible to agrobacterial infection. Fortunately, knowledge of the molecular mechanisms underlying the interaction between plants and agrobacteria has progressed remarkably in recent years, making it possible to genetically transform an increasing number of plant species (Lacroix and Citovsky 2013). One of the first factors to consider is the choice of the *A. rhizogenes* strain. Several strains need to be tested to identify the bacteria capable of inducing hairy roots with a phenotype that is as close as possible to the one of the non-transformed roots. A very pronounced hairy root phenotype can profoundly alter root architecture and biotic interactions, thus affecting conclusions drawn concerning the function of the candidate genes (Dolatabadian et al. 2013). In the actinorhizal shrub *Discaria trinervis*, for example, both A4RS and ARqua1 strains induced transgenic roots at the site of infection, but they differed in their phenotype (Imanishi et al. 2011). Since the hypervirulent strain A4RS had a strong impact on root architecture, further experiments with composite plants of *D. trinervis* were conducted with Arqua1. The second factor involved in a successful gene transfer is indeed linked to the host, which needs to provide the appropriate signaling molecules to activate the virulence genes of *A. rhizogenes*. The addition of exogenous phenolic compounds such as acetosyringone can sometimes improve the dialogue between the host and the agrobacterium (Lacroix and Citovsky 2013).

The co-cultivation phase, during which the host and the bacteria are usually in contact for 1–6 days, is another critical step in the interaction. It has been shown that the T-DNA transfer and integration can be affected by bacterial density, plant age and growth conditions, light, gas exchange, nutrient medium, growth regulators, pH, and humidity, among other factors (Karami 2008). Unfortunately, optimal conditions for the genetic transformation have to be studied empirically, requiring intensive work to combine the different factors.

12.2.2 In Vitro and Ex Vitro Transformation Procedures

Both in vitro and ex vitro techniques have been successfully used to generate composite plants. In the in vitro process, plants have to be germinated, grown, and manipulated in aseptic conditions. When young seedlings are used, inoculations with *A. rhizogenes* can be achieved by wounding the hypocotyls with a needle dipped in a fresh colony (or culture in exponential growth) of the chosen *A. rhizogenes* strain (i.e., Diouf et al. 1995). It has also been reported that the agrobacterium culture can be injected directly into the stem (Markmann et al. 2008). An alternative procedure consists in sectioning the radicle of the seedlings with a scalpel and then coating the sectioned surface with *A. rhizogenes* (Boisson-Dernier et al. 2001). The rate of development of hairy roots varies considerably depending on the method of infection. For instance, in the tropical tree *Casuarina glauca*, hairy roots developed in less than 30% of the plants with a sectioned hypocotyl, whereas when hypocotyls were inoculated with a needle, hairy roots developed on 95% of the plants (Svistoonoff et al. 2010). To obtain the composite plants, the normal non-transformed root system is removed about 3 weeks after inoculation with *A. rhizogenes*, and when possible, co-transformed roots containing the newly introduced genes from the appropriate binary vector are selected. It should be noted that, even though the shoot is not transgenic, composite plants sometimes exhibit an altered aerial part with shorter internodes. This alteration of phenotype is probably linked to the modification of the hormonal balance in the transgenic root system displaying the hairy root phenotype.

Ex vitro procedures may be preferred to avoid the constraints and costs linked to tissue culture and aseptic conditions. This technique was first reported in 2005 with the introduction and expression of the reporter gene *gfp* in hairy roots induced in 14 different plant species belonging to five different orders including nine plant families (Collier et al. 2005). Apical stems from young plants were directly inserted into rockwool cubes containing the *A. rhizogenes* inoculum. After 3 weeks to 2 months, hairy roots were observed on 56–100% of the inoculated stems, depending on the plant species. The major challenge of this simple procedure is preventing the dispersal of the transgenic pathogenic rhizobacteria in the environment, thus requiring an appropriate confined growth chamber or glasshouse.

12.2.3 Selection of Co-transformed Hairy Roots

One advantage of *A. rhizogenes*-mediated transformation is that transgenic roots can be obtained without using a selection agent. Hairy root morphology is used for the primary selection of transgenic roots. However, when performing co-transformation with an *A. rhizogenes* strain harboring a genetically engineered gene construct in a binary vector, a selection procedure with either a reporter gene or an antibiotic resistance gene is usually required to detect the co-transformed roots and to facilitate molecular and phenotypic analyses of the composite plants.

Based on experience gained with the actinorhizal tree *C. glauca*, and the analysis of several hundred composite plants, the rate of co-transformed hairy roots can vary from 20% to 65%. In order to identify the co-transformed roots, a constitutively expressed reporter gene such as ß-glucuronidase (GUS) (Jefferson et al. 1987), DsRED1, or green fluorescent protein (GFP) gene (Haseloff and Siemering 2006) was included in the T-DNA of the binary vector. Reporter genes encoding fluorescent proteins appeared to be the best candidates since their gene products could be visualized in roots under UV light without affecting the viability of plant tissues. Interestingly, the intensity of fluorescence was generally correlated with the level of expression of the other genes stacked on the T-DNA of the binary vector. In RNAi experiments designed to downregulate symbiotic genes in *C. glauca*, hairy roots displaying a high level of fluorescence also exhibited a strong extinction of the candidate symbiotic gene, as determined by q-RTPCR (Gherbi et al. 2008a).

Kanamycin selection of co-transformed roots has occasionally been performed using the *nptII* gene in the transferred T-DNA. A range of kanamycin concentrations has to be tested to inhibit the growth of non-co-transformed hairy roots on the agar plates. After 2 or 3 weeks of incubation with kanamycin, the non-co-transformed roots stop growing, turn brown, and do not penetrate the agar nutrient medium, whereas the co-transformed roots continue to grow rapidly on the agar. Once the hairy roots have developed, the antibiotic has to be rapidly removed to avoid a negative impact on the growth of the non-transformed aerial part of the composite plants.

12.3 Functional Analyses of Plant Genes in Composite Plants

In recent years, a wide diversity of composite plants have been used to improve the functional analysis of plant genes expressed in roots, the largest number of publications being in the area of plant-microbe interactions (Table 12.2). As illustrated below, the use of *A. rhizogenes* together with that of RNA interference (RNAi) has proven to be very useful to study gene function using reverse genetics.

12.3.1 Study of Interactions Between the Host Plant and Nitrogen-Fixing Microorganisms

Due to the difficulty in obtaining transgenic legumes using *A. tumefaciens*, composite plants were rapidly used to characterize the plant genes involved in the symbiotic process with nitrogen-fixing rhizobia. *A. rhizogenes* transformation was first described for *Lotus corniculatus* (Jensen et al. 1986) and subsequently used in the two model plants *Medicago truncatula* (Boisson-Dernier et al. 2001) and *Lotus*

Table 12.2 Examples of gene function analysis using composite plants obtained after genetic transformation using *Agrobacterium rhizogenes*

Plant	Gene	Function	References
Aeschynomene indica	*gus*	Reporter gene	Bonaldi et al. (2010)
Allocasuarina verticillata	*gus*	Reporter gene	Gherbi et al. (2008b)
Arabidopsis thaliana	*KOJAK* *gfp*	Root hair development Reporter gene	Limpens et al. (2004) Collier et al. (2005)
Arachis hypogaea	*gfp, gus* *Cry8Ea1* *AdEXLB8* *EXLB*	Reporter genes Toxicity against white grubs Nematode resistance Stress-responsive expansin gene	Sinharoy et al. (2009) Geng et al. (2013) Guimaraes et al. (2017a) Guimaraes et al. (2017b)
Camellia sinensis	*gus*	Reporter gene	Alagarsamy et al. (2018)
Casuarina glauca	*gus* *SYMRK* *CHS* *CCaMK*	Reporter gene Root nodulation Flavonoid pathway Root nodulation	Diouf et al. (1995) Gherbi et al. (2008a) Abdel-Lateif et al. (2013) Svistoonoff et al. (2013)
Coffea arabica	*gus*	Reporter gene	Alpizar et al. (2006)
Cucurbita pepo	*DR5-gus, DR5-gfp*	Reporter genes driven by an auxin-responsive promoter	Ilina et al. (2012)
Discaria trinervis	*ENOD11-gus*	Marker of *Frankia* infection	Imanishi et al. (2011)
Eucalyptus camaldulensis	*gfp*	Reporter gene	Balasubramanian et al. (2011)
Eucalyptus grandis	*CCR1*	Lignin biosynthesis	Plasencia et al. (2016)
Glycine max	*IFS* *TIP1* *EXPB2* *SPX3* *Fib-1* *Y25C1A.5*	Isoflavone synthase Salt tolerance Cell wall ß-expansin Phosphorous signaling pathway Nematode reproduction and fitness	Subramanian et al. (2004, 2006), White et al. (2015) An et al. (2017) Guo et al. (2011), Li et al. (2015) Yao et al. (2014) Li et al. (2010)
Lotus japonicus	*gus, luc*	Reporter genes	Stiller et al. (1997)
Lupinus albus	*LaMATE*	Phosphorous stress	Uhde-Stone et al. (2005)
Lycopersicon esculentum	*gfp*	Reporter gene	Collier et al. (2005)
Medicago truncatula	*ENOD11* *ENOD8* *ROP9* *RbohE* *ABCG10* *RDN*	Root infection by *Rhizobium* Root nodulation Oomycete colonization Role in arbuscule development ABC transporter of the G subfamily Root nodulation	Boisson-Dernier et al. (2001, 2005) Coque et al. (2008) Kiirika et al. (2012) Belmondo et al. (2016) Banasiak et al. (2013) Kassaw et al. (2017)
Populus spp.	*YFP*	Reporter gene	Neb et al. (2017)

(continued)

Table 12.2 (continued)

Plant	Gene	Function	References
Persea americana	*gus, gfp*	Reporter genes	Prabhu et al. (2017)
Phaseolus vulgaris	*NIN* *gus, gfp*	Root nodulation Reporter genes	Nanjareddy et al. (2017) Colpaert et al. (2008)
Pisum sativum	*LYK9*	Control of plant immunity	Leppyanen et al. (2017)
Prunus cerasifera	*Ma*	Nematode resistance	Claverie et al. (2011)
Prunus spp.	*Egfp*	Reporter gene	Bosselut et al. (2011)
Solanum tuberosum	*gus*	Reporter gene	Horn et al. (2014)
Vicia hirsuta	*gus*	Reporter gene	Quandt et al. (1993)
Vigna unguiculata	*RSG3-301*	Resistance to *Striga gesnerioides*	Mellor et al. (2012)
Zea mays	*gfp*	Reporter gene	Runo et al. (2012)

In this table, functional analyses include promoter studies, overexpression of the candidate genes, or RNAi experiments to downregulate the genes studied. Reporter genes were usually used to establish the proof of concept of the technology.
ABCG, ATP-binding cassette transporter of the G family; ABC transporter; CCaMK, calcium/calmodulin-dependent kinase; CCR1, cinnamoyl-CoA reductase 1; CHS, chalcone synthase; CRY8Ea1, CRY protein from *Bacillus thuringiensis*; DR5, auxin-responsive promoter; ENOD8, nodule-specific esterase; ENOD11, early nodulin; EXL, expansin-like protein; EXP, expansin gene; gfp, green fluorescent protein gene; gus, ß-glucuronidase gene; IFS, isoflavone synthase gene; KOJAK, cellulose synthase-like protein; LaMATE, *Lupinus albus* multidrug and toxin efflux; luc, luciferase; LYK9, Lys-M-receptor like kinase; Ma, R protein; NIN, nodule inception gene; RbohE, NADPH oxidase; RDN, root-determined nodulation protein; ROP9, GTPase; SPX, protein related to phosphate homeostasis and signaling; SYMRK, symbiosis receptor kinase; TIP, tonoplast intrinsic protein; Yfp, yellow fluorescent protein gene

japonicus (Stiller et al. 1997). Composite plants have also been reported in *Glycine max* (Kereszt et al. 2007; Cao et al. 2009), *Vicia hirsuta* (Quandt et al. 1993), *Vigna aconitifolia* (Lee et al. 1993), *Phaseolus vulgaris* (Estrada-Navarrete et al. 2006; Colpaert et al. 2008), *Trifolium rubens* (Diaz et al. 1989), and *T. pratense* (Diaz et al. 2000).

Composite plants have largely contributed to a better understanding of the symbiotic dialogue established between the host and nitrogen-fixing rhizobial strains, in legumes which develop determinate or indeterminate nodules, and which undergo either an intracellular or intercellular infection process. Whereas mutants in the model plants *M. truncatula* and *L. japonicus* led to the identification of the so-called common symbiotic pathway (CSP) (Gueurts et al. 2016), RNAi experiments in composite plants confirmed that the CSP was also involved in the nodulation process of legumes in which the infection process does not proceed via root hair infection, such as *Sesbania rostrata* (Van de Velde et al. 2003), *Arachis hypogea* (Sinharoy et al. 2009), and *Aeschynomene indica* (Bonaldi et al. 2010).

Major advances have also been made in actinorhizal plants which develop nitrogen-fixing nodules following interaction with the gram-positive actinobacteria

Frankia. Since it takes about 12 months to obtain transgenic nodulated plants of *C. glauca* resulting from a T-DNA transfer by *A. tumefaciens* (Smouni et al. 2002), composite plants were used to generate data on a large number of co-transformed hairy roots more rapidly. This method was first used in 1995 (Diouf et al. 1995) to demonstrate that a promoter from a legume hemoglobin gene kept its spatiotemporal pattern of expression in an actinorhizal nodule, thus suggesting the conservation of molecular mechanisms underlying the nodulation process between actinorhizal plants and legumes. With the development of the RNAi technology, downregulation of two genes isolated from *C. glauca* and sharing homology with the receptor-like kinase SYMRK and the calcium- and calmodulin-dependent kinase CCaMK genes from the CSP in legumes revealed that this pathway was also required by *Frankia* for root infection and nodulation (Gherbi et al. 2008a; Svistoonoff et al. 2013). Additional data obtained in composite plants of two other actinorhizal plants *D. trinervis* (Imanishi et al. 2011) and *Datisca glomerata* (Markmann et al. 2008), which cannot be transformed by *A. tumefaciens*, have also considerably enriched our knowledge of the original nodulation process resulting from *Frankia* intercellular infection.

12.3.2 Plant Mycorrhizal Interactions

Arbuscular mycorrhiza is a major widespread mutualistic association that concerns 80% of land plants and involves fungi of the phylum Glomeromycota. The plant provides carbohydrates to the fungus which, in return, supplies the host with mineral nutrients, especially phosphate, and improves water absorption and disease resistance (Lanfranco et al. 2016).

In *M. truncatula*, together with the possibility to obtain nitrogen-fixing nodules on composite plants, it has been shown that these roots can be colonized by endomycorrhizal fungi, even when the hairy roots are excised from the composite plants and propagated as independent organs (Boisson-Dernier et al. 2001, 2005; Mrosk et al. 2009). With the actinorhizal plant *C. glauca*, it was not possible to grow hairy roots independently, but mycorrhization by *Rhizophagus irregularis* occurred on the hairy roots of composite plants (Gherbi et al. 2008a). Composite legume and actinorhizal plants were further used to compare gene expression during the symbiotic process with rhizobium and/or *Frankia* and in endomycorrhizal associations. These experiments, together with the study of legume mutants, have provided evidence that the common signaling pathway involved in the nodulation process is necessary for all root endosymbioses involving rhizobium, *Frankia*, and AM fungi (Gherbi et al. 2008a; Markmann et al. 2008).

Composite plants have also been used to characterize some candidate genes potentially involved in specific stages of the endomycorrhization process. For example, co-transformed hairy roots of *M. truncatula* highlighted the role of a NADPH oxidase encoded by the gene *RbohE* during arbuscule accommodation within cortical root cells (Belmondo et al. 2016).

12.3.3 Plant Nematode Interaction

Meloidogyne species of root-knot nematodes (RKN) attack the roots of most vegetable, fruit, and ornamental crops under Mediterranean and tropical climates. Infested roots become distorted and develop rounded or irregular galls which alter water and nutrient uptake, thereby reducing plant growth and yield (Fosu-Nyarko and Jones 2016). Composite plants for studying nematode resistance have been documented in *Lycopersicon esculentum* cv. (Collier et al. 2005), *Glycine max* (Li et al. 2010), *Prunus* spp. (Claverie et al. 2011), *Arachis hypogaea* (Guimaraes et al. 2017a), and *Persea americana* (Prabhu et al. 2017).

In *Prunus* species, composite plants have been used to validate the function of the candidate gene *Ma* isolated in *Prunus cerasifera* (Claverie et al. 2011). When co-transformed roots expressed the *Ma* genomic sequence under the control of its native promoter, a high level of resistance was obtained to the three major RKNs *Meloidogyne incognita*, *M. arenaria*, and *M. javanica*.

Several RKN species are pathogenic on *A. hypogaea* and cause considerable yield losses in Africa every year. In wild-type *Arachis* species which are resistant to a number of pests and diseases, transcriptomic studies have identified candidate genes that could contribute to resistance to *M. arenaria*. Since peanut is recalcitrant to genetic transformation, *A. rhizogenes* was tested as an alternative to develop the functional analysis of plant genes. Using the *A. rhizogenes* strain K599, the candidate gene for nematode resistance *AdEXLB8* was overexpressed in hairy roots induced on the peanut cultivar "Runner." Two months after *M. arenaria* infection, a reduction of 98% in the number of galls and egg masses was observed compared to the control hairy roots (Guimaraes et al. 2017b).

12.3.4 Interactions of Hairy Roots with Parasitic Plants

Striga is one of the most important genera of parasitic plants and causes devastating losses in cereal yields in sub-Saharan Africa. It is an obligate hemiparasitic parasite that attaches to host roots, forms a haustorium, and penetrates the root cortex of potential hosts. It then damages cereal crops by draining off water and nutrients, impairing photosynthesis, and having a phytotoxic effect (Yoshida et al. 2016). The combination of these factors severely reduces the growth of the crops and causes the subsequent failure to set seeds. Understanding the molecular mechanisms underlying the plant-parasitic weed interaction is essential for the identification of genes that could improve crop yield via biotechnological or marker-assisted breeding strategies.

The possibility for *Striga* to parasite hairy roots of composite plants has been demonstrated. *Striga gesnerioides* (L.) is a major parasite of the grain legume cowpea (*Vigna unguiculata*) in Africa. Following infection with the *A. rhizogenes* strain R1000, composite plants of *V. unguiculata* were obtained using an ex vitro

protocol, and co-transformed roots were selected using *gfp* as biomarker (Mellor et al. 2012). Up to 80% of the inoculated plants developed at least one transgenic root. When subjected to *Striga*, hairy roots of composite plants responded similarly to wild-type roots of the susceptible cowpea cultivar, allowing the formation and growth of parasite tubercles on the legume transformed roots. When the gene *RSG3-301* encoding a resistance (R) gene to *Striga* was co-transformed in the hairy roots of a susceptible cowpea genotype, its expression resulted in the acquisition of a resistant phenotype. These data demonstrate that the expression of the oncogenes from *A. rhizogenes* has no impact on the cowpea-*Striga* interaction.

Runo et al. (2012) reported a similar approach using *Zea mays*. Using the strain K599, composite maize plants with co-transformed roots were obtained in vitro on 85.3% of the inoculated seedlings. Two weeks after inoculation with *Striga hermonthica*, the number and size of *S. hermonthica* individuals infecting transformed or wild-type roots of maize were identical. Microscopic examination of the infected roots further confirmed that the timing and characteristics of the infection process were not altered in the hairy roots. These data confirm that composite plants will be suitable for the characterization of plant genes which play a critical role in parasitism or host defense.

12.3.5 Hairy Roots for the Study of Wood Formation

Since the regeneration of transgenic forest trees is limited to a small number of species due to poor regeneration ability and difficulty to achieve T-DNA transfer by *A. tumefaciens*, *A. rhizogenes* appeared to be a viable alternative. Composite plants have now been reported in several forest trees including *Eucalyptus camaldulensis* (Balasubramanian et al. 2011) and *E. grandis* (Plasencia et al. 2016) and recently in poplar (Neb et al. 2017).

Whereas poplar has been the main forest tree used to advance our knowledge of the lignification process in forest trees, other trees such as eucalyptus are of major economic value. With the release of the *Eucalyptus grandis* genome sequence, many candidate genes involved in wood formation have been identified, paving the way for functional analysis. Due to the recalcitrance of *E. grandis* to *Agrobacterium*, the hypervirulent A4RS strain had to be used to obtain efficient transformation (62% on average) (Plasencia et al. 2016). Microscopic examination showed that xylem development was similar in both hairy and wild-type roots. A proof of concept of the composite plant approach was obtained with the downregulation of the cinnamoyl-CoA reductase1 gene (*EgCCR1*) in *E. grandis*, encoding a key enzyme from the lignin biosynthetic pathway. As expected, the expression of an *EgCCR1* antisense construct led to a decrease in lignin content. The authors also demonstrated that composite plants were suitable for the analysis of the expression pattern conferred by promoters from genes involved in the lignin biosynthetic pathway.

12.4 Genome Editing in Transgenic Roots of Composite Plants

The first reports of CRISPR/Cas9 editing in plants appeared in 2013, with successful application for both transient expression and recovery of stable transgenic lines. In addition to demonstration of efficacy in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* (Li et al. 2014), there have also been many reports on different crop species including rice (Miao et al. 2013), maize (Liang et al. 2014), and wheat (Wang et al. 2014). Because of the ease of use and low cost, CRISPR/Cas9 has rapidly become the tool of choice for gene editing and creating knockout mutants in plants (Belhaj et al. 2015; Liu et al. 2016; Nogué et al. 2016). However, a prerequisite for the application of the technology is the ability to deliver guide RNAs (gRNAs) and the CRISPR-associated protein 9 to the target cells either stably or transiently. When it is not possible to regenerate edited plants after transient expression of the gRNA and Cas9 in protoplasts and a genetic transformation procedure with *A. tumefaciens* is not available either, composite plants offer an alternative for creating mutations in root-expressed genes (Table 12.3).

The potential of the CRISPR/Cas9 system to induce gene mutations using hairy root transformation was first tested in tomato and targeted the *SHORT-ROOT* (*SHR*)

Table 12.3 Use of genome editing for gene function analysis in composite plants

Plant	Gene	Function	References
Brassica carinata	*FLA1*	Adhesion molecule	Kirchner et al. (2017)
Glycine max	*FEI1, FEI2* *SHR* *GS1* *CHI* *PDS* *Rfg1*	Plant signaling Seed coat development Nitrogen metabolism Flavonoid pathway Carotenoid biosynthesis pathway Resistance to nodulation	Cai et al. (2015) Jacobs et al. (2015) Du et al. (2016) Fan et al. (2017)
Lotus japonicus	*SYMRK*	Symbiotic nitrogen fixation	Wang et al. (2016)
Medicago truncatula	*gus*	Proof of concept	Michno et al. (2015)
Salvia miltiorrhiza	*CPS1* *RAS*	Diterpenoid biosynthesis Phenolic acid biosynthetic pathway	Li et al. (2017) Zhou et al. (2018)
Solanum lycopersicum	*SHORT-ROOT, SCARECROW*	Root development	Ron et al. (2014)
Taraxacum koksaghyz	*1-FFT*	Inulin biosynthesis	Iaffaldano et al. (2016)

1-FFT, fructan 1-fructosyltransferase; CHI, chalcone-flavone isomerase; CPS1, diterpene synthase; FEI1 and FEI2, leucine-rich receptor kinase; FLA1, fasciclin-like arabinogalactan protein 1; GS, glutamine synthase; gus, ß-glucuronidase; PDS, phytoene desaturase; RAS, rosmarinic acid synthase; Rfg1, plant resistance protein; SCARECROW and SHORT-ROOT, Gras transcription factors regulating root patterning; SHR, seed coat wrinkling; SYMK, symbiosis receptor kinase

sequence expressed in root vascular tissue and encoding a transcription factor (Ron et al. 2014). Several hairy roots genetically transformed with a gRNA targeting the coding sequence of *SHR* were obtained and characterized by a short meristem. Sequence analysis of the targeted gene in putatively edited roots confirmed that the *SHR* coding region contained a variety of insertion and deletion (indel) mutations. The alterations in the root phenotype were the result of defects in stem cell division and cell patterning and were consistent with the phenotype of *Arabidopsis shr* mutants. From these data, it was concluded that SHR function was conserved between tomato and *Arabidopsis*.

Similar experiments were performed on the nitrogen-fixing legumes *M. truncatula* (Michno et al. 2015), *G. max* (Cai et al. 2015; Du et al. 2016; Jacobs et al. 2015; Michno et al. 2015; Sun et al. 2015), and *L. japonicus* (Wang et al. 2016). Previously reported ex vitro or in vitro composite plant transformation assays were used to introduce T-DNA gene constructs with the designed gRNA and codon-optimized gene encoding the Cas9 protein. Sequencing of the targeted genes revealed mutations induced by the CRISPR/Cas9 system. Following the analysis of 11 targeted loci in soybean, DNA mutations mainly consisting of small deletions were detected in 95% of the hairy roots (Jacobs et al. 2015). One limitation of the CRISPR/Cas9 system is possible off-target mutations that may alter the expression of genes that were not originally targeted. Experiments on soybean composite plants indicate that off-target mutations do occur, although at low rates (Jacobs et al. 2015).

The CRISPR/Cas9 system is also effective for the study of biosynthetic pathways. Two genes isolated in the Chinese medicinal plant *Salvia miltiorrhiza* coding for water-soluble phenolic acids have been successfully targeted (Zhou et al. 2018). When the diterpene synthase gene *SmCPS1* from the tanshinone biosynthetic pathway was edited, a mutation rate of 42.3% was obtained in the hairy roots, and tanshinone was absent in the homozygous plants (Li et al. 2017). The second gene targeted was rosmarinic acid synthase (*SmRAS*). The level of *RAS* expression was reduced in successfully edited plant roots, revealing a promising potential method to regulate plant metabolic networks and improve the quality of medicinal herbs.

From these recent studies, it can be concluded that CRISPR/Cas9 and related genome editing methods will facilitate a wide range of functional analyses in roots of composite plants, since specific mutations and knockout mutants can be easily obtained, even in non-model plants (Wang et al. 2017).

12.5 Conclusions

The first composite plant obtained after T-DNA transfer with *A. rhizogenes* was reported more than 20 years ago in the legume *L. corniculatus*, the aim being to study genes involved in nodulation with rhizobia. The feasibility and potential of this simple low-cost approach have now been demonstrated in numerous legume and nonlegume plant species. Experiments show that *A. rhizogenes* is a useful tool to rapidly test gene expression and function in the context of root development and in

response to the biotic and abiotic environment. Furthermore, recent findings demonstrate that the CRISPR/Cas9 technology can also be used to induce targeted indel mutations in the root system of composite plants.

While studies on hairy roots advance the speed of the investigations in plants which are also amenable to genetic transformation by *A. tumefaciens*, sometimes they are the only way to obtain gene transfer in plant species that remain recalcitrant to in vitro regeneration and/or T-DNA transfer by *A. tumefaciens* or direct gene techniques. In the future, this system will thus certainly continue to be a valuable way to advance functional genomic research and to improve our knowledge of the molecular mechanisms underlying a wide range of processes in root-microbe and root-parasitic interactions, root development, and root adaptation to abiotic stress.

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Part III
Novel Approaches and Future Prospects

Chapter 13
An Update on Transcriptome Sequencing of Hairy Root Cultures of Medicinally Important Plants

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Abstract Hairy root cultures induced by *Agrobacterium rhizogenes* infection have been recognized as promising and attractive alternative source of secondary metabolites owing to several advantageous features like genetic stability, comparable biosynthetic capabilities to the native plant root, and sizable biomass production. Hairy root cultures are reported to produce all major classes of secondary metabolites, such as isoprenoids/or terpenoids, alkaloids, phenolics, and flavonoids. So far, hairy root cultures have been established from a variety of plants providing commercially valuable products, such as artemisinin (*Artemisia annua*), astragalosides (*Astragalus membranaceus*), acteoside (*Rehmannia glutinosa*), centellosides (*Centella asiatica*), resveratrol (*Arachis hypogaea*), camptothecin (*Camptotheca acuminata*), vinblastine, vincristine (*Catharanthus roseus*), and kutkin, iridoid glycosides (*Picrorhiza kurroa*). In hairy root cultures, these specialized metabolites are produced via complex network of several distinctive biochemical pathways operating in an integrated manner. However, biochemical pathways and genes involved in production of many phytochemicals have not been completely elucidated. Transcriptome sequencing of hairy root cultures by next-generation sequencing techniques has been proven to be an excellent approach in elucidation of biosynthetic pathways and genes of phytochemical production. Newly emerged next-generation sequencing techniques like Roche/454 and Illumina/Solexa have greatly facilitated sequencing of transcriptome of hairy root cultures. At present, transcriptome sequence datasets of hairy root cultures of only a limited numbers of plants, viz., *C. roseus*, *P. ginseng*, *A. membranaceus*, *R. glutinosa*, *C. asiatica*, etc., are available. Thorough analyses of transcriptome sequence datasets of hairy root cultures have unraveled many biosynthetic pathways and genes responsible for the biosynthesis of commercially important phytochemicals. The present chapter

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provides an up-to-date information of transcriptome sequencing of hairy root cultures of important plants performed by next-generation sequencing techniques.

Keywords *Agrobacterium rhizogenes* · Elicitors · Hairy root cultures · Methyl jasmonate · Next-generation sequencing · Secondary metabolites · Transcriptome

13.1 Introduction

Transcriptome sequencing has emerged as an excellent method to provide genome data, large expressed sequence tag (EST) sequences, and molecular markers. Currently, genome sequences of more than 180 plants are available (http://www.plabipd.de/portal/sequence-timeline). Emerging next-generation sequencing technologies (NGS) have triggered an explosion of available genomic and transcriptomic resources in plant sciences (Bolger et al. 2014). NGS technologies provide cutting-edge approaches for high-throughput sequence generation (Egan et al. 2012) and allow rapid and comprehensive analyses of plant genomes and cost-effective means of analyzing transcripts (Wei et al. 2015; Wen et al. 2015). Most important NGS techniques like Roche/454 pyrosequencing, SOLiD/ABI (supported oligonucleotide ligation and detection/Applied Biosystems), and Illumina/Solexa have led to tremendous advancements in genomics by providing cheaper and faster delivery of sequencing information (Morozova and Marta 2008). Now, more powerful third-generation sequencers have arrived, such as Helicos from Heliscope, PacBio from Pacific Biosciences, and Nanopore from Oxford Nanopore Technologies (ONT), which can sequence single-molecule templates directly with no PCR (polymerase chain reaction) amplification. For details on NGS and its applications in transcriptome sequencing, refer to the article by Chen and Luo (2014). Initially, Roche/454 sequencer has been successfully used for transcriptome sequencing of *Brassica napus* (Trick et al. 2009), *Artemisia annua* (Wang et al. 2009), *Eucalyptus grandis* (Novaes et al. 2008), *Olea europaea* (Alagna et al. 2009), *Arabidopsis thaliana* (Jones-Rhoades et al. 2007; Weber et al. 2007; Zhou et al. 2012), *Medicago truncatula* (Hsiao et al. 2008), and other plant species (Varshney et al. 2009). Currently, Illumina/Solexa RNA-sequencer is the most widely used platform for transcriptome sequencing with higher read depth and prediction accuracy capabilities (Yu et al. 2014; Song et al. 2017). The Illumina/Solexa RNA-seq is a highly sensitive, powerful, and cost-efficient technique for discovering gene expression, novel genes, and differentially expressed genes, as well as the detection of low-abundance transcripts. Due to its higher accuracy and dynamic range, it has replaced other methods for quantifying gene expressions. Further, RNA-seq has been useful in non-model plants that lack a reference genome, such as olive (Alagna et al. 2009), chestnut (Barakat et al. 2009), and tea (Shi et al. 2011) because here the aim of sequencing is to focus on the coding regions. It has also been used for model agriculture crops, such as *Zea mays* and *Glycine max* (Tuan et al. 2015). Transcriptome sequencing using NGS techniques has been proven to be a very

useful tool to investigate biochemical pathways and search genes related to novel bioactive phytochemicals in a number of medicinal plants. In all studies of transcriptome sequencing by NGS techniques aimed to elucidate secondary metabolite biosynthetic pathways and genes, the developmental stages of the concerned plant, plant parts, and tissue types (roots, hairy roots, leaves, stems, etc.) have been taken into account as they significantly influence levels of secondary metabolites in plants. Hairy roots are manifestation of a disease caused by *Agrobacterium rhizogenes* and characterized by a proliferation of excessively branching roots (Mehrotra et al. 2010; Georgiev et al. 2012). Literature survey revealed that HRCs have been established in more than 400 plant species and are continually being induced from new plants (Porter and Flores 1991; Ono and Tian 2011). Hairy root cultures established for many plants have been used for production of secondary metabolites, such as alkaloids, terpenoids, flavonoids, saponins, and other novel metabolites (Srivastava and Srivastava 2007; Mehrotra et al. 2010; Sharma et al. 2013). Also hairy root cultures have been developed as biotechnological factories with potential for the production of valuable phytochemicals through chemical transformations aided by its inherent enzyme resources (Banerjee et al. 2012). Production of secondary metabolites in HRCs can be enhanced through metabolic engineering of the concerned pathways (Mehrotra et al. 2010). In view of the rapidly increasing use of HRCs for production of valuable phytochemicals, sequencing of the transcriptome of HRCs has become highly desirable to gain deeper insight into biochemical pathways and putative genes involved in the biosynthesis of phytochemicals. Most of the studies on transcriptome sequencing of HRCs have revealed an important fact that HRCs have been treated with hormonal elicitors, methyl jasmonate (MeJA), and/or salicylic acid (SA) for better and comprehensive understanding of the gene expression profiles. Till date, transcriptome sequencing of HRCs of only limited plants, namely, *Ophiorrhiza pumila* (Yamazaki et al. 2013), *Centella asiatica* (Kim et al. 2014), *Salvia miltiorrhiza* (Gao et al. 2014; Xu et al. (2015)), *Panax ginseng* (Cao et al. 2015), *Astragalus membranaceus* (Tuan et al. 2015), *Catharanthus roseus* (Sun et al. 2016), and *Rehmannia glutinosa* (Wang et al. 2017), have been performed. In the future, numbers of transcriptomics dataset of HRCs will increase as HRCs for new commercially important plants are currently being established. In this chapter, we have discussed the progress made on transcriptome sequencing of HRCs of medicinal plants. Also, we have discussed in the nutshell about HRCs and elicitors, methyl jasmonate (MJ), and salicylic acid (SA) emphasizing their effects on production of phytochemical in HRCs.

13.2 Hairy Roots

The name "hairy root" was first introduced by Stewart et al. (1900). Hairy roots are disease manifestations developed by plants that are wounded and infected by *A. rhizogenes*. When a large number of small, fine, hairy roots covered with root hairs originate directly from the explant in response to *A. rhizogenes* infection, it is

called "hairy root." The main advantage of hairy roots is that they often exhibit about the same or greater biosynthetic capacity for phytochemical production as compared to their mother plants (Kim et al. 2002). *Agrobacterium rhizogenes* has root-inducing plasmid (Ri) harboring root loci (rol) genes, which get inserted into the nuclear genome of the host plant eventually causing neoplastic root and root hair proliferation. It is believed that Rol genes affect the growth and development of the transformed roots and trigger secondary metabolite synthesis by turning on the transcription of defense genes. The transformed root cells produce low-molecular-weight molecules known as opines, such as agropine, mannopine, and cucumopine, which are metabolized by *A. rhizogenes*. Hairy roots are highly differentiated and can proliferate on phytohormone-free media, which distinguishes them from undifferentiated plant cell cultures. It is reported that plants of a number of families, such as Balsaminaceae, Chenopodiaceae, Compositae, Juglandaceae, Labiatae, Moraceae, Ranunculaceae, Solanaceae, Asteraceae, Cucurbitaceae, Plumbaginaceae, Apocynaceae, Asclepiadaceae, and Umbelliferae, induce hairy root disease symptoms on infection with *A. rhizogenes* (Porter and Flores 1991; Giri and Narasu 2000; Ono and Tian 2011).

The mechanism of diseases caused by hairy root proliferation by *A. rhizogenes* has been exploited to develop HRCs in plants for large-scale production of valuable phytochemicals. HRCs have already been established in more than 400 plant species and are continually being induced from new plants (Porter and Flores 1991; Ono and Tian 2011). Hairy root cultures of *Artemisia annua* (artemisinin), *Arachis hypogaea* (resveratrol), *Catharanthus roseus* (indole alkaloids), and *Camptotheca acuminata* (camptothecin) are occupying increasing significance due to their valuable phytochemicals (Shivkumar 2006; McCoy and O'Connor 2008). Because of their genetic stability, comparable biosynthetic capacity to the native plant root, and sizable biomass production, HRCs offer promising alternative source of phytochemicals. Furthermore, HRCs often accumulate phytochemicals at a higher level than cell/callus cultures that contain undifferentiated cells. Till date, many research papers, reviews, books, and chapters have been published covering every aspects of HRCs, including its applications and future prospects (Srivastava and Srivastava 2007; Mehrotra et al. 2010, 2015; Ono and Tian 2011, Georgiev et al. 2012; Srivastava et al. 2012, 2013; Mehrotra et al. 2015). Biotechnological applications of HRCs, such as production of recombinant proteins, phytoremediation, molecular breeding, rhizosphere physiology, biochemistry, and metabolic engineering, have been discussed in detail (Srivastava and Srivastava 2007; Mehrotra et al. 2010; Ono and Tian 2011, Georgiev et al. 2012). Production of important secondary metabolites in HRCs can also be enhanced through metabolic pathway engineering (Mehrotra et al. 2010). Besides, because of the potential of HRCs for production of phytochemicals, they have been used as a tool for studying functional genomics and unravelling biochemical pathways (Ono and Tian 2011; Sharma et al. 2013). Banerjee et al. (2012) have highlighted biotransformation capabilities of HRCs, which have been exploited for generating novel pharmaceutical compounds by chemical transformations of parent skeleton through its inherent enzyme resources. The potential of *Atropa belladonna* hairy roots in the production of industrially important

cosmeceutical and pharmaceutical derivatives of betuligenol and to transform carbonyl compounds through oxidation and reduction reactions have also been reported (Srivastava et al. 2012, 2013).

13.2.1 Roles of Elicitors

Elicitors are the most effective substances, which can enhance the production of secondary metabolite by several folds when added to HRCs. Elicitors have been categorized into abiotic and biotic types on the basis of their origin (Table 13.1). Elicitors if added even in a very small concentration to the HRCs may trigger chemical defense system to stimulate physiological and morphological responses resulting in increased biosynthesis and accumulation of secondary metabolites. In view of elicitor's stimulating effects on production of secondary metabolites, they have tremendous significance in biotechnological production of commercially valuable secondary metabolites. Despite the fact that HRCs are a promising source of valuable secondary metabolite accumulation, HRCs developed for many plants do not produce significant levels of secondary metabolites. Thus, addition of elicitors to such HRCs may significantly enhance production of secondary metabolites. A number of reports and reviews have been published on elicitors (biotic and abiotic) and their applications to enhance phytochemical production from HRCs (Dicosmo and Misawa 1985; Ebel and Casio 1994; Namdev 2007; Goel et al. 2011; Ramirez_estrada et al. 2016; Wang and Wu 2013; Naik and Alkhayri 2016). Here, we have discussed mainly MeJA and SA because they were used in most of the studies on transcriptome sequencing of HRCs. The rationale behind the treatment of HRCs with MeJA is that the treated HRCs are ideal models to learn the complex biochemical variation in secondary metabolism and discover novel genes related to secondary metabolite biosynthesis (Yan et al. 2014). Elicitation studies in combination of *in silico* approaches could be a more useful practice for a better understanding and identification of the rate-limiting steps of biosynthetic pathways existing in HRs (Goel et al. 2011). Jasmonates are plant-specific signaling molecules that regulate various physiological and developmental processes (Pauwels et al. 2009). Salicylic acid is a small molecule, which has a vital role in plant defense regulatory system. It

Table 13.1 Classification of elicitors

Elicitors			
Abiotic			Biotic
Physical	Chemical	Hormonal	
Salinity	Heavy metals	Methyl jasmonate	Polysaccharides
UV radiation	Mineral salts	Salicylic acid	Yeast extracts
Osmotic stress	Gaseous toxins	Gibberellic acid	Fungal
Thermal stress		Brassinosteroids	Bacterial
Drought			

induces systemic-acquired resistance against many pathogens. Salicylic acid has been reported as an effective elicitor of secondary metabolite in several plant species. Derivatives and analogs of salicylic acid such as 2,6-dichloroisonicotinic acid and benzothiadiazole have also been used as chemical inducers of systemic-acquired resistance (Hayat et al. 2010; Pieterse and van Loon 1999; Durrant and Dong 2004). Other biotic and abiotic elicitors (Table 13.1) have also been reported to increase production of secondary metabolites in HRCs (Guillon et al. 2006). Wu and Shi (2008) have reported the use of sorbitol in conjunction with yeast extract as an elicitor on production of a diterpenoid, tanshinone in *S. miltiorrhiza* HRCs. Production of tanshinone has been enhanced by 100fold in fed-batch cultures as compared to the controls. In *C. asiatica*, MeJA markedly influence the biosynthesis of a triterpene, saponin, by upregulation of the terpenoid pathway. Additionally, MeJA treatment increases the transcript levels of squalene synthase and dammarenediol synthase genes, which are associated with the triterpenoid pathway in *C. asiatica* (Kim et al. 2005a, b; Kim et al. 2009). In *S. sclarea* and *S. miltiorrhiza* HRCs, addition of MeJA elicits defense responses resulting in elevated production of useful secondary metabolites (Kuzma et al. 2009). Methyl jasmonate has also been used as a tool for identification and characterization of novel genes involved in phenylpropanoid biosynthesis in *S. miltiorrhiza* (Hou et al. 2013). It suppresses cell growth and decreases the biomass yield in *S. miltiorrhiza* cell cultures (Zhao et al. 2010). In recent times, high-throughput sequencing has facilitated transcriptome sequencing of *S. sclarea* (Legrand et al. 2010) and *S. miltiorrhiza* (Li et al. 2010; Wenping et al. 2011) and also made it possible to perform transcriptome-wide investigation of MeJA-induced plant responses. In *Hyoscyamus albus*, treatment of HRCs with combination of copper sulfate and MeJA has dramatically altered the phytochemical profiles of four new sesquiterpene phytoalexins (Kawauchi et al. 2010). The use of elicitors, such as SA, MeJA, AgNO3 (Ag^+), and putrescine, in HRCs of *R. glutinosa* significantly affected the biosynthesis of acteoside, which is attributed for several bioactive properties, such as antioxidant, antinephritic, anti-inflammatory, hepatoprotective, immunomodulatory, and neuroprotective (Wang et al. 2017). Among these elicitors, SA (25 μmol/L) has been found to enhance the synthesis of acteoside in hairy roots.

13.3 Transcriptome Sequencing of Hairy Roots

Advancements in the molecular biology techniques mainly the NGS techniques have contributed tremendously in understanding secondary metabolism pathways and genes involved therein. Several review articles have discussed technical aspects of NGS techniques and its biotechnological applications in genome/transcriptome sequencing (Egan et al. 2012; Chaudhary and Sharma 2016). Undoubtedly, NGS techniques have revolutionized our understanding about the secondary metabolism by generating genome/transcriptome sequence databases (Chaudhary and Sharma 2016). Transcriptome is defined as a set of RNA molecules in one cell or a

population of cells, whereas transcriptomics is the study of the expression of RNA level in a population of a cell. The total set of RNA sequences in a cell determines why and how the gene will turn on or off in the cells and tissues of an organisms. In principle, any high- throughput sequencing technology can be used for RNA-seq, and the Illumina IG (Nagalakshmi et al. 2008; Wilhem et al. 2008; Mortazavi et al. 2008; Lister et al. 2008; Marioni et al. 2008; Morin et al. 2008), A Biosystems SoliD22, and Roche 454 Life science systems have already been applied for this purpose. Following sequencing, the resulting reads are either aligned to a reference genome or reference transcripts or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and level of expression for each gene (Ku et al. 2012).

In early 2010, Illumina launched HiSeq 2000, which uses solid-phase bridge amplification in which 5′ and 3′ adapters are ligated to each end of a DNA template (http://www.illumina.com). The library with fixed adapters is denatured to single strands and grafted to the flowell, followed by bridge amplification to form clusters, which contains clone, DNA fragments. Before sequencing, the library splices into single strands with the help of linearization enzyme (Madris 2008) and then four kinds of nucleotides (ddATP,ddGTP, ddCTP, ddTTP), which contain different cleavable fluorescent dyes, and a removable blocking group would complement the template one base at a time, and the signal could be captured by a (charge-coupled device) CCD.

So far, transcriptome sequence databases have been constructed and annotated for a number of plants, namely, *Taxus mairei* (Hao et al. 2011), *Glycyrrhiza uralensis* (Ramilowski et al. 2013), *Lycium chinense* (Zhao et al. 2013), *Litsea cubeba* (Han et al. 2013), *Opium Poppy* (Gurkok et al. 2014), *Panax notoginseng* (Liu et al. 2015), and *Gastrodia elata* (Tsai et al. 2016). Researchers exploit these transcriptome sequence dataset to unravel the biochemical pathways of secondary metabolites on a transcriptome-wide scale in non-model plant species, particularly medicinal plants, for which the complete genome sequences and annotation are not yet available. In the past few years, transcriptome sequencing of HRCs of several plant species, *viz*., *O. pumila* (Yamazaki et al. 2013), *C. asiatica* (Kim et al. 2014), *S. miltiorrhiza* (Gao et al. 2014; Xu et al. 2015), *P. ginseng* (Cao et al. 2015), *A. membranaceus* (Tuan et al. 2015), *C. roseus* (Sun et al. 2016), and *R. glutinosa* (Wang et al. 2017), have been carried out using Roche454 and Illumina/Solexa NGS techniques. Information of their transcriptome sequence dataset has been summarized in Table 13.2. For the first time, Illumina platform was used for sequencing of transcriptome of hairy roots and cell suspension cultures of *O. pumila*, which generated 2 Gb of sequences (Yamazaki et al. 2013). Transcriptome sequence data provided detail insight into the genes involved in the biosynthetic pathway for the camptothecin, anthraquinones, and chlorogenic acid as well as genes involved in post-strictosamide biosynthetic events. Thus, combined transcriptome and metabolome datasets have facilitated the identification of genes and intermediates involved in the biosynthesis of camptothecin in *O. pumila*. Few studies have used combination of transcriptome sequencing and metabolic profiling to gain deeper insight into biosynthesis and regulation of phytochemicals in HRCs. In *I. indigotica*,

Table 13.2 Sequencing data of transcriptomes of hairy root cultures (HRCs) of some medicinal plants

Plant	Family	Major metabolites	Elicitors	Sequencing technique	Sequence summary	References
Ophiorrhiza pumila	Rubiaceae	Camptothecin	MeJA	Illumina	Size: 2 Gb Nonredundant unigenes: 35,608	Yamazaki et al. (2013)
Centella asiatica	Umbelliferae	Triterpenoids	MeJA	Illumina	ESTs: 4381 Contigs: 376 Sigletones 2461 Total unique annotated sequences: 2420	Kim et al. (2014)
Salvia miltiorrhiza	Labiatae	Tanshinones	MeJA	Solexa	Nonredundant genes: 20,972 Differential expressed genes: 6358	Gao et al. (2014)
Panax ginseng	Araliaceae	Ginsenoside	MeJA	Roche 454	All-unigenes: 71,095 Total annotated unigenes: 56,668	Cao et al. (2015)
P. vietnamensis var. fuscidiscus	Araliaceae	Majonoside	–	Illumina	Unigenes: 126,758 Annotated unigenes: 85,214	Zhang et al. (2015)
Astragalus membranaceus	Leguminosae	Astragaloside, calycosin Calycosin-7-O-β-D-glucoside	MeJA	Illumina/Solex	Assembled Transcripts: 48636 Annotated genes: 19940	Tuan et al. (2015)
Catharanthus Roseus	Apocynaceae	Vinblastine Vincristine	MeJA	Illumina	Unigenes: 30,281 Differentially expressed transcripts: 2853	Sun et al. (2016)
Isatis indigotica	Brassicaceae	Lignan	MeJA	Illumina	Total isogenes: 65196 Differently expressed genes: 984–1583	Zhang et al. (2016)
Rehmannia glutinosa	Scrophulariaceae	Acteoside	SA	Illumina	Total differentially expressed transcripts (DETs): 10,449	Wang et al. (2017)

transcriptome sequencing coupled with metabolic profiling has successfully revealed genes involved in the biosynthesis of the active compounds (Chen et al. 2013). Combination of transcriptome and metabolite profiling has identified *Ii*PLR1 gene that plays important role in lariciresinol accumulation in *I. indigotica* (Xiao et al. 2015). Another similar study with *I. indigotica* has provided detail insights into the biosynthetic pathway and regulation of lignin (Zhang et al. 2016). There are total 17 major genes involved in the biosynthesis of lignan, but only 4*CL3* has greater impact on lignin biosynthesis. These results have been used for improving production of lignan-like compounds in *I. indigotica*. Application of the MeJA in HRCs facilitated the discovery of the potential key catalytic steps and metabolic/transcriptional changes and candidate genes that might be playing key roles in lignan biosynthesis. Previously, Chen et al. (2013) have also demonstrated the beneficial effect of MeJA on lignan biosynthesis in *I. indigotica* HRCs. Gao et al. (2014) used a combination of metabolomics and transcriptomics to investigate the inducible biosynthesis of the bioactive diterpenoid, namely, tanshinones in *S. miltiorrhiza* (Danshen), a Chinese medicinal herb. *S. miltiorrhiza* produces and accumulates tanshinone pigments in the root periderm. A combination of NGS and single-molecule real-time (SMRT) sequencing technique was applied to various root tissues, mainly the periderm, which was found more efficient providing a complete view of the *S. miltiorrhiza* transcriptome, with deeper insight into tanshinone biosynthesis (Xu et al. 2015). Also, the use of SMRT long-read sequencing has the ability to examine alternative splicing, which was found to occur in approximately 40% of the detected gene loci, including several genes involved in isoprenoid/terpenoid metabolism.

Analysis of expressed sequence tags from *C. asiatica* urban hairy roots elicited by MeJA has led to the discovery of genes related to cytochrome P450s and glucosyltransferases involved in the biosynthesis of the centellosides (Kim et al. 2014). *C. asiatica* is a perennial plant, which is used in wound-healing due to its strong anti-inflammatory properties. The sequencing revealed that asiatic and madecassic acids are biosynthesized from α-amyrin by cytochrome P450 hydroxylase and carboxylase (P450). Asiatic and madecassic acids are used as precursors for the biosynthesis of asiaticoside and madecassoside. The UDP-glucosyltransferases catalyze synthesis of two centellosides from asiatic acid and madecassic acid.

Cao et al. (2015) have studied the transcriptome of *P. ginseng* adventitious roots following treatment with MeJA. The *P. ginseng* is an elite member of family Araliaceae, which produces ginsenoside used in many herbal formulations. Transcriptome sequencing of *P. ginseng* hairy roots revealed that putative genes involved in the biosynthesis and transport of ginsenoside showed a wide range of expression levels. Sequencing analyses provided information about 749 ginsenoside biosynthetic genes and 12 promising pleiotropic drug resistance (*PDR*) genes related to ginsenoside transport. Transcriptome sequencing of (roots, hairy roots, and stems) of another species of ginseng *P. vietnamensis* var. *fuscidiscus* has been carried out using Illumina HiSeq™ 2000 sequencing platform (Zhang et al. 2015). Transcriptome sequence analyses have revealed 15 candidate cytochrome P450 genes and 17 candidate UDP-glycosyltransferase genes most likely to be involved

in triterpenoid saponin biosynthesis pathway. Further, it provided information on SSR markers, which may be utilized to facilitate the marker-assisted breeding in *Panax* species. *P. vietnamensis* var. *fuscidiscus* has been considered as a suitable medicinal herbal plant to study biosynthesis of ocotillol-type saponins as no genomic information is available about this important herbal plant. *P. vietnamensis* var. fuscidiscus is reported to contain high percentage of ocotillol-type saponin, majonoside R2. The transcriptome of *Astragalus membranaceus* hairy roots treated with MeJA has been sequenced by Illumina/Solexa HiSeq 2000 platform (Tuan et al. 2015). The study of transcriptome sequence data led to the identification of most of the genes related to biosynthesis and regulation of astragaloside, calycosin, and calycosin-7-O-β-D-glucoside. Treatment of HRCs with MeJA caused upregulation of 2127 genes and downregulation of 1247 genes. The study also identified 17 novel astragaloside biosynthetic genes and seven novel calycosin and calycosin-7-O-β-D-glucoside biosynthetic genes. Transcriptome analyses findings may be exploited for molecular characterization of astragaloside, calycosin, and calycosin-7-O-β-D-glucoside biosynthetic pathways leading to new approaches to enhance their production and biomass productivity in the hairy roots of *A. membranaceus* (Tuan et al. 2015).

Sun et al. (2016) have investigated transcriptional response of overexpressing anthranilate synthase in the hairy roots of an important medicinal plant *C. roseus* by Illumina sequencing and RT-qPCR. *C. roseus* is an important medicinal plant, which provides two well known anticancer compounds, vinblastine and vincristine. In *C. roseus*, vinblastine and vincristine are biosynthesized *via* the terpenoid indole alkaloid pathway. An enzyme anthranilate synthase identified as a regulatory enzyme of this pathway controls and regulates the overall production of terpenoid indole alkaloids. Also, it stimulates overall stress response and affects the metabolic networks in *C. roseus* hairy roots. A study in *C. roseus* that has been engineered with anthranilate synthase (αβ-subunit) revealed that jasmonic acid signal transduction is involved in the upregulation of endogenous jasmonate biosynthesis (Sun et al. 2016).

Chakrabarty et al. (2015) have performed transcriptome sequence analyses in two strategically selected and contrasting morphotypes of vetiver, one representing the North Indian type having thick, smooth, and fast growing roots, and the other the South Indian type having thin, hairy, and more roots. They used Illumina paired-end sequencing technology to characterize the root transcriptome of vetiver and developed SSR markers. This was the first study in vetiver providing comparative molecular analysis of root transcriptome from two distinct morphotypes of vetiver. The vetiver root transcriptome may serve as a public information platform for further studies of gene expression, genomics, and functional genomics in vetiver. This study also provided an important starting point for further discovery of genes related to root oil quality in different ecotypes of vetiver (Chakrabarty et al. 2015). Wang et al. (2017) have performed transcriptome sequencing of *R. glutinosa* hairy roots treated with SA. *R. glutinosa* is a valuable medicinal plant, which accumulates high amount of acteoside responsible for several bioactive properties, such as antioxidant, antinephritic, anti-inflammatory, hepatoprotective, immunomodulatory, and

neuroprotective. Analyses of the hairy root transcriptome indicated 219 putative unigenes involved in acteoside biosynthesis of which 54 are upregulated.

13.3.1 Advantages and Limitations of NGS

The main advantage of the genome/transcriptome sequence datasets is that these datasets can be used to characterize genes and biochemical pathways involved in the biosynthesis of valuable bioactive phytochemicals. Moreover, it provides deeper insight into gene expression profiles and molecular regulatory mechanisms underlying biochemical pathways, which control and regulate biosynthesis and accumulation of secondary metabolites (Hao et al. 2011). This information can be exploited for metabolic engineering of biochemical pathways in HRCs to enhance production of the phytochemicals of interest. Transcriptome sequencing of HRCs of medicinal plants may also underpin some breakthroughs for the discovery of new phytomedicines and development of pharmacological and biological resources. Transcriptome sequencing could also be useful in identification of novel transcript involved in the metabolism of bioactive compounds and finding alternate splice form of genes (Wang et al. 2009). Most importantly, NGS techniques have overcome the drawbacks of EST sequencing, such as low-throughput data generation, high cost, lack of quantification of gene expression, bias in cDNA library composition, and inadequate representation of un-clonable transcripts (Mortazavi et al. 2008; Morozova et al. 2009; Shi et al. 2011). Despite, numerous advantages of NGS techniques, limitations in sequencing and assembling of complex genomes are major challenges need to be overcome in the future.

13.4 Conclusion

Hairy root culture is a diverse technique with a wide breadth of applications that continue to multiply with the development of novel gene manipulation techniques. So far, HRCs have been established for a number of plants serving as biotechnological factories for the production of valuable phytochemicals. Establishment of HRCs involves optimization of several factors, each of which varies greatly between species. Seeking out the best of conditions based on projected uses and goals is the key maximizing the functionality of this approach. Besides, a better understanding of biosynthetic genes and biochemical pathways is a prerequisite for metabolic engineering to enhance production of valuable phytochemical in HRCs. Sequencing of transcriptome of HRCs by NGS techniques has greatly facilitated our understanding of biochemical pathways in plants. Some researchers have also used transcriptomics in combination with metabolomics thus squeezing more detailed information about metabolic pathways and gene expression profiles in plants. In the present chapter, we have discussed progress on transcriptome sequencing of HRCs of important

medicinal plants. Transcriptome sequence datasets have proven to be a valuable source of information, thus facilitating elucidation of biochemical pathways and identification of candidate/putative genes in HRCs of plants. The knowledge of biochemical pathways and their genes is crucial for metabolic pathway engineering in HRCs in order to enhance production of valuable metabolites. A number of studies undertaken for transcriptome sequencing have demonstrated potential of Illumina/Solexa platforms as a fast, reliable, simple experimental design and cost-effective approach for transcriptome characterization and gene discovery in HRCs. In the future, transcriptome sequencing studies will extend to HRCs of a large number of important medicinal plants and accelerate the progress of novel gene discovery program. In this chapter, for the first time we have provided an in-depth information on contribution of NGS techniques for sequencing of transcriptome of HRCs of important medicinal plants, which will be highly useful for researchers working in areas of metabolomics and transcriptomics researches.

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Chapter 14
Strategies for Monitoring and Modeling the Growth of Hairy Root Cultures: An In Silico Perspective

Mandavi Goswami, Salman Akhtar, and Khwaja Osama

Abstract Hairy roots have been identified as a good source of secondary metabolites in plants. These secondary metabolites in the genera of phytochemicals have been used by humans since long in the form of drugs, flavors, colors, and others. Thereby, large-scale culture of hairy roots, its management, and production have been conferred as most important and critical steps at industrial scale. Conversely, culture of hairy roots in bioreactors at industrial scale has proven to be a tedious job and requires continuous monitoring and precise control of the system. These challenges for hairy roots owe to their heterogeneous nature. Conventional methods for monitoring of such cultures have failed to work well within this system. So, indirect methods are being used for continuous monitoring of growth and metabolite content in hairy roots. Efficiency and efficacy of these indirect methods depend largely upon models of hairy root growth, product synthesis, and substrate utilization. Several mathematical and computational models have been developed to explain hairy root growth. Some of these models are complex mathematical equations which are based on physical principles, while others are computational models derived from empirical data. This chapter intends to outline and explain some of the prominent models for hairy root growth and their mode and mechanism of action in large-scale bioreactors.

Keywords Artificial neural network · Genetic algorithm · Hairy root culture · Hidden Markov model · Image analysis · Mathematical model · Metabolic flux analysis

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14.1 Introduction

Hairy roots are plant disease syndrome caused by the infection of soil-borne bacterium *Agrobacterium rhizogenes* to the higher plants. Upon infection *A. rhizogenes* transfers a DNA segment (T-DNA) from its root-inducing (Ri) plasmid into the genome of the host plant. Set of genes, carried by T-DNA segment, codes for the enzymes which interfere into the auxin and cytokine in the biosynthesis of the host (Chandra 2012; Mehrotra et al. 2015; Srivastava et al. 2016). Due to this reason, the hormonal imbalance at the wounded site occurs that causes the rapid growth of hairy rootlike mass at the wounded sites. These are called hairy roots and are prominent disease syndrome of higher plants (Georgiev et al. 2012). The hairy roots are characterized by high growth rate, genetic stability, and growth in hormone-free media. These roots are very much similar to the native roots of the host plant in terms of their capacity to produce similar or much higher amount of secondary metabolites that are normally produced by the plant (Pistelli et al. 2010). In laboratories, hairy roots are cultured to explore secondary metabolite synthesis both in terms of their production for commercial purposes and to investigate their biochemical and molecular aspects. Hairy root cultures have proven their worth for the production of commercially valuable secondary metabolites. This property of hairy roots is of particular interest for researchers who strive for large-scale production of these metabolites. Secondary metabolites are the chemicals produced by plants for which no role has been found in growth, photosynthesis, reproduction, or other primary functions. Humans use some of these compounds as medicines, flavorings, fragrance, recreational drugs, biopesticides, nutrients, cosmetic additives, etc. The procurement of these phytochemicals at large scale from plants requires vast agricultural land, time and labour. Further, their chemical synthesis is a costly and labor-intensive affair. Therefore, the constant use, ever-increasing demand, and less availability of these important phytochemicals from natural sources are the driving efforts to develop new ways to optimize their alternative production. In this reference, hairy root-based production of plant-derived metabolites needs scientific consideration. Another commercially important aspect of hairy root is their ability to produce proteins. Although there are some problems associated with production of foreign proteins by hairy roots like low accumulation levels, instability of proteins, etc., hairy roots have great potential for large-scale production of proteins (Doran 2006). Furthermore, hairy root cultures are known for their use in value-added applications like phyto-/rhizoremediation of toxic compounds, biotransformation of exo-/endogenously supplemented substrates into commercially more valuable compounds, etc. (Mehrotra and Srivastava 2017; Srivastava et al. 2017).

To sum up, hairy root cultures have gained popularity as one of the most suitable biological systems to fulfill various biotechnological objectives. For this purpose, the establishment and maintenance of the culture system is a prerequisite. Further, before using any hairy root culture system, sometimes there is a need to know about their biological behavior and their responses to various environmental factors as these factors determine their overall growth and productivity. Practically, in

biological systems it is troublesome and tedious to optimize all the factors every time before using a system for any purpose as biological behaviors are nonlinear and nondeterministic. For this question, the use of modeling of biological systems to investigate their growth patterns, responses to their physical and chemical environment, production potential, etc. can provide an answer. The upcoming text provides condensed information about modeling of biological system and their applications with particular reference to hairy root cultures.

14.2 Modeling of Biological Systems

Advances in various fields of biology and information technology have produced huge amounts of data. Accumulation of this data is day to day increasing and is in continuous need of interpretation and investigation in order to understand the behavior of biological systems. These advances are changing the way biological research, development, and applications are conducted. Modeling is the human activity which includes representation, manipulation, and communication with real-world life objects. A model can be defined as a portrayal of a system (comprised of many integral parts) in terms of its constitutive parts and their association/interactions, where the portrayal itself is decodable or interpretable by humans. Biological processes are nonlinear and complex because of their collective behavior and changes in various phases of development. They depend upon different external and internal factors (Gago et al. 2009). The challenge in modeling any biological process is to find a model which is accurate and able to provide deep insight of the process. Because of the complexity of biological data, simple algorithms or mathematical equations cannot be used to describe the process (Osama et al. 2015). Deterministic models of biological process have been developed based on physical interactions. These models are helpful in providing underlying information of the process. However, due to complexity of biological systems, these models are difficult to develop and are very complex to interpret and solve. Modeling the biological processes requires accounting for action and feedback involving a wide range of spatial and temporal scale. The upcoming text provides an informative description upon various approaches for modeling in hairy root cultures to explore their growth and production phenomenon. Some models of hairy root growth in bioreactors have been summarized in Table 14.1.

14.3 Statistical and Mathematical Models for Hairy Root Growth

Statistical designs can be used to model relationships of different factors on hairy root growth. These designs are simple and easy but require some prior data. Bhadra and Shanks (1995) used statistical design to model the effect of inoculum conditions

Table 14.1 Summary of reported models for the hairy root growth

S. no.	Model	Hairy roots	Effects of model	References
1.	Box-Behnken design (BBD)	*Isatis tinctoria*	Secondary metabolites (rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, and isoliquiritigenin)	Gai et al. (2015)
2.	Agent-based modeling (ABM) approaches	*Beta vulgaris*	Total root length, branching point distribution, segment distribution, and secondary metabolite accumulation	Lenk et al. (2014)
3.	Artificial neural network in combination with hidden Markov model	*Rauwolfia serpentina*	Overall productivity of a bioprocess	Mehrotra et al. (2013)
4.	Artificial neural network-based model	*Artemisia annua*	Effect of different reactor parameters on hairy root biomass	Osama et al. (2013)
5.	Artificial neural network (ANN)	*Glycyrrhiza glabra*	Prediction of optimal culture conditions for maximum hairy root biomass yield	Prakash et al. (2010)
6.	Mathematical model	*Artemisia annua*	On and off cycle in a nutrient mist reactor	Ranjan et al. (2009)
7.	Multi-scale mathematical model	*Ophiorrhiza mungos* Linn.	Temporal evolution of biomass increase and nutrient uptake	Bastian et al. (2008)
8.	Structured nutritional model	*Catharanthus roseus*, *Daucus carota*	Secondary metabolites (nitrogenous compounds and storage carbohydrates, recombinant protein)	Cloutier et al. (2008)
9.	Feed-forward back propagation neural network-based model	*Glycyrrhiza glabra*	Optimum culture condition on biomass growth	Mehrotra et al. (2008)
10.	Population-based model	*Helianthus annuus*	Biomass increase based on age distribution of cells and branching	Han et al. (2004)
11.	Kinetic model for pigment associated with root growth	*Beta vulgaris*	Kinetic behavior of root and pigmentation based on hairy root growth	Kino-oka et al. (1995)
12.	Aerosol model	*Artemisia annua*	Deposition of mist droplets on root hairs in a nutrient mist reactor	Wyslouzil et al. (1997)
13.	Branching number and age	*Tagetes erecta*		Kim et al. (1995)

(continued)

Table 14.1 (continued)

S. no.	Model	Hairy roots	Effects of model	References
	distribution-based model		Specific growth rate of hairy root based on branching dynamics	
14.	Image analysis	*Brassica napus, Brassica campestris*	Assessment of phenotypic effects of expressing foreign genes in plant root systems	Coles et al. (1991)
15.	Comprehensive model based on conductivity	*Coffea arabica*, *Nicotiana tabacum*, *Withania somnifera*, *Catharanthus roseus*	Dry weight of hairy root based on conductivity	Taya et al. (1989a)
16.	Kinetic model for branching	*Daucus carota* *Armoracia lapathifolia* *Cassia torosa* *Ipomoea aquatica*	Increase in hairy root biomass	Taya et al. (1989b)

on growth of hairy roots of *Catharanthus roseus*. In this study, a two-level factorial design was used to study the effect of a number of root tips inoculated, the length of inoculated root tips, and the initial volume of media. Experimental and statistical analysis demonstrated that hairy root growth is highly influenced by the inoculum conditions particularly with the length of root tips which were the dominant variable without any clonal variability. Statistical designs have also been extensively used in media optimization. Growth media composition plays most an important role for both growth and productivity in any culture practice. Thus, during initiation and maintenance of culture, variables of chemical and physical conditions as well play a dominant role. Additionally, the biological condition of culture initiating material (explant) also plays a definite role in growth, development, and productivity. Therefore, optimization of these culture conditions is a prerequisite. Traditionally, optimization of media in biological analyses has been carried out by monitoring the influence of one factor at a time. This technique is called one-variable-at-a-time. However, the main disadvantage of this method is that it does not include any interactive effects among the variables studied. Further, it requires a large number of experimental set, labor and cost inputs, and finally time consumption (Bezerra et al. 2008). To avoid all these limitations, statistical methods for optimization of various culture conditions have been introduced in various culture systems including hairy root cultures (Toivonen et al. 1991; Srivastava and Srivastava 2012). Among initial studies, the effect of sucrose, phosphate, nitrate, and ammonia concentrations on growth and indole alkaloid production of *C. roseus* hairy root cultures was investigated with the help of statistical experimental designs and linear regression analysis (Toivonen et al. 1991). Interestingly, a contradictory effect of these nutrients on growth and indole alkaloid production in *Catharanthus* hairy roots was found. Statistical medium optimization for enhanced azadirachtin production from

the hairy root cultures of *Azadirachta* is one of the most cited examples of the use of statistical methods in media optimization (Srivastava and Srivastava 2012). Plackett-Burman experimental design protocol was used to identify dominating medium components and their concentrations to support high root biomass production and azadirachtin accumulation in hairy roots. The overall exercise has resulted in increased azadirachtin production by 68% in *Azadirachta indica* hairy roots. RSM are efficient tools for optimization, and an increase in productivity by more than 200% can be achieved. In another study, medium optimization for hairy root cultures of *Stizolobium hassjoo* producing secondary metabolites was studied through statistical experimental design (Sung and Huang 2000). The increased production of L-DOPA from hairy roots by 280% was obtained by optimizing medium components using steepest ascent method with central composite design. Also, the study reported 18% increase in the biomass of *Stizolobium hassjoo* hairy roots from the basal media.

Response surface methodology (RSM) has evolved as the most popular optimization method having versatile applicability in various disciplines. RSM is a well-known, dynamic, and efficient mathematical approach which comprises of statistical experimental designs and multiple regression analysis which are the best combination for the formulation of constrained equations (Bezerra et al. 2008). RSM has often applied for the optimization of the fermentation as well as hairy root cultivation (Amdoun et al. 2010; Latha et al. 2017; Singh et al. 2017; Adebo et al. 2018).

RSM is a combination of mathematical and statistical techniques used for modeling of process based on empirical parameters. This method optimizes the response (output variable) which is dependent upon several independent variables (input variable). RSM requires a careful design of experiment; most commonly central composite design or Box-Behnken design is used. In this technique a second-order mathematical model is developed to relate response and independent variables. The model is then differentiated to find the global maxima or minima in order to optimize response (Amdoun et al. 2010). RSM allows the researcher to study the interactive effect between the independent variables. RSM has also been used to optimize the culture medium composition for the growth of elicited *Datura stramonium* L. hairy roots to improve the production of hyoscyamine (Amdoun et al. 2010). In B5 medium the content of nitrate, calcium, and sucrose was optimized to get the best hyoscyamine production. In continuation of the study, the use of the RSM was also made in biological factors, like plant material, to establish a predictive model with the planning of experiments, analysis of the model, and interpretation of the accuracy of the model. Also, the effect of nitrogen, phosphorus, potassium, calcium, and magnesium ions on production of tropane alkaloids from *Datura stramonium* hairy roots was investigated with the help of RSM. The model was developed to study the effect of ions on production of hyoscyamine from *Datura stramonium* hairy roots with elicitation and without elicitation (Amdoun et al. 2009, 2010).

In a recent study, *A. rhizogenes*-mediated hairy root cultures of *Portulaca oleracea* were established for which Box-Behnken model of response surface methodology (RSM) was employed to optimize B5 medium for the growth and noradrenaline production. Upon experimental validation, the optimal conditions for

growth and metabolite production predicted by RSM were confirmed as appropriate for the enhancement of overall productivity (Ghorbani et al. 2015). Further, in a similar study, one-factor model of RSM was utilized to formulate L-arginine amino acid levels along with bacterial strains (ATCC 15834, C58C1, and R1000), type of explant (leaf and stem), and co-cultivation medium (B5 and MS) as three different variables for hairy root induction in *Rubia tinctorum* (Ghorbani et al. 2014). According to the results, L-arginine concentration of 1.00 mM, bacterial strain C58C1, leaf explant, and B5 medium were found optimal for best results. These optimal conditions predicted by RAS were validated and confirmed experimentally to enhance hairy root induction and its implementation for increased metabolite production.

In this context, growth monitoring of hairy roots in liquid medium is a major point of concern. Basically, the growth monitoring in liquid medium is required during large-scale cultures of hairy roots in bioreactors for various purposes. In a culture vessel, during running culture one can visually observe the growth and distribution of hairy root tissue throughout the vessel. However, it is not possible to measure the biomass accurately like this. Several mathematical models have been developed for the estimation of biomass and related metabolite accumulation in hairy root cultures keeping in mind the bioreactor type and culture vessel configuration. The complex interplay of variables like dissolved O_2, temperature, aeration and agitation rates, pH, etc. is monitored, and values are inserted to mathematical models. These models interpret the values and predict the results very near to accuracy in a very short time. The synergistic and individual roles of various variables are thus defined, and in this way growth of subjected root is monitored and maintained throughout culture duration. This ultimately leads to desired productivity. An online monitoring of growth characteristics of hairy root cultures was done by the measurement of conductivity in the bioreactor system (Taya et al. 1989a). A comprehensive model was developed based on conductivity measurements to assess the biomass concentration of hairy root cultures of *Coffea arabica*, *Nicotiana tabacum*, *Withania somnifera*, *and Catharanthus roseus*. A linear relationship between dry cell mass and conductivity for all the root cultures was observed. This method provided an effective means of in situ monitoring of hairy growth in the culture. Later this method was used to determine the biomass concentration of horseradish and carrot in stirred tank and airlift loop bioreactors (Taya et al. 1989c). During the cultivation of high-density biomass, the volume of liquid media reduces with the growth. This change in media volume was considered as a parameter for estimation of biomass growth. Jung et al. (1998) used this method for estimation of biomass of *Catharanthus roseus* hairy root in a 2 liter bubble column bioreactor. It was observed that this method was more accurate in biomass prediction than the conventional method using electrical conductivity as the only parameter. However, in another study, electrical conductivity (EC) in the media along with kinetics of changes in ion concentrations and sugar was monitored to understand the relationship between growth, ginsenoside production, and nutrient partitioning with the help of gaseous composition gradient in terms of O_2, CO_2, and ethylene content (Jeong et al. 2006). In another study, a 3 L nutrient trickling reactor was operated on the

basis of online monitoring of conductivity, pH, and dissolved oxygen. An enhanced production of L-DOPA was observed from hairy root culture of *Stizolobium hassjoo* (Huang et al. 2004). In an earlier study, Wyslouzil et al. (1997) developed an aerosol model for deposition of mist droplets on root hairs in nutrient mist reactor. The *Artemisia annua* hairy root bed was assumed as a fibrous filters, and a model was prepared for mist deposition on the single root fiber. In this study, the deposition of mist across a packed bed of roots was modelled as a function of droplet size, bed length, and gas flow rate. The predictions of the aerosol deposition model were validated with experimental measurements which were found similar.

In nutrient mist reactor, the time of mist on and off cycle is very important. If on cycle is long, it results in accumulation of media on hairy roots causing a gas-phase nutrient deficiency. While during long off cycle, roots can be starved of liquid-phase nutrients. A mathematical model for the mist on and off cycle was prepared by Ranjan et al. (2009), for maximum root density and root growth in nutrient mist reactor. If the mist flow rate in the on cycle is low and the rate of drainage of the media from the bed is equal to the rate of mist deposition, then the reactor could be run in a continuous on cycle. In nutrient mist reactors, to study the kinetic growth of hairy roots, a discrete model was developed (Ranjan et al. 2015). The elongation rate is modeled as exponential growth with the growth coefficient being dependent on mass transfer coefficient, nutrient concentration difference, and distribution of nutrients in growth and sustenance requirements. The experimentally validated results have shown that the primary root growth is reduced by one-fifth of its initial growth rate due to the branching process, and the growth of new branches is significantly faster than its primary root growth due to internal transport of nutrients. Bastian et al. (2008) used a multi-scale approach to simulate hairy root growth. They treated root bulk as a macroscopic porous filter of varying porosity, and all processes were defined in its continuum. The growth was assumed to depend upon nutrient concentration in the medium and inside the root. On microscopic scale the structure of root affects the flow and transport process of nutrients around the root network.

Attempts have been made to model the branching pattern in hairy roots. Hairy roots generally grow by elongation of nodes. New nodes develop and on elongation they form new branches. A kinetic model of branching in hairy roots was developed and simulated for different root cultures in shake flask (Taya et al. 1989b). The model was found to fit the experimental results and was used to estimate different kinetic parameters of hairy roots. Kim et al. (1995) developed a mathematical model for describing branching patterns in hairy roots. This model was then combined with age distribution balance to give a model of age distribution in root culture. Similarly, Han et al. (2004) modeled hairy root growth based on population balance approach. The model proposed that growth of hairy roots depends on formation of new branches and elongation of existing branches. Although probability of formation of new branch is high at a certain age, some lateral branches can develop over distribution of ages of the parent branch.

The growth of hairy roots can also be monitored by the pigment production. A kinetic model was developed for pigment production associated with growth of red beet hairy roots (Kino-oka et al. 1995). The model was based on concept of

distribution of age of cells in hairy roots. The model was able to describe the kinetic behaviors of growth and pigmentation during hairy root growth.

Mass and oxygen transfer rates have a crucial role in the growth of hairy roots in liquid medium. Different workers have proposed methods to investigate and optimize this event for desired productivity. In a study, a mathematical model is developed that defines the oxygen transfer kinetics in the cultured *Azadirachta indica* hairy root matrix as a case study for offline simulation of process control strategies ensuring non-limiting concentrations of oxygen in the medium throughout the hairy root cultivation period. The unstructured model simulates the effect of oxygen transfer limitation in terms of efficiency factor on specific growth rate of the hairy root biomass. The model is able to predict effectively the onset of oxygen transfer limitation in the inner core of the growing hairy root matrix such that the bulk oxygen concentration can be increased so as to prevent the subsequent inhibition in growth of the hairy root biomass due to oxygen transfer (diffusional) limitation (Palavalli et al. 2012).

14.4 Image Analysis

Image analysis is the procurement of meaningful information from images particularly from digital images with the help of digital image processing techniques. In recent years, many image analysis softwares have been designed for more detailed root morphological and architectural measurements. One example of such software is WinRHIZO root-scanning software (Regent Instruments Inc., Ottawa, ON Canada). This software has the ability of rapid measurement of multiple root parameters such as root length, volume, surface area, diameter, tips, and crossings and has been widely used in research related to plant root growth and responses (Aryal et al. 2015; Kadam et al. 2017). WinRHIZO, however, is a costly software, and several freeware like ROOTEDGE are also available which are highly efficient (Kaspar and Ewing 1997). Image analysis provides an efficient way for noninvasive and nondestructive monitoring of hairy root growth kinetics on the basis of their morphological characteristics (Coles et al. 1991). A manual imaging method "PetriCam" along with an image processing algorithm was initially proposed by Lenk et al. (2012, 2014) to assess the growth performance and secondary metabolite production in *Beta vulgaris* hairy root cultures growing in petri plates. The uniqueness of the method is its ability to take images from the closed petri plates without destroying the culture. However, as the image consists of planner 2D growth pattern, the major limitation of this method is that it does not provide any information regarding the distribution of hairy roots in z-axis. Considering the fact that different HR morphologies result in dissimilar levels of secondary metabolite production, the effect of morphological features on growth and production potential needs proper attention. As two HR clones with a similar biomass but different root architectures could have completely different product yields, it becomes important to investigate their differential production. Image analysis has been successfully adapted by many

researchers for measurement of root length, diameter, and other morphological characteristics (Cai et al. 2015). These workers demonstrate a near to perfect numerical scheme for accurate, detailed, and high-throughput image analysis of plant roots. Involvement of image analysis methods provides better results in terms of accuracy, robustness, and the ability to process root images under high-throughput conditions (Flavel et al. 2017). In context of hairy roots, Berzin et al. in 1997 developed a morphological structured model of *Symphytum officinale* hairy roots using a desktop scanner for image analysis and NIH image program which is a public image processing and analysis program developed by the National Institutes of Health. In another study, nondestructive measurement of the chlorophyll pigment based on color image analysis was done for the assessment of herbal toxicity in photoautotrophic hairy roots of *Ipomoea aquatic* (Ninomiya et al. 2003a). Furthermore, the elongating behavior of *Ipomoea aquatica* hairy roots exposed to external herbicidal stimuli was evaluated by automatic tracing of the root tip point employing computer-aided image analysis (Ninomiya et al. 2003b). Such results advocated that the system developed could be a useful tool for the assessment of herbicidal toxicity in the hairy roots.

Once the images are taken, they are needed to be processed to procure numerical information such as segment length, branching point distribution, metabolite accumulation patterns, etc. In recent years, several efficient open-source and commercial solutions have been reported by several researchers for image processing purposes (Lobet et al. 2011; Clark et al. 2013).

Image analysis can also be used for estimation of secondary metabolite in hairy root cultures (Lenk et al. 2012). Due to accumulation of secondary metabolites, the morphological characteristics of hairy roots change. This change can be analyzed by image analysis to predict secondary metabolite concentration. For the estimation of secondary metabolite, color image analysis is used. Smith et al. (1995) introduced hue-saturation-intensity (HSI) color coordinate system and developed equations for converting the red-blue-green color coordinate system to HSI. They proposed that HSI color coordinate is better for image analysis of hairy roots. Berzin et al. (1999) developed a nondestructive method, based on the analysis of scanned images in HIS color space, for determining local and overall levels of secondary pigment metabolites in hairy root cultures of *Beta vulgaris*. Modified saturation values (saturation divided by dimensionless root diameter) were found to be proportional to pigment concentration. The analysis was carried out manually for each local point of the root, and morphological measurements were performed separately. RHIZOSCAN is semiautomated software for root image analysis. It provides typical measurement analysis, such as root axis length (primary, secondary, total) and comparative plots. It measures root thickness, volume, length, etc. of each lateral of hairy roots and also can be used for estimation of secondary metabolite from scanned images. Berzin et al. (1999) tested this software for characterization of morphology of hairy roots of *Beta vulgaris* and estimation of secondary metabolite concentration. They concluded that RHIZOSCAN is a reliable tool for analysis of root architecture and determination of secondary metabolite in hairy roots.

14.5 Genetic Algorithm (GA)

Economic viability of secondary metabolite production in hairy root cultures depends largely on the kinetic growth model and efficient scale-up in bioreactor designs. A population-based model as genetic algorithm has seen its significant implications in modeling of specifically hairy root cultures owing to its capacity to search solutions in large hyperspace applying schemata theorem and optimizing to its nearest best solutions in the best possible time (Han et al. 2004; Arab et al. 2016). Based on the nature's natural process of evolution, GAs involve a class of computational models lying on the principle of natural selection and survival of the fittest phenomenon (Fogel et al. 1975). GAs have been prominently used as a suitable function in the determination of the optimum concentration of the medium components in hairy root bioreactor design.

Conceptually, genetic algorithms begin with a set of solutions encoded like genes on the chromosomes called population. Motivated by a hope that the new population will be better than the old solutions, individuals from one population are selected on the basis of fitness functions (given more chances to reproduce) and are used to form a new population by the application of natural selection operators. Natural selection is usually applied through stochastic or remainder stochastic sampling techniques. This is followed by the F2 generation (offspring) by the application of recombination operator using one-point or two-point crossover to introduce the genetic diversity in the solutions to the current population. Mutation operation is often implied involving a single flip of bit in binary encoding of individual fitness values which tends to introduce novel solution to the current population (Fig. 14.1). This is repeated to several generations until we move toward to better population of individuals with improved solutions than parent populations (Fogel et al. 1975; Davis 1991). GAs have been continuously applied to solve many search and optimization problems involving non-differentiable, discontinuous, stochastic, or highly nonlinear objective functions, which are normally not well suited for standard optimization algorithms.

GAs in hairy root cultures are based on the fact that age distribution of cell in cell cultures is not uniform and evolves with time, and thus the dependent variable in this population-based model tends to be the biomass at a time t or the number of cells at the time t. A variable X is defined to denote the biomass weight distribution function for hairy roots of age A at culture time. The age of the oldest cell from a branch is taken as the age of the specific branch. The total biomass of hairy root at any time t tends to be a function of X(t, A) where Amax is the greatest possible age of hairy root culture during harvesting time. As branching rather than root lengthening accounts for the maximum production of biomass in hairy root cultures, GA model has been specifically designed for branching studies as a function of time and age (Han et al. 2004; Arab et al. 2016). One of the most critical advantages of GA seen is its capacity in handling a large number of data including previous data assumed at each generation in the direction of producing the optimized result.

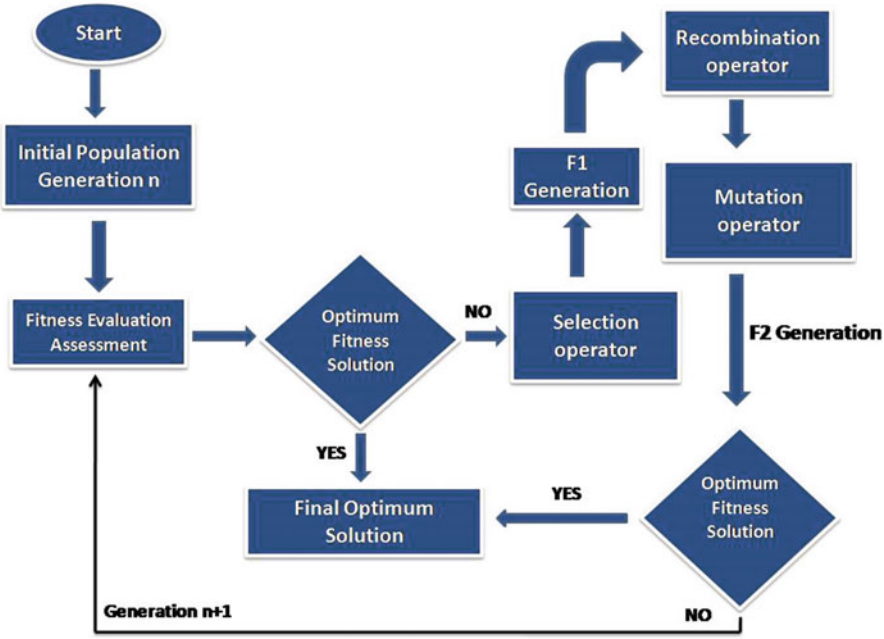

Fig. 14.1 Working of genetic algorithm

14.6 Artificial Neural Network

An artificial neural network (ANN) is a mathematical or computational model that mimics the structural and functional behavior of biological neural network. Consisting of a set of precisely designed artificial neurons, ANN works in unison to solve a specific problem. ANNs have been broadly applied with great success for system designing, modeling, optimization, and control mainly due to its capacity to learn noise filter signals and generalize information through a systematic training procedure (Singh et al. 2009). ANN appeared to be a feasible method for modeling hairy root growth and culture conditions. Neural networks are typically implemented in the estimation and multistep prediction problems even with unknown solutions but can also be used as controllers directly or as an adjuster of any process parameter for a traditional controller. In ANN artificial neurons are arranged in input, hidden, and output layers (Fig. 14.2). Almost all the computations are done in the hidden layer.

Neural networks are "trained" using a data set and then used to foretell new data points. The prior knowledge is not essential for this training as the network and system remain as a black box to the user and provide the result through its own artificial intelligence. Notable characteristics of ANNs are that they can work steadily with large amounts of data which outshine at complex pattern recognition, involve real-time operations, possess fault tolerance potential, and require no

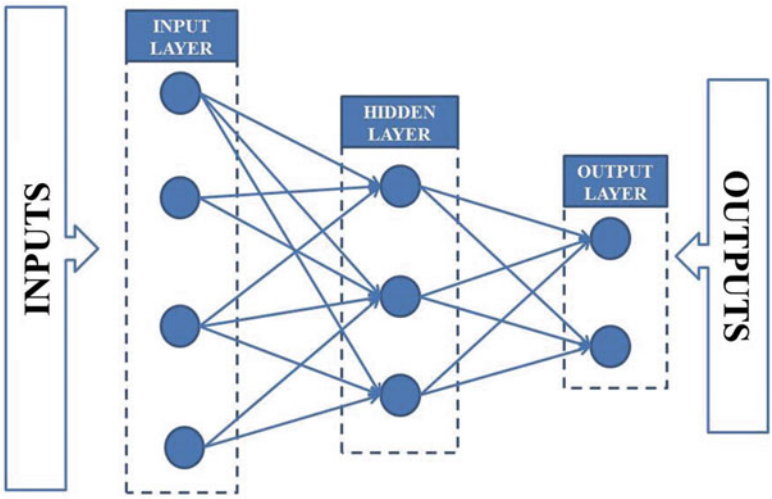

Fig. 14.2 Architecture of feed-forward artificial neural network

mechanistic description of the system. ANN is well suited for media design, as it generates a large amount of data that contains the hidden model. The "learning conditions" of neural networks are categorized into three combinations as follows:

1. Administered (associative), where the neural network is trained by providing it with input and output experimental data,
2. Self-organization in which output unit is trained to respond against clusters of pattern within the input. Different from the organized, there is no previous set of groups in which the patterns are to be classified and then the system must develop its representation of the input stimuli.
3. Support where training may consider as an ordinary form of the above two classes of learning.

ANN has been found to be well suited for hairy root growth in nutrient mist reactor as reported by Osama et al. (2013). Combination of ANN and genetic algorithm (GA) has also been found to be very effective for maximizing the native concentration and shelf life of secondary metabolites (Khan and Tripathi 2011). The ANN can perform well on nonlinear program problems and can continue working without any difficulty by their parallel nature even when an element of the neural network fails (Vaidya et al. 2003). ANNs can be implemented in a wide range of problems and do not need to be reprogrammed at every step of solution providing.

The major limitation of artificial neural networks is that they require prior data of the process. The data set used for training is very important; it decides the quality of network prediction. If the training data set is incomplete or contains wrong values, the training will be incomplete. The network will give faulty outputs.

The growth pattern in biological system is complex, nonlinear, and difficult to predict and cannot be controlled by our will. These processes are controlled by

genetic and environmental factors which are highly inconsistent (Mehrotra et al. 2008). Hairy roots are considered to be an alternate source of secondary metabolite production. In large-scale culture and production of hairy roots, the cost and the culture duration for production are very important. The conventional modeling techniques often become ineffective in monitoring and predicting the growth pattern of hairy roots. Hairy roots form heterogeneous clumps, and therefore direct monitoring of growth parameters is difficult. There is a need for indirect strategies of monitoring their growth. These may include development of models of the developmental pattern of hairy roots which can then be used for monitoring of growth. Mehrotra et al. (2008) used a feed-forward back propagation neural network to predict in vitro culture conditions for optimum biomass growth of *Glycyrrhiza glabra* plant. In other study regression and back propagation neural network was used to predict the culture parameters for maximum biomass yield for hairy root of *G. glabra* (Prakash et al. 2010). They used inoculum density, pH, and volume of growth medium per culture vessel and sucrose content of the growth medium as parameters to predict hairy root biomass. The neural network model was very efficient and was able to explain over 98% of the variations in the kinetic data. This approach was also used for modeling growth of hairy root of *Artemisia annua* in a nutrient mist reactor (Osama et al. 2013). Different architectures of ANN were compared to model reactor and several reactor parameters. All the network models were found to be efficient in modeling the nutrient mist reactor.

Artificial neural networks have also been used in combination with hidden Markov model for predicting optimum conditions for maximum biomass of hairy roots (Mehrotra et al. 2013). Five culture conditions were taken as input parameters to predict hairy root biomass. The input parameters were fed to the neural network through five HMM models. The combinatorial model proved to be efficient in predicting hairy root biomass.

14.7 Conclusion

Hairy roots cultures are fast growing and have high capacity of production of secondary metabolites. They possess several qualities due to which they are very promising candidate for large-scale production of phytochemicals. For large-scale production of hairy roots, constant monitoring of growth and phytochemical production is essential. Direct monitoring of hairy root growth in the reactor system is difficult, time taking, and labor intensive. Several indirect techniques have been proposed for monitoring hairy root growth in bioreactors. Most of these techniques require good understanding of the biological processes and effect of environmental conditions on hairy root growth. Apart from this, a good understanding of the effect of hairy root growth on their environment is also essential. Several mathematical models and machine learning-based models have been proposed for this. Machine learning techniques prove to be a promising tool for modeling complex biological process.

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Chapter 15
Engineering in Hairy Roots Using CRISPR/Cas9-Mediated Editing

Anshu Alok, Jitesh Kumar, and Santosh Kumar Upadhyay

Abstract *Agrobacterium rhizogenes* is a well characterized bacterium for "hairy root induction" due to presence of Ri plasmid. Ri plasmid has been modified and engineered with required foreign genes and used as a binary vector for plant genetic transformation. *A. rhizogenes*-mediated hairy root induction and cultures of recalcitrant plant species are useful in genetic and metabolic engineering for secondary metabolite and recombinant protein production. With the advancement of CRISPR/Cas9 genome editing tools, plant genome can be easily manipulated for metabolic engineering. However, CRISPR/Cas9-mediated genome editing requires efficient *A. rhizogenes*-mediated genetic transformation and selection. In this chapter, we discussed the different essential component of CRISPR/Cas9 editing tools. Different types of CRISPR/Cas9 vectors are now available for various purposes such as disruption, replacement, transcriptional activators, and inhibitors of desired gene.

Keywords *Agrobacterium rhizogenes* · CRISPR/Cas9 · Genome editing · Hairy root · Transformation

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15.1 Introduction

Genetic engineering in whole plants or any of its organs has become a very interesting approach for functional genomics and metabolic engineering. Various tools related to knock-in and knock-out of genes within a plant genome are available and repeatedly used for multiple plant species. These tools have been applied in numerous important crop and medicinal plants to increase nutritional and pharmaceutical values, respectively (Newell-McGloughlin 2008; Hefferon 2015). These plant engineering tools can be applied in specific organs such as leaves, roots, tubers, etc. in addition to whole plant for the targeted production of important metabolites. Numerous binary vectors and genetic transformation methods, for example, electroporation, PEG mediated, bombardment, and nanoparticle mediated have been used for plant genetic engineering. However, it becomes easier and efficient to use *Agrobacterium*-mediated gene transfer, which is "nature's genetic engineer" (Nester 2011, 2015). Naturally, *Agrobacterium* species infect wounded plants that results in the formation and outgrowth of tissue which is commonly known as "crown gall disease" or "hairy root disease." *A. rhizogenes*, also known as *Rhizobium rhizogenes* (Young et al. 2001; Ron et al. 2014), is a gram-negative soil-borne bacterium. It transfers a part of its extrachromosomal DNA, known as root-inducing (Ri) plasmid, which integrates into the plant genome as transfer DNA (T-DNA) (Chilton et al. 1982; Kumar and Mitra 2017). T-DNA consists of *rol* gene that is responsible for formation of root like structures well known as hairy roots, on the site of infection of host tissues. The recombinant DNA engineering within the wild-type Ri plasmid has done for proficient transformation of different plant species. In case of *A. tumefaciens*-mediated genome modification, it needs a fast and reproducible protocol for recalcitrant plant species to explore gene function. Due to this, *A. rhizogenes*-mediated "hairy root induction and culture" has become a very useful model system for studying the gene function and valuable secondary metabolite production (Mehrotra et al. 2015; Srivastava et al. 2016).

Various approaches of plant genetic engineering such as overexpression, RNA-mediated interference (RNAi), virus-induced gene silencing (VIGS), T-DNA insertion mutagenesis, and genome editing tools requires genetic transformation and simultaneously regeneration of transformed tissues of plant species. *A. rhizogenes*-mediated overexpression and RNAi constructs have been successfully applied in different lines of hairy roots of *Coleus blumei* (Hücherig and Petersen 2013). The modification of soybean genome with zinc-finger nucleases (ZFNs) to knock out dicer-like genes and other genes involved in RNA silencing has been done. The targeted transgene in addition to nine endogenous soybean genes by preparing zinc-finger arrays (Curtin et al. 2011). On the other hand clustered regularly interspaced short palindromic repeats/(CRISPR)-associated nuclease 9(CRISPR/Cas9)-mediated editing has gained tremendous attention for genome modification (Upadhyay et al. 2013; Cong et al. 2013). This tool is more precise and easy to design as compared to ZFNs and transcription activator-like effector nucleases

(TALENs) (Gaj et al. 2013). CRISPR/Cas9 editing tool was initially demonstrated in protoplast, which was further applied to germline cells, callus, and leaf tissues in various plant species. Genome modifications were efficiently demonstrated within hairy roots of tomato using *A. rhizogenes* carrying CRISPR/Cas9 vector (Ron et al. 2014).

15.2 *Agrobacterium rhizogenes* Strains and Its Specificity Toward Different Plant Species

A. rhizogenes are mainly grouped into four categories: agropine, mannopine, cucumopine, and mikimopine types. The structural arrangement of genes within Ri plasmid may vary with the different bacterial strains. The T-DNA which consists of few genes transfers from bacterium into plant cells and then integrates into the host genome. The T-DNA consists of three regions, namely, right, left, and central T-DNA regions commonly denoted as T_R-, T_L-, and T_C-DNA, respectively (Offringa et al. 1986). Left and right borders having a size of 25 bp nucleotide sequences are similar in both Ri plasmid and Ti plasmid of *A. rhizogenes* and *A. tumefaciens*, respectively (Slightom et al. 1986). T_L-DNA consists of *rol* gene with different open reading frames (orf), whereas T_R-DNA consists of *mas*, *aux*, and *ags* genes (Slightom et al. 1986; Camilleri 1991). The *rol* gene of Ri plasmid is mainly responsible for hairy root induction (Mehrotra et al. 2015). For example, strains A4 and HRI are agropine-type *A. rhizogenes* which consist of 18 orf in their Ri plasmid (Slightom et al. 1986). Various strains have been identified and characterized for inducing hairy roots in plants species such as R1000, A4, LBA9340, ATCC15834, etc. (Table 15.1).

Table 15.1 *A. rhizogenes* strains being used for hairy root induction

Bacterial strain	Chromosomal background	Ri plasmid	Opine classification	Antibiotic resistance	References
A4	NA	pRiA4	Agro	Neo, Rif	Tiwari et al. (2008), and Hosokawa et al. (1997)
LBA9402	NA	NA	Agro	Neo, Rif	Tiwari et al. (2008)
R1000	C58	pRiA4b	NA	Neo, Rif	Tiwari et al. (2008)
K599 or NCPPB2659	Biovar 1	pRi2659	Cucumopine	NA	Mankin et al. (2007)
ARqua1	Smr derivative of A4T strain	NA	NA	NA	Plasencia et al. (2016)
A4RS	A4 derivative	NA	NA	Rif, Spec	Plasencia et al. (2016)

Agro agropine, *Rif* rifampicin, *Neo* neomycin, *Spec* spectinomycin, *NA* not available

15.3 CRISPR/Cas9 Components

CRISPR/Cas9 plant transformation vectors consists of mainly two components, i.e., *Cas9* and guide RNA (*gRNA*), along with essential component of binary vectors such as selectable marker, origin of replication, and T-DNA border. A schematic map of CRISPR/Cas9 plant genome editing vector is depicted in Fig. 15.1. In this section we have mentioned different types of Cas9, gRNA, and transformant selection genes which are available in various vectors.

15.3.1 Cas9 Nucleases and Its Variants

Cas9 gene is located within the genome of few bacteria, which encode Cas9 endonuclease. This nuclease recognizes the target sequence with the help of gRNA and cut both strands of the DNA (Esvelt et al. 2013). Plant cells have their own DNA repair mechanism correct the break either by inserting or deleting few nucleotides, which subsequently leads to mutation. The size of *Cas9* varies between the bacteria and has been reported to be of 3.1 kb and 4.3 kb. *Cas9* has been reported from *Streptococcus pyogenes* and *Staphylococcus aureus*, respectively (Fonfara et al. 2014). Using mutagenesis technique different kinds of changes have been done in the *Cas9*, which altered its properties. The mutation within RuvC domain

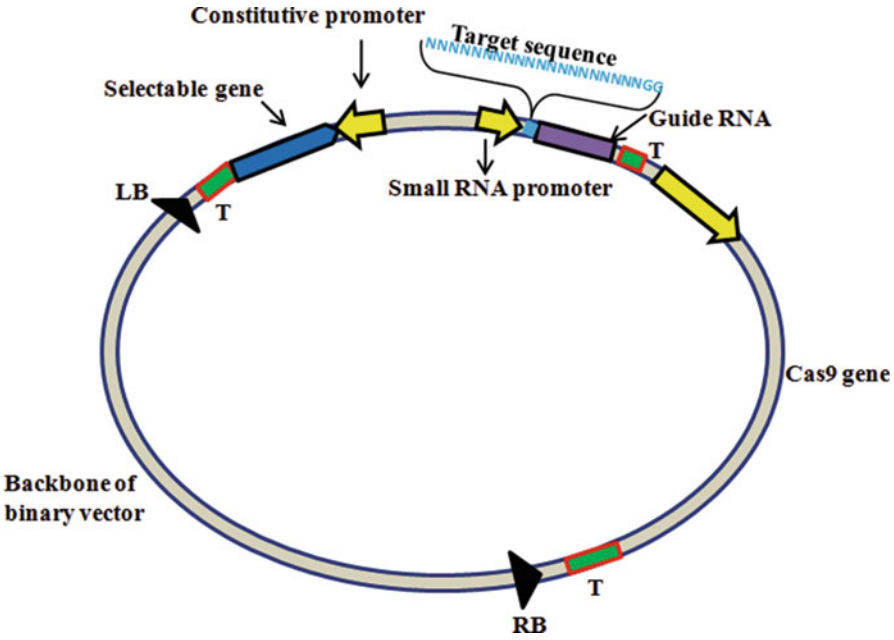

Fig. 15.1 Schematic map of CRISPR/Cas9 plant editing vector

with D10A and D31A in *S. pyogenes* and *Streptococcus thermophilus* Cas9, respectively, leads to cleavage of only sense strand (non-complementary) of DNA. Therefore this type of Cas9 variants is called as "nickase Cas9" (Jinek et al. 2012; Xu et al. 2014). Similarly, mutation within HNH domain of Cas9 with H840A and N891A of *S. pyogenes* and *S. thermophilus* resulted into variant that cleaves only antisense strand of DNA (Xu et al. 2014). Mutation in both RuvC and HNH domains forms a dead Cas9 (dCas9), which only recognizes and binds to the gRNA, but cannot cut any strand of the target DNA. The fusion of desired molecule with dCas9 can also be used for different applications, for instance, transcriptional activator, suppressor and base editor, etc.

S. pyogenes Cas9 nucleotide sequences were altered to substitute specific amino acid instead of its naturally existing amino acids without changing Cas9 activity. These types of Cas9 were designated as high-fidelity eSpCas9 (1.0) and eSpCas9 (1.1), which are very efficient and have less off-target effect shown in plant (Zhang et al. 2017).

15.3.2 Guide RNA (gRNA)

It is a chimera sequence of naturally existing crRNA and trans-activating crRNA (tracrRNA) of bacteria, which provide binding ability to Cas9 endonuclease at the target site within the genome (Hsu et al. 2013). The synthetic gRNA does not exist in nature, but it mimics the natural crRNA-tracrRNA hybrid where Cas9 acts and leads to DNA break. The gRNA consist of 20 bp nucleotide target sequence which is complementary to the sequence of the target gene. The target sequence within DNA essentially consists of 5'-NGG-3′ which is also known as protospacer adjacent motif (PAM) for *S. pyogenes* Cas9 activity. The 5'-NGG-3′ requirement of the PAM limits the target sites within plant genome by SpCas9. The PAM recognition site varies according to Cas9 of different bacteria.

15.3.3 Plant Selectable Marker

Plant selectable markers are very important criteria for the CRISPR/Cas9 vector selection. Dose of selection agent within the media may cause different kinds of lethality for plant cells which diminish plant cell's ability to grow into whole plants. Different markers have been explored and used to generate whole plants from transformed tissue on selective media (Breyer et al. 2014). Most of the available CRISPR/Cas9 vectors consist of *NptII*, *HptII*, and *Bar* gene as selectable marker. For instance, pRGEB31 and pRGEB32 have *HptII*, while pBUN411 consists of *Bar* gene.

15.4 Types of CRISPR/Cas9 Vectors

Various modifications have been done in basic CRISPR/Cas9 vectors according to the need of researcher and the applications in plants. In this section we will focus on these available vectors and their applicability.

15.4.1 Knockout CRISPR/Cas9 Vectors

These vectors mainly consist of wild-type *Cas9* gene of bacteria and synthetic gRNA within the T-DNA border. The main function of this vector is to create double-strand break which leads to mutation within target sequence and finally "knock out the gene function." Therefore they are called as knockout CRISPR/Cas9 vectors, for example, pRGEB32, pHSE401, and pBUN411.

15.4.2 Knock-In CRISPR/Cas9 Vectors

In this system, the basic component of CRISPR/Cas9 is along with donor construct which has homologous arm similar to the sequence where it has to be incorporated within the genome. This "donor construct" or "donor vector" consists of gene of interest flanked with upstream (left homology arm) and downstream sequence (right homology arms) of the target sequence where this has to be inserted. The donor construct may reside onto the same T-DNA along with essential editing components or onto additional vectors. These are generally used for either gene correction or "knock-in" of gene of interest within the genome. For example, pTC217 carry essential components which target to create double-strand break within ANT1 locus, whereas donor construct carries Pnos:NptII as 5′ homology arm and 35S: ANT1 as 3′ homology arm (Čermák et al. 2015).

15.4.3 CRISPRa and CRISPRi Vectors

Here the *dCas9* is fused with transcriptional activator or repressor and therefore termed as CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) vectors. These fused Cas9 along with transcriptional activators, for example, VP64 and p65AD, target the upstream region (promoter) of desired gene and enhance the transcription. These vectors such as pYPQ152, pHSN6A01, pBUN6A11, pdCas9 (GB1079), and pD10AH840AhCas9 (GB1041) are available with multiple types of activators. Krüppel-associated box (KRAB) is used as repressor along with dCas9. This fusion recruits heterochromatin-forming complex, and due to this histone

methylation as well as deacetylation occurs and finally the silencing of targeted gene (Thakore et al. 2015).

15.4.4 CRISPR/Cas9 Vectors for Visualization and Purification

The fluorescent proteins are generally fused to dCas9 and further used to locate the target location within chromosome or whole genome (Chen et al. 2013). Similarly, specific tags are also fused to dCas9 to purify the desired DNA fragments (Fujita et al. 2016).

15.4.5 CRISPR/Cas9 Base Editor

Cytidine deaminases are known to be responsible for conversion of cytosine (C) to uracil (U) residues by removing the amino group within pyrimidine ring of cytidine (Betts et al. 1994). These cytidine deaminases as well as uracil DNA glycosylase inhibitor, upon fusion with dCas9, act as base editor. This base editor can change DNA bases without creating break (Liang et al. 2017). The base editor can deaminate C to U without any break within DNA and consequently occur in the target DNA as C to T (or G to A) conversion sequence. Recently, proficient base editing in *Arabidopsis* has been reported (Chen et al. 2017). These base editor vectors are available with different selectable markers such as pHSE901, pnCas9-PBE, and pH-nCas9-PBE.

15.5 CRISPR/Cas9-Mediated Genome Editing in Hairy Root

The genome alteration using this technique within hairy root genome of plants requires an efficient protocol for *A. rhizogenes*-meditated genetic transformation of desired plant species. Selections of bacterial strain, CRISPR/Cas9 vector, and selection medium are important parameters to produce mutated hairy root lines. The detailed steps required for CRISPR/Cas9-mediated genome editing in hairy root is mentioned below and also being given in Fig. 15.2.

1. Select target region within the genome of interested plant.
   - This 20 bp sequence followed by 5'-NGG-3′ (PAM region) within gene to be modified/deleted.
   - Online off-target prediction tools should be used to minimize the off-target.

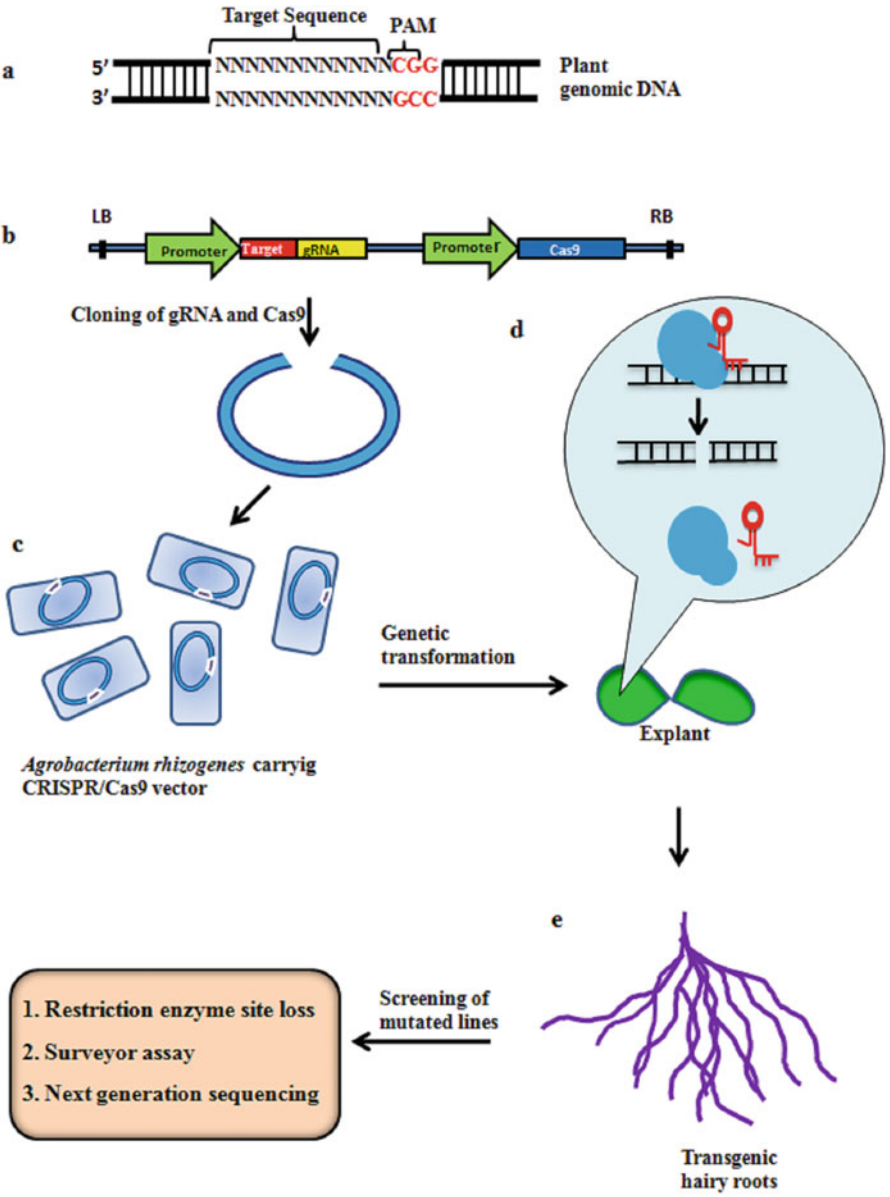

Fig. 15.2 Schematic representation of CRISPR/Cas9-mediated editing in hairy roots. (**a**) Target identification within genome, (**b**) construction of vector, (**c**) *Agrobacterium rhizogenes* carrying construct, (**d**) editing mechanism within the transformed explants, and (**e**) hairy root induction

2. Designing of single or multiple gRNAs.
   - The gRNA is generally regulated by small RNA promoter, such as U6 or U3 promoters.
   - The multiple gRNA targeting various sites can be assembled using glycine tRNA-processing system or cys4 spacers.
3. Choose the desired CRISPR/Cas9 plant genome editing vector.
   - Carefully choose the plant selectable marker gene present in vector.
   - Promoter used to regulate Cas9 depends upon the plant of interest.
4. *A. rhizogenes*-mediated genetic transformation.
   - Strain of bacterium and different parameters are important for this step.
5. Generation and confirmation of transgenic hairy root lines on selection medium.
   - Genomic DNA isolation from different lines
   - PCR-based screening of transgenic lines
6. Detect the mutation at target site within genome.
   The mutation can be detected by the following approaches:
   - Loss of restriction site near target sequence
   - Surveyor assay
   - Next-generation sequencing
7. Sequencing analysis and calculation of indel and mutation frequencies.

The mutation frequencies are extremely unpredictable according to plant species and type of bacterial strain used for transformation. For example, soybean transgenic hairy roots generated with strain K599 have shown that 54% of 170 roots were mutated with indel frequencies varying between 0.6% and 95.0% (Cai et al. 2015).

15.6 Editing in Hairy Root Genome for Functional Genomics

Functional genomics in plants to explore the function of unknown gene requires genetic transformation and successively regeneration of transformants. This genome editing tool has greatly excited the researcher with its various properties and ease to apply in contrast to other available tools. This CRISPR/Cas9 tool was successfully applied in hairy roots produced by *A. rhizogenes* to know the function of transcription factors *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*) in tomato and other plants species (Table 15.2). This targeted editing showed mutations with diverse types of insertion or deletion in the *SHR* gene of tomato. In tomato, this targeted editing causes alterations in hairy root phenotype which was similar to *Arabidopsis shr* mutants (Ron et al. 2014).

Table 15.2 CRISPR/Cas9-mediated genome editing in hairy roots

Plant name	Vector	Selectable marker	Strain	Target genes	Purpose	Reference
Glycine max	p201N/ pSPH2	*NptII*	K599	GFP, Glyma07g14530, Glyma06g14180, Glyma01g38150, Glyma11g07220, Glyma04g36150, miR1509, miR1514	Functional genomics	Jacobs et al. (2015)
Glycine max	pCambia3301	*NptII*	K599	Glyma08g02290, Glyma12g37050, Glyma06g14180	Functional genomics	Sun et al. (2015)
Glycine max	SgRNA: Cas9 expression vector	*Gfp*	K599	*FEI1*(Glyma01g35390), *FEI2* (Glyma09g34940), *SHR*, *Bar*	Functional genomics	Cai et al. (2015)
Brassica carinata	CRISPR+35S::GFP	*Gfp*	*A. tumefaciens* C58 (ARqua1)	*BcFLA1*	Functional genomics	Kirchner et al. (2017)
Salvia miltiorrhiza	AtU6-26SK, 35S-Cas9-SK, pCambia1300	*HptII*	Accc10060	*CPS1*	Metabolic engineering	Li et al. (2017)
Solanum lycopersicum and *Lepidium hyssopifolium*	pMR093	*NptII* or *bar* or *HptII*	ATCC15834	*SHR*	Functional genomics	Ron et al. (2014)
Rubber dandelion	*pFGC-pcoCas9 FFT,*	*Not used*	*K599*	*1-FFT*	Metabolic engineering	Iaffaldano et al. (2016)

Hairy roots of recalcitrant plant species has become a good platform for the functional validation of genes. Cai et al. (2015) used *A. rhizogenes* rather than *A. tumefaciens* for soybean genetic transformation due to less transformation efficiency. In vitro regeneration of soybean is a long, labor-intensive procedure and requires expert skills. Therefore, they used *A. rhizogenes* and the CRISPR/Cas9 vector to generate genome-edited hairy roots. In this study, they efficiently edited the targeted soybean *FEI2* and *SHR* endogenous gene as well as exogenous *bar* gene for editing in hairy roots.

CRISPR/Cas9-mediated genome editing is very useful as compared to other loss of function approach tools in case of polyploid plant species, where most genes have homoeologs. Most of homoeologous genes have similar nucleotide sequences with very less variation, and therefore targeting by RNAi is difficult to knock down these genes. CRISPR/Cas9 editing tools might be efficiently used to target a single or multiple homoeologous genes at the same time (Jacobs et al. 2015). In soybean hairy roots, individual gRNA were designed to target *DDM1* gene which was located on chr1 and chr11 and edited with 21% and 8.9% mutation frequency, respectively. Another gRNA which was targeting both homoeologous of *DDM1* gene at a time were also demonstrated to edit with less mutation frequency (Jacobs et al. 2015). *A. rhizogenes* and CRISPR/Cas9 tools offer a fast and proficient means to explore the role of gene of interest within hairy root of various plant species.

15.7 Conclusions

The CRISPR/Cas9 editing tool may well assist the development of homology-directed repair using dsDNA donor templates. This approach can be used for gene or nucleotide insertions, disruption, and replacements within hairy root. Additionally, this system has been also used for transcriptional activation and inhibition in plants. Hairy roots can easily and rapidly grow from recalcitrant plant species, which mimic natural root systems. Therefore, important metabolic pathways can be altered in hairy root for production of important metabolites.

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