CHAPTER 7

Green Factories: Plants As A Platform For Cost-effective Production of High-value Targets

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Abstract: Transgenic plants have been developed since the early 1980s, when researchers were able to transform a piece of foreign DNA into a plant genome. Since then, the technology has expanded enormously, giving rise to many private and public ventures in the field of plant-based recombinant technology. The technology has helped in crop improvement against various biotic and abiotic stresses such as insect resistance and herbicide tolerance, as well as improving their nutritional values, for example, Golden rice. In addition to crop improvement, the technology has enabled plants to be used as green factories for the production of recombinant proteins. Several platforms are available for the heterologous expression of foreign proteins, each of which represents its own set of advantages and limitations. Plants offer many advantages for inexpensive yet large-scale production of high-value targets, making them extremely attractive for commercial applications. In this chapter, we briefly discuss the need for using plants as solar-powered cellular factories to produce recombinant proteins. We provide a snapshot of different expression systems and argue that the plant-based expression system is highly commercially feasible not only for the production of high-value targets but also to help address global challenges like Covid-19.

Keywords: Biopharming, High-Value Targets, Green Factories, Plants.

3. WHY PLANT-BASED EXPRESSION SYSTEMS?

Heterologous expression of recombinant proteins for different applications has become a focus of intensive research for a while, paving the way for another revolution in the area for the development of new production technologies. The demand for cost-effective yet large-scale production of protein and secondary metabolites for various purposes, such as medical reagents, cosmetic products,

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and industrial enzymes, in terms of quantity, diversity, and, most importantly, quality has dramatically increased since the past decade [1]. The gap between demand and supply has further increased due to inefficient yet highly expensive production systems [2, 3]. Several systems, including bacteria, yeasts, animal cells, transgenic animals, plant cells, and transgenic plants, are available for the heterologous production of high-value targets [4, 5]. All available expression systems have their pros and cons in terms of cost, time, efficiency, product size, growth conditions, yield, post-translational modification, downstream processing, and regulatory approval [6]. The advantages of plant expression platforms are cited in several earlier reports [7 - 12]. Table **1** shows head-to-head comparisons of all existing platforms. Transgenic plants have become a focus of interest as new generation bioreactors mainly due to: i) reduced up-front production costs, ii) lower risk of endotoxins as well as human pathogen contamination, iii) scalability, iv) availability of existing infrastructure for the cultivation of transgenic plants, v) assemble complex protein with eukaryotic-like post-transcriptional modifications. However, plants lack the human-like N-glycosylation mechanism for protein processing that has been overcome by engineering tactics to ensure the authentic quality, homogeneity, and quantity [13]

Table 1. Comparison of different expression system [10].

PARAMETER	BACTERIA	YEAST	INSECT CELLS	MICROALGAE	MAMMALIAN CELLS	TRANSGENIC PLANTS
Capital cost	Medium	Medium	High	Medium	Very high	Low
Operating cost	Low	Medium	High	Low	Very high	Low
Production scale	Short	Short	Medium	Short	Long	Long
Speed	Fast	Fast	Medium	Fast	Slow	Slow
Multigene engineering	Yes	No	No	Yes	No	Yes
Glycosylation	Absent	Incorrect	Yes	Yes, absent in chloroplast	Yes	Yes, absent in chloroplast
Contamination risk	High	Medium	High	Low	High	Low
Multimeric assembly	No	No	No	Yes	No	Yes
Protein folding	Low	Medium	High	High	High	High
Protein yield	High	Moderate high	Medium	High	Medium	Low-High
Scale up cost	High	High	Very high	Medium	Very high	Very low
Safety	Low	Unknown	Medium		Low	High

(Table 1) cont.....

PARAMETER	BACTERIA	YEAST	INSECT CELLS	MICROALGAE	MAMMALIAN CELLS	TRANSGENIC PLANTS
Storage	Very cheap	Costly	Expensive	Low	Very expensive	Very cheap
Distribution	Easy	Feasible	Difficult	Easy	Difficult	Easy

Plant molecular farming (PMF) is termed as the technique of producing high-value proteins recombinantly in plants without disturbing their phenotype, metabolism, or performance. The proteins have been produced by this technique for more than 30 years, either in purified form, crude extract, or in planta [3, 14]. The idea of molecular farming based on the genetic transformation of plants was first proposed in the 1980s [15], which has now become a reality and is often termed as the 3rd generation of biotechnology [6]. The first examples of molecular farming using transgenic plants and plant cell suspension cultures involved the production of a human growth hormone, Nopaline synthase [16], and an antibody IgG_1 (6D4) [17]. However, the commercial application of this platform came years later when avidin was recombinantly produced in transgenic maize [18]. The breakthrough to commercial success for plant-derived biologics culminated in 2012 when the first plant-made pharmaceutical, Taliglucerase alfa, commercially known as Elelyso®, was developed by Protalix BioTherapeutics, was approved by the US Food and Drug Administration [19]. Elelyso® is a recombinant human glucocerebrocidase used for the treatment of Gaucher's disease (lysosomal storage disorder) [20].

The use of plants for the production of valuable proteins has been refined and improved over the years due to advancements in knowledge and technology. This has led to a major paradigm shift in the pharma sector, as the potential drawbacks associated with the early stages of PMF, including high expression level and efficient downstream processes, have been attained [6]. The product portfolio ranges from pharmaceutical therapeutics to non-pharmaceutical products such as antibodies, vaccine antigens, enzymes, growth factors, research or diagnostic reagents, and cosmetic ingredients. A number of 'proof-of-concept' studies have been performed to evaluate the potential of different plant species as hosts for molecular pharming [21, 22]. The host cells or the plant used for molecular farming purposes, depending upon target protein and its application, range from crop plants (rice, maize, tobacco, alfalfa, safflower, and lettuce) to pondweed, algae, microalgae, and mosses. The *Nicotiana* genus has been widely used for genetic transformation studies as it is easily genetically manipulated and has a fast growth rate. Two species, *Nicotiana benthamiana* and *Nicotiana tabacum* are considered as 'biological warehouses' for the production of many pharma or non-pharma products by the stable and transient expression [21]. Many plant-based proteins, including antibodies, either pharmaceutical or nonpharmaceutical, are in

the pipeline of clinical or preclinical trials, and some are in the developmental stage for commercialization (Table **2** & Table **3**).

Table 2. Selected list of antibodies expressed in plants against various diseases.

RECOMBINANT PROTEIN	PATHOGEN/DISEASE	HOST PLANT	TRANSFORMATION METHOD	EXPRESSION LEVEL	REFERENCES
cT84.66	Cancer (tumor marker)	*Nicotiana tabacum*	Transient expression	1 mg/kg FW	[66]
scFvT84.66	Cancer (tumor marker)	*Nicotiana tabacum*	Transient expression	5 mg/kg FW	[66]
scFvT84.66	Cancer (tumor marker)	*Oryza sativa*	Nuclear transformation	3.8 _g/g FW	[67]
scFvT84.66	Cancer (tumor marker)	*Wheat and rice*	Nuclear transformation	30 _g/g FWY	[68]
BR55-2	Human colorectal cancer	*Nicotiana tabacum*	Nuclear transformation	30 mg/kg FW	[69]
2F5	HIV	*Nicotiana benthamiana*	Nuclear transformation	0.01% of TSP	[70]
2G12	HIV	*Nicotiana benthamiana*	Transient expression	0.3 g/kg FW	[71]
2G12	HIV	*Nicotiana benthamiana*	Nuclear transformation	8 mg/L culture medium	[72]
6D8	Ebola virus	*Nicotiana benthamiana*	Transient expression	0.5 mg/g FW	[73]
6D8	Ebola virus	*Lettuce (L. sativa)*	Transient expression	0.23–0.27 mg/g FW	[74]
CO17-1AK	Human colorectal cancer	*Nicotiana tabacum*	Nuclear transformation	0.25 mg/kg FW	[75]
Palivizumab-N	Respiratory syncytial virus	*Nicotiana benthamiana*	Transient expression	180 mg/kg FW	[76]
E559	Rabies	*Nicotiana tabacum*	Nuclear transformation	1.8 mg/kg FW (0.04% of TSP	[77]
pE16	West Nile virus	*Nicotiana benthamiana*	Transient expression	0.74 mg/g FW	[78]
pE16scFv-CH	West Nile virus	*Nicotiana benthamiana*	Transient expression	0.77 mg/g FW	[78]
E60	Dengue virus	*Nicotiana benthamiana*	Transient expression	120 _g/g FW	[79]
2G12	HIV	*Oryza sativa*	Nuclear transformation	46.4 g g DW (seed)	[80]
8B10	Chikungunya virus	*Nicotiana benthamiana*	Transient expression	20–30 mg/kg FW	[81]
5F10	Chikungunya virus	*Nicotiana benthamiana*	Transient expression	20–30 mg/kg FW	[81]
SO57	Rabies virus	*Nicotiana tabacum*	Transient expression	0.014–0.019% of TSP	[82]

(Table 2) cont.....

RECOMBINANT PROTEIN	PATHOGEN/DISEASE	HOST PLANT	TRANSFORMATION METHOD	EXPRESSION LEVEL	REFERENCES
cD5	Enterovirus 71	*Nicotiana benthamiana*	Transient expression	50 _g/g FW	[83]
PD1	Cancer	*Nicotiana benthamiana*	Transient expression	140 _g/g FW	[84]
c2A10G6	Zika virus	*Nicotiana benthamiana*	Transient expression	1.47 mg/g FW	[85]
6D8	Ebola	*Nicotiana benthamiana*	Transient expression	1.21 mg/g FW	[85]
HSV8	Herpes simplex virus	*Nicotiana benthamiana*	Transient expression	1.42 mg/g FW	[85]
CHKV mab	Chikungunya virus	*Nicotiana benthamiana*	Transient expression	100 _g/g FW	[86]
2C10	Porcine epidemic diarrhea virus	*Nicotiana benthamiana & (L. Sativa)*	Transient expression	NR	[87]
KPF1-Antx	Influenza	*Nicotiana benthamiana*	Transient expression	NR	[88]

Table 3. Plant-derived antibodies in clinical stages of development or on market.

PRODUCT	DISEASE	PLANT	CLINICAL TRIAL STATUS	COMPANY	REFERENCE
CaroRX	Dental caries	Tobacco	EU approved as medical advice	Planet Biotechnology, USA	www.planetbiotechnology.com
DoxoRX	Side-effects of cancer therapy	Tobacco	Phase I completed	Planet Biotechnology, USA	www.planetbiotechnology.com
RhinoRX	Common cold	Tobacco	Phase I completed	Planet Biotechnology, USA	www.planetbiotechnology.com
Fv antibodies	Non-Hodgkin's lymphoma	Tobacco	Phase I	Large Scale Biology, USA	www.lsbc.coma
IgG (ICAM1)	Common cold	Tobacco	Phase I	Planet Biotechnology, USA	www.planetbiotechnology.com
Antibody against hepatitis B	Vaccine purification	Tobacco	On market	CIGB, Cuba Kaiser	www.planetbiotechnology.com/ Kaiser,2008.

The PMF, with more than 120 companies, universities, and research institutes, is involved in realizing the fullest potential of this area. Therefore, it has emerged as

a vibrant segment of the biotech industry [14]. [10] have critically reviewed the strengths and bottlenecks of the commercial potential of plant-based expression platforms. The pharmaceuticals products which are endorsed from 2014 to mid of 2018 are 7 vaccines, 9 enzymes, 16 coagulating factors, 23 hormones, and 68 monoclonal antibodies [23]. There are several reports to prove the significant efficiency of plant-based pharmaceuticals compared with mammalian cell-based protein, which provides efficacious and less costly strategies to treat emerging infectious diseases. The plant-based expression system can be quickly up-scaled to satisfy the sudden and unexpected arising demands such as Covid-19 pandemic crisis (Covid-19) [24].

This chapter is aimed at describing the principles, current advancements in methodology for plant molecular pharming. We argue that plant molecular pharming has presented itself as a viable as well as a competitive platform to produce recombinant proteins inexpensively at a large scale. Different strategical system for plant transformation and expression is discussed, that have been developed to produce commercially important proteins. The advantages and disadvantages of each system have been well considered. The chapter also reviews the high-value bio-products (pharmaceutical or non-pharmaceutical) that are successfully being produced in the established and emerging plant systems and are in the pipeline of commercialization. The final section focuses on the outlook and perspective of plant molecular pharming as a potential therapeutic intervention against the ongoing human pandemic – COVID19 (SARS-nCo--2019).

4. DEVELOPMENT OF TRANSGENIC PLANTS

Traditionally, crop plants have been improved through artificial selection and breeding based on phenotypic characteristics such as reduced susceptibility to biotic and abiotic stresses, plant height, grain size, and higher yield [25]. This approach has been quite instrumental in attaining supply-demand equilibrium, but the continuous increase in human population and a decline in arable land area necessitates to devise of new strategies for crop plant improvement. Conventional methods are limited by a narrow gene pool and lengthy procedures of selection [26]; however, transgenic technology offers the ability to develop transgenic plants with novel traits from varied taxonomic groups [27]. In this scenario, several candidate genes conferring novel traits such as insect resistance, salt tolerance, herbicide tolerance, heat tolerance, biofortification, and value addition have been identified and evaluated in model systems for their functioning into higher plants [28 - 63]. In addition, plants can also be developed having improved nutritional status [64] or value-addition such as delayed fruit ripening to improve

the shelf life of perishable commodities such as tomatoes, so that they can reach the consumer intact preserving their taste, smell, color, and texture [65]. Transgenic plants can also be developed to produce high-value targets which are cheaper and are in larger quantities, such as recombinant proteins and metabolites of industrial importance, including antibodies (Table **2**, Table **3**), vaccine antigens (Table **4**), industrial enzymes (Table **5**), non-pharmaceutical recombinant proteins (Table **6**, Table **7**), human therapeutics (Table **8**) and even nutraceuticals (Table **9**). In this section, we will discuss, briefly, that how transgenic plants can be developed, and the advantages and challenges of each methodology used for the development of transgenic plants.

Table 4. Selected vaccine antigens produced in plants.

Expressed Protein	Disease	Gene	Plant	Expression level	Reference
Cholera toxin B subunit	Cholera	*Codon optimized CTB* of *Vibrio cholerae*	*Nicotiana tabacum*	4.1%	[89]
Bovine group A rotavirus VP6	Rotavirus	*VP6*	*Nicotiana tabacum*	3%	[90]
Canine parvovirus; Cholera toxin B subunit	Parvovirus and Cholera	*CTP-2L21*	*Nicotiana tabacum*	31.3%	[91]
Anthrax protective antigen	Anthrax	*pagA*	*Nicotiana tabacum*	14.2%	[92]
Bacterial lipoprotein A	Lyme disease	*OspA*	*Nicotiana tabacum*	10%	[93]
Cysteine rich region of lectin	*Entamoeba histolytica*	*LecA*	*Nicotiana tabacum*	6.3%	[94]
Cholera toxin B subunit–human proinsulin	Cholera	*CTB-Pins*	*Nicotiana tabacum*	16%	[95]
Cholera toxin B subunit–human proinsulin	Cholera	*CTB-Pins*	*Lactuca sativa*	2.5%	[95]
Human papillomavirus L1 protein	Cervical cancer	*L1* HPV-16	*Nicotiana tabacum*	24%	[96]
VP1 of the foot and mouth disease virus	Foot and Mouth Disease	*VP1*	*Stylosanthes guianensis*	0.1-0.5%	[97]
A27L of vaccinia virus	Orthopoxviruses (OPVs)	*A27L*	*Nicotiana tabacum*	18%	[98]
Heat labile toxin B subunit and heat-stable toxin	Enterotoxigenic Escherichia coli (ETEC)	*LTB:ST*	*Nicotiana tabacum*	2.3%	[99]

(Table 4) cont.....

Expressed Protein	Disease	Gene	Plant	Expression level	Reference
LTB–HN-neutralizing epitope	New Castle Disease Virus (NDV)	*LTB-HNE*	*Nicotiana tabacum*	0.5%	[100]
Cholera toxin-B subunit fused with apical membrane antigen-1	*CTB:AMA1*	Cholera and Malaria	*Nicotiana tabacum*	13.17%	[101]
Cholera toxin-B subunit fused with merozoite surface protein-1	*CTB:MSP1*	Cholera and Malaria	*Nicotiana tabacum*	10.11%	[101]
Cholera toxin-B subunit (CTB) fused with apical membrane antigen-1	*CTB:AMA1*	Cholera and Malaria	*Lactuca sativa*	7.3%	[101]
Cholera toxin-B subunit (CTB) fused with merozoite surface protein-1	*CTB:MSP1*	Cholera and Malaria	*Lactuca sativa*	6.1%	[101]
human b-site APP cleaving enzyme	*BACE*	Alzheimer disease	*Nicotiana tabacum*	2%	[102]
GRA4 antigen	*GRA4*	*Toxoplasma gondii*	*Nicotiana tabacum*	0.2%	[103]
Hemagglutinin (HA) proteins	*HA*	Influenza virus	*Nicotiana benthamiana*	1300mg/kg	[104]
Mtb72F fused with cholera toxin B-subunit	*CTB-Mtb72F*	*Mycobacterium tuberculosis*	*Nicotiana tabacum*	1.2%	[105]
ESAT-6 fused with cholera toxin B-subunit	*CTB-ESAT6*	*Mycobacterium tuberculosis*	*Nicotiana tabacum*	7.5%	[105]
ESAT-6 fused with cholera toxin B-subunit	*CTB-ESAT6*	*Mycobacterium tuberculosis*	*Lactuca sativa*	0.75%	[105]
Tetra-epitope antigen	*cE-DI/IIp*	Dengue virus	*Lactuca sativa*	-	[106]
Hepatitis C virus E1E2 heterodimer	*HCVE1E2*	Hepatitis C virus (HCV)	*Lactuca sativa*	1.6 ug/mL	[107]
M2e Peptide fused with Ricin Toxin B Chain	*M2e*	Avian Influenza Virus	*Wolffia globosa*	0.01%	[108]
E2 protein of classical swine fever virus	*E2*	classical swine fever virus (CSFV)	*Arabidopsis thaliana*	0.7%	[109]
Human Papillomavirus (HPV) type 16 E7 protein	*E7*	Human Papillomavirus (HPV)	*Solanum lycopersicum*	35.5 µg/g	[110]
Enterotoxin B subunit	*LTB-Syn*	Parkinson's disease	*Nicotiana tabacum*	0.27ug/g	[111]

(Table 4) cont.....

Expressed Protein	Disease	Gene	Plant	Expression level	Reference
A region of PAc protein and cholera toxin B subunit	*PAcA-ctxB*	Streptococcus mutans	*Solanum lycopersicum*	-	[112]
VP1 capsid protein of FMDV serotype O	*VP1*	Foot and Mouth Disease (FMDV)	*Nicotiana tabacum*	0.72%	[113]
non-toxic carboxylterminal domain of a-toxin (PlcC) and attenuated mutant of NetB (NetB-W262A)	*PlcC-NetB*	Clostridium perfringens	*Nicotiana benthamiana*	20%	[114]
LamB outer membrane protein of Vibrio bacteria	*LamB*	*Vibrio alginolyticus*	*Wolffia globosa*	-	[115]
Four African horse sickness (AHS) capsid proteins	*AHSV-1 VP2, VP5, VP3 and VP7*	African horse sickness virus (AHSV)	*Nicotiana benthamiana*	-	[116]

Table 5. Industrial enzymes and biomaterials obtained via chloroplast production in tobacco.

GENE/S	PRODUCT	HOST PLANT	EXPRESSION LEVEL	REFERENCE
bgl-1	β-Glucosidase	Tobacco Chloroplast	44.4 U/g FW	[117]
bgl1, celA, celB	β-Glucosidase, Cellulases	Tobacco Chloroplast	9.9–58.2 U/mg of TSP	[118]
endo, celB, xyn	Cellulases, Xylanase	Tobacco Chloroplast	0.38–75.6% TSP	[119]
bgl1C, cel6B, cel9A, xeg74	Cell wall-degrading enzyme	Tobacco Chloroplast	5–40% TSP	[120]
manI	β-Mannanase	Tobacco Chloroplast	25 U/g FW	[121]
xynA, xyn10A, xyn11B	Xylanase	Tobacco Chloroplast	0.2–6% TSP	[122]
UbiC	p-Hydroxybenzoic acid	Tobacco Chloroplast	25% DW	[123]
PHB pathway genes	Polyhydroxybutyrate	Tobacco Chloroplast	18.8% TSP	[124]
xynA	xylanase	Tobacco Chloroplast	6% TSP	[125]
xyn2	xylanase	Tobacco Chloroplast	421 U/mg TSP	[126]

(Table 5) cont.....

GENE/S	PRODUCT	HOST PLANT	EXPRESSION LEVEL	REFERENCE
xyl10B	xylanase	Tobacco Chloroplast	13% TSP, 61.9 U/mg D	[127]
E1	Endo- β -1,4-glucanase	Tobacco Chloroplast	1.35% TSP	[128 - 130]
E1	Endo- β -1,4-glucanase	Potato chloroplast	2.6% TSP	[131]
EG	Endo- β -1,4-glucanase	Sugarcane chloroplast	Avg 223.8 ng/mg,	[132]
CBH I, CBH II	Cellobiohydrolase	Sugarcane chloroplast	-	[132]
Cel6A,Cel6B	Endo- β -1,4-glucanase	Tobacco chloroplast	2-4% TSP	[133]
BglB	Betaglucosidase	Tobacco chloroplast	5.8% TSP	[134]
BglB	Betaglucosidase	Tobacco chloroplast	9.3% TSP	[135]
XylII	Endo-1,4-β –xylanase	Arabidopsis Chloroplast	3-4.8% TSP	[136]
XynII	Endo-1,4-β –xylanase	Arabidopsis Chloroplast	1.4-3.2% TSP	[137]
Chitinase	Chitinase	Tobacco Chloroplast	0.8-1% TSP	[138]
Chitinase	Glucanase	Tobacco Chloroplast	0.3% TSP	[139]

Table 6. Selected list of various non-pharmaceutical proteins produced in plants.

RECOMBINANT PROTEIN	HOST PLANT	TRANSFORMATION METHOD	EXPRESSION LEVEL	REFERENCES
Human serum albumin	*Solanum tuberosum*	Nuclear transformation	0.25 g/mg (0.02% of TSP)	[140]
Erythropoietin	*Nicotiana tabacum*	Nuclear transformation	4.6–5.7 mg/g dry cell	[141]
1-antitrypsin	*Oriza sativa japonica*	Nuclear transformation	4.6–5.7 mg/g dry cell	[142]
Aprotinin	*Zea mays*	Nuclear transformation	0.069% of total seed protein	[143]
Human-secreted alkaline phosphatase	*Nicotiana tabacum*	Nuclear transformation	1.1 _g/g FW (3% of TSPs	[144]
Collagen	*Nicotiana tabacum*	Nuclear transformation	0.03 g/kg powdered plants	[145]
Human somatotropin	*Nicotiana tabacum*	Chloroplast transformation	>7% of TSP	[146]

(Table 6) cont.....

RECOMBINANT PROTEIN	HOST PLANT	TRANSFORMATION METHOD	EXPRESSION LEVEL	REFERENCES
Bacillus thuringiensis (Bt) cry2Aa2	*Nicotiana tabacum*	Chloroplast transformation	5 mg/g FW (45.3–46.1% of TSPs	[147]
Human serum albumin	*Nicotiana tabacum*	Chloroplast transformation	11.1% of TPs	[148]
Human epidermal growth factor	*Nicotiana tabacum*	Nuclear transformation	34.2 _g/g FW	[149]
Human basic fibroblast growth factor	*Glycine max*	Nuclear transformation	2.3% of TSP	[150]
Type I interferon (IFN_2b)	*Nicotiana tabacum*	Chloroplast transformation	3 mg/g FW (20% of TSP	[151]
Human growth hormone	*Oryza sativa*	Nuclear transformation	57 mg/L culture medium	[152]
PlyGBS lysin	*Nicotiana tabacum*	Chloroplast transformation	>70% of TSP	[153]
Human growth hormone	*Tobacco BY-2 cells*	Nuclear transformation	35 mg/L or 2-4% of TSP	[154]
Human basic fibroblast growth factor	*Oryza sativa*	Nuclear transformation	185.66 mg/kg	[155]
Lumbrokinase	*Helianthus annuus*	Nuclear transformation	5.1 g/kg seeds	[156]
Human acidic fibroblast growth factor 1	*Salvia miltiorrhiza*	Nuclear transformation	272 ng/g FW	[157]
Glucocerebrosidase	*Nicotiana benthamiana*	Nuclear transformation	68 _g/g FW (1.45% of TSP	[158]
Human acid alpha glucosidase	*Nicotiana tabacum*	Chloroplast transformation	6.38 _g/g FW	[159]
Human basic fibroblast growth factor	*Nicotiana tabacum*	Chloroplast transformation	0.1% of TSP	[160]
Endo-_-1,4-xylanase	*Nicotiana tabacum*	Chloroplast transformation	35.7% of TSP	[119]
Glucosidase	*Nicotiana tabacum*	Chloroplast transformation	>75% of TSP	[119]
Osteopontin	*Nicotiana benthamiana*	Transient expression	100 ng/g FW	[87]
Dentin matrix protein-1	*Nicotiana benthamiana*	Transient expression	0.3 _g/g FW	[161]

Table 7. Commercial development of nonpharmaceutical proteins produced in plants.

PRODUCT	APPLICATION	PLANT SPECIES	PROCESSING DEGREE	ADVANTAGE	DEVELOPMENT STAGE	COMPANY	SOURCE
Trypsin	Technical reagents	Maize seeds	Purified	Cost, animal-free	Commercialized	ProdiGene/ Sigma-Aldrich, United States	www.sigmaaldrich.com
Avidin	Technical reagents	Maize seeds	Purified	Cost, animal-free	Commercialized	ProdiGene/ Sigma-Aldrich, United States	www.sigmaaldrich.com
Endo-1,4-β-D-glucanase	Technical reagents	Maize seeds	Purified	Cost, animal-free	Commercialized	ProdiGene/ Sigma-Aldrich, United States	www.sigmaaldrich.com
Cellobiohydrolase I	Technical reagent	Maize seeds	Purified	Cost, integrated production	Commercialized	Infinite Enzymes/ Sigma Aldrich, United States	www.sigmaaldrich.com
Growth factors	Research reagents	Tobacco leaves, transient	Purified	Cost, animal-free	Commercialized	Agrenvec, Spain	www.agrenvec.com
Cytokines	Research reagents	Tobacco leaves, transient	Purified	Cost, animal-free	Commercialized	Agrenvec, Spain	www.agrenvec.com
Thioredoxin	Research reagents	Tobacco leaves, transient	Purified	Cost, animal-free	Commercialized	Agrenvec, Spain	www.agrenvec.com
TIMP-2	Research reagents	Tobacco leaves, transient's	Purified	Cost, animal-free	Commercialized	Agrenvec, Spain	www.agrenvec.com
Growth factors	Research reagent	Barley seeds	Purified	Cost, animal-free	Commercialized	ORF Genetics, Iceland	www.orfgenetics.com
Cytokines	Research reagent	Barley seeds	Purified	Cost, animal-free	Commercialized	ORF Genetics, Iceland	www.orfgenetics.com
Epithelial growth factor	Cosmetics	Barley seeds	Purified	Cost, animal-free	Commercialized	Sif Cosmetics, Iceland	www.sifcosmetics.com
Albumin	Research reagents	Rice seeds	Purified	Cost, animal-free	Commercialized	Ventria Bioscience/ InVitria, United States	www.invitria.com
Lactoferrin	Research reagents	Rice seeds	Purified	Cost, animal-free	Commercialized	Ventria Bioscience/ InVitria, United States	www.invitria.com www.invitria.com
Lysozyme	Research reagents	Rice seeds	Purified	Cost, animal-free	Commercialized	Ventria Bioscience/ InVitria, United States	www.invitria.com
Transferrin	Research reagents	Rice seeds	Purified	Cost, animal-free	Commercialized	Ventria Bioscience/ InVitria, United States	www.invitria.com
Insulin	Research reagents	Rice seeds	Purified	Cost, animal-free	Commercialized	Ventria Bioscience/ InVitria, United States	www.invitria.com

(Table 7) cont.....

PRODUCT	APPLICATION	PLANT SPECIES	PROCESSING DEGREE	ADVANTAGE	DEVELOPMENT STAGE	COMPANY	SOURCE
Aprotinin	Research reagent	Tobacco leaves, transient	Purified	Cost	Commercialized	Kentucky Bioprocessing, United States	www.kbpllc.com
Collagen	Research reagent, tissue culture, health applications	Transgenic tobacco	Purified	Cost, animal-free	Commercialized	CollPlant, Israel	www.collplant.com
Trypsin	Research reagents, cosmetic ingredients	Rice cell suspension	Purified	Cost, animal-free	Commercialized	Natural BioMaterials, South Korea	www.nbms.co.kr
Enterokinase	Research reagents, cosmetic ingredients	Rice cell suspension	Purified	Cost, animal-free	Commercialized	Natural BioMaterials, South Korea	www.nbms.co.kr
Growth factors	Research reagents, cosmetic ingredients	Rice cell suspension	Purified	Cost, animal-free	Commercialized	Natural BioMaterials, South Korea	www.nbms.co.kr
Cytokines	Research reagents, cosmetic ingredients	Rice cell suspension	Purified	Cost, animal-free	Commercialized	Natural BioMaterials, South Korea	www.nbms.co.kr
Antibody	Purification of a hepatitis B vaccine	Transgenic tobacco	Purified	Cost	Commercial application	Center for Genetic Engineering and Biotechnology, Cuba	gndp.cigb. edu.cu
A-Amylase	Bioethanol production	Maize seeds	Biomass extract	Cost, integrated production	Commercialized	Syngenta, United States	www.syngenta.com
Phytase	Feed	Maize seeds	Delivered in biomass	Increased mineral availability, integrated production	Commercialization pending	Origin Agritech, China	www.originseed.com.cn
Growth factors	Tissue culture reagent	Tobacco leaves, transient	Purified	Cost, animal-free	Commercialized	NexGen, South Korea	www.exgen.com

Table 8. Plant-derived therapeutic human protein in clinical stages of development or on market.

PRODUCT	DISEASE	PLANT	CLINICAL TRIAL STATUTS	COMPANY	REFERENCE
Gastric lipase, Merispase®	Cystic fibrosis	Maize	On market	Meristem Therapeutics France	www.meristem-therapeutics.com
α-Galactosidase	Fabry disease	Tobacco	Phase I	Planet Biotechnology, USA	www.planetbiotechnology.com
Lactoferon™ (α-interferon)	Hepatitis B and C	Duckweed	Phase II	Biolex, USA	www.biolex.com

(Table 8) cont.....

PRODUCT	DISEASE	PLANT	CLINICAL TRIAL STATUTS	COMPANY	REFERENCE
Fibrinolytic drug (thrombolytic drug)	Blood clot	Duckweed	Phase I	Biolex, USA	www.biolex.com
Human glucocerebrosidase	Gaucher ‘s disease	Carrot suspension cells	Awaiting USDA's approval	Protalix Biotherapeutics, Israel	www.protalix.com
Insulin	Diabetes	Safflower	Phase III	SemBioSys, Canada	www.sembiosys.com
Apolipoprotein	Cardiovascular	Safflower	Phase I	SemBioSys, Canada	www.sembiosys.com

Table 9. Plant-derived nutraceuticals in advanced clinical stages of development or on market.

PRODUCT	DISEASE	PLANT	CLINICAL TRIAL STATUTS	COMPANY	REFERENCE
ISOkine™	Human growth factor	Barley	On market	ORF Genetics	www.orfgenetics.com
DERMOkine™	Human growth factor	Barley	On market	ORF Genetics	www.orfgenetics.com
Human intrinsic factor	Vitamin B12 deficiency	Arabidopsis	On market	Cobento Biotech AS	www.cobento.dk
Coban	Vitamin B12 deficiency	Arabidopsis	On market	Cobento Biotech AS	www.cobento.dk
Human lactoferrin	Anti-infection, anti-inflammatory	Rice	Advanced stage	Ventria, USA	www.ventriabio.com
Human lysozyme	Anti-infection, anti-inflammatory	Rice	Advanced stage	Ventria, USA	www.ventria.com
Immunosphere™	Food additive for shrimps	Safflower	On market	SemBioSys, Canada	www.sembiosys.com

Genetic engineering of plants comprises of several steps: identifying and isolating the gene of interest, choice of the promoter, construction of expression and selection cassette, selection of a suitable cloning and expression vector, an appropriate method for stable or transient DNA introduction into the plant genome, tissue culture system that allows the regeneration of whole plants, selection pressure for the distinction of transgenic plants from non-transformants followed by biochemical methods to detect the expression of foreign genes.

The plant genome is compartmentalized into the nucleus, mitochondria, and plastids; each of which possesses its genome and genetic machinery. The

development of transgenic plants depends on recombinant DNA technology for the stable or transient expression of foreign gene(s) into any of the plant genomes. Stable transformation refers to the permanent integration of exogenous gene(s) into the plant genome while transient expression refers to a temporary high-level transgene expression, generally but not limited to validate the constructs. Transformation of the nuclear genome is now a routine in tailoring agronomical traits [31, 54, 62, 162] while that of organellar genomes, mitochondrial or plastids, is also emerging as an alternative target for the transformation process. Mitochondrial genome transformation is relatively a new concept and very limited success has been achieved so far, however, the transformation of the plastid genome has become an established platform for the production of commercially important compounds due to high expression and confinement into bona fide structure [153]. Plastids are a group of semi-autonomous organelles found in green plants, algae, and cyanobacteria that possess a great capacity for differentiation, de-differentiation, and re-differentiation. Their major roles include photosynthesis, storage of various products, and synthesis of key molecules which maintain the basic architecture and operation of cells. These organelles vary in size, shape, content, and function. Pro-plastids are the precursors of all plastids and are present in the meristematic regions of the plant. The plastid DNA (ptDNA) is present in the form of DNA-protein complexes known as plastid nucleoids and are attached to the inner membrane of the plastids. Each plastid nucleoid possesses 10-100 copies of ptDNA. The size of chloroplast genomes is ~140 kbp in higher plants while <200 kbp in unicellular eukaryotes. The copy number of the chloroplast genome is variable and ranges between 1,000-10,000 per plant cell. Transformation of both the chloroplast genome as well as the nuclear genome offers its own set of features which are discussed in Table **1**.

TABLE 7. Comparison of nuclear and chloroplast expression approaches.

FEATURE	NUCLEAR TRANSFORMATION	CHLOROPLAST TRANSFORMATION
Copy number	Low copy number Specific chromosome number in each species	High copy number 2 Inverted repeats (IR)/ptDNA 10-100 ptDNA/plastid 10-100 plastid/cell ~20,000 transgene copies/cell
Level of gene expression	Low 1-2% of TSP	High 5-25% of TSP
Integration	Random May interrupt expression of other genes	Site specific No effect on the expression of other genes due to homologous recombination

FEATURE	NUCLEAR TRANSFORMATION	CHLOROPLAST TRANSFORMATION
Gene silencing	Yes Natural phenomenon of RNAi in eukaryotes	No Prokaryotic in nature
Gene containment	Transgene escape Non-maternal mode of inheritance	No gene pollution Maternal mode of inheritance
Formation of disulphides	Yes	Yes
Toxic proteins	No Severe pleiotropic effects	Yes Plastid expressed proteins are contained within the plastid
Gene expression	Monocistronic	Polycistronic (Operon)

There are various methods to introduce transgene into the plant genome, however, in this chapter, we will discuss the commonly used system such as *Agrobacterium*-mediated transformation, biolistic, and viral (transient expression).

Agrobacterium is a gram-negative soil bacterium that causes crown gall or hairy roots in most of the dicotyledonous plants to produce amino acids and sugar-phosphate compounds known as opines for its energy source. The cells of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* contain Ti (tumor-inducing) plasmid and Ri (roots-inducing) plasmids, respectively. Commonly *Agrobacterium tumefaciens* is used for plant transformation by replacing the T-DNA region with the gene of interest and selection cassette. Injured plant tissues release a phenolic compound (acetosyringone) that triggers *Agrobacterium* recognition in which VirA protein acts as an antenna and autophosphorylates itself consequently phosphorylation VirG [163]. This triggers a cascade of chemical reactions wherein type IV secretion system is formed consisting of VirD4 and 11 VirB proteins [164]. T-DNA is replicated to produce T-DNA/VirD2 complex; Vir protein complex (VirB2, VirB5, VirB7) work together to allow VirD2-DNA complex (immature T-DNA complex) to enter the cytoplasm of the recipient cell, VirE2 is combined with T-strand to form mature T-DNA complex [165], which passes through the cytoplasm to reach the nucleus; the T-DNA complex enters the nucleus of the recipient cell through the nuclear pore targeting the integration site. The T-complex removes the guard protein, and the T-DNA is integrated into the nuclear genome. The gene expression regulatory sequence in the T-DNA region is like that of eukaryotes, so it can be expressed in plant cells. *Agrobacterium*-mediated transformation is a method of choice for transformation of the nuclear genome of dicotyledonous plants due to simple operation, low cost, high success rate [166]. However naturally the bacterium offers limited host range and organellar genomes cannot be transformed. Different

steps involved in the *Agrobacterium*-mediated transformation procedure are shown in Fig. (**1**) .

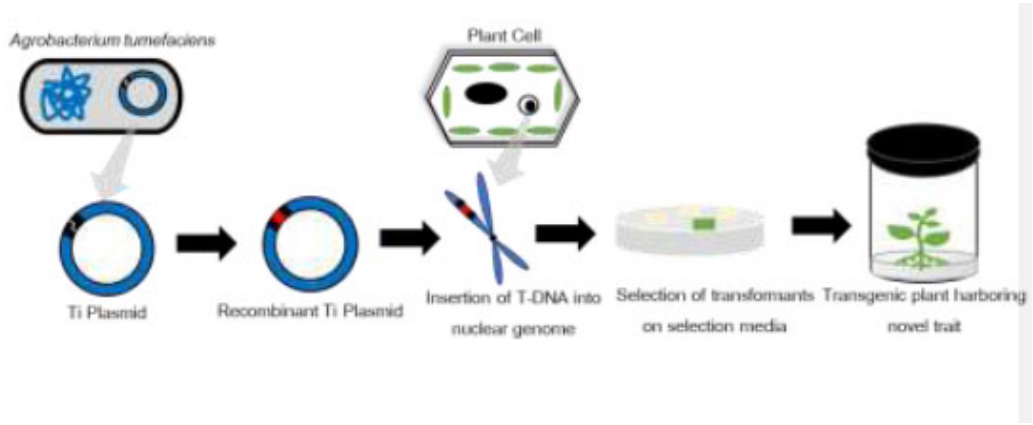

Fig. (1). Illustration of different steps involved in the development of transgenic plants using nuclear transformation.

As illustrated in Fig. (**2**) , the microprojectile bombardment method employs physical means of transforming cells; also known as biolistics or gene-gun method. It accelerates the metal particles (tungsten, gold) coated with foreign DNA so that they can penetrate the cell wall [167]. It can be used to introduce foreign genes into plant cells, tissues, and organs. PDS-1000/He uses rupture discs of different thicknesses to regulate the helium pressure. When the helium pressure reaches the capacity of rupture disc it bursts to generate a strong acceleration of macrocarrier to carry the microcarriers at high speed. When it encounters the rigid blocking mesh, the macrocarrier is blocked while microcarriers use inertia to continue to move forward at high speed, bombarding target cells or tissues, thereby carrying foreign genes into the cells. The gene bombardment method is not restricted by the recipient's genotype, a wide range of explants, rapid and simple operation, and can effectively transform plastids, thus turning a new page in the genetic transformation of plants. However, this technology is expensive, and large DNA fragments can be easily sheared during the transformation process, multiple insertions are also a limitation.

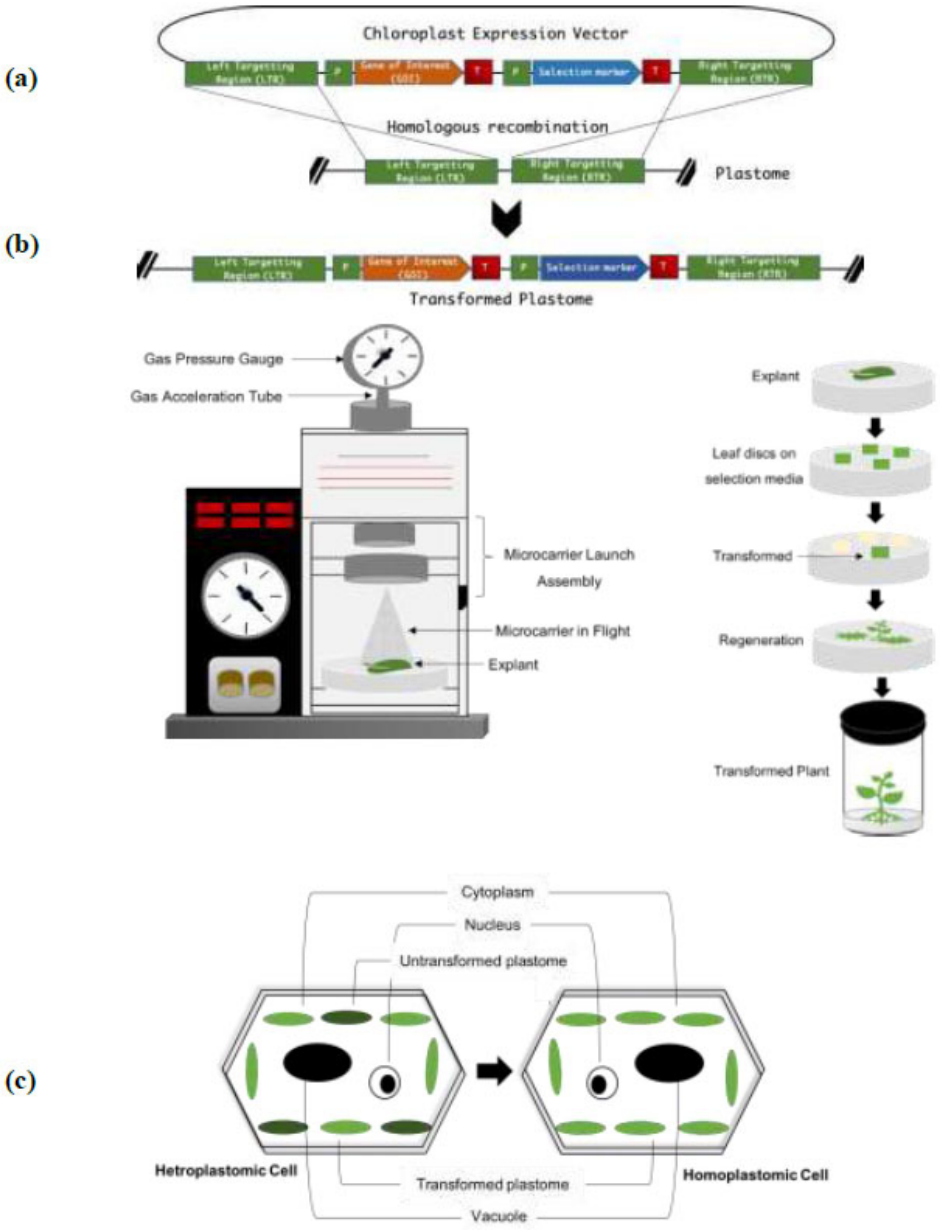

Fig. (2). Development of transgenic plants via transformation of the chloroplast genome. **a)** Displays a typical vector construction scheme and the homologous recombination for the integration of transgene into chloroplast genome at the chosen location. **b)** Shows the delivery of transgene into chloroplast genome via particle bombardment system using a gene gun. **c)** Demonstrates the progression of homoplasmy (right; in which all chloroplasts are transformed) a heteroplasmic cell (left; in which untransformed chloroplasts are also present).

Plant viruses such as Tobacco Mosaic Virus (TMV), and Potato Virus X (PVX) can also be used for plant transformation exploiting their natural capability [168]. Viruses can spread rapidly and produce a large number of foreign protein(s) due to their efficient self-replication and expression ability [169]. The introduction and expression of new genes can be achieved in most cells of a mature plant without the need to undergo a long-term transformation process from explants to regenerated plants. This method is used for transient expression since the transgene is not integrated into plant genome and therefore, cannot be transferred to next generation [168]. It will not affect the expression of other functional genes in the recipient plant. Two types of viral expression systems are available: i) independent viral vectors-harboring the transgene in place of capsid protein [170] or alongside all required viral proteins. However, this system suffered from transgene size limitation, ii) minimal viral vectors-harboring the transgene with minimal viral proteins capable of accommodating larger transgene at the cost of systemic infection. This was compensated by the transformation of agrobacterium with minimal viral vector and infiltrating the inoculum into the host plant leaf using vacuum. It helps to quickly validate the cassette for its successful expression in plants. However, there are several limitations associated with virus-mediated plant transformation such as viral vectors cannot integrate foreign genes into chromosomes, so they cannot be passed to the offspring according to Mendelian laws, and they have no advantage in long-term expression of foreign proteins. The frequency of mutations in the genome is relatively high. The instability of the viral vector itself can easily cause the loss of foreign genes. In view of this, viral vector-mediated genetic transformation is mainly used in two fields: the application of virus-induced gene silencing (VIGS) and high efficiency transient expression of foreign protein(s).

5. SUMMARY AND OUTLOOK

It has now been established that plants are capable of producing recombinant proteins of industrial importance at commercially feasible levels. Plants offer several advantages compared to conventional systems. Although, the lengthy procedures to produce transgenic plants are considered a big hurdle at present, several tissue-culture independent methods such as the floral dip method to generate transgenic plants have been devised. However, they are currently limited to few plant species such as Arabidopsis. The success of such methods depends on the use of an efficient selectable marker system that could effectively suppress the growth of non-transformed plants at the seedling stage. Such methods have been attempted for other species as well however, they have not been successful yet. Once, the transgenic plants have been developed, then they offer significant advantages compared to other systems particularly in terms of scaling up. With

minimum efforts and existing infrastructure deployed, transgenic plants can be grown on several thousand hectares in one planting season.

In situations like Covid-19 pandemic calling for sudden, huge, and cheap production of vaccines, the plant-based production system of recombinant vaccines also holds great promise. Covid-19, a novel coronavirus with the potential of lethality has created an alarming situation at a global scale. The virus was first identified in late December 2019 in Wuhan, China, and has been declared a serious global health concern by WHO [171]. The virus is responsible for acute respiratory (pneumonia-like) infection characterized by different symptoms. Critical cases can cause respiratory failure, septic shock, or organ failure which then requires intensive care support. The rapid outbreak of this deadly virus through human transmission has provoked governments across the world to ensure and address the emergency control and containment measures, treating patients with quarantine facilities and vaccine development. As the virus has emerged suddenly with no available vaccination and other treatments, prevention of infection is the current priority to control the pandemic. This outbreak with massive mortalities and newly reported cases has created an urgent demand for vaccine development. Although the traditional expression system is flexible for biopharmaceutical production the transient expression in plants has carved a niche in the biopharmaceutical sector for producing biopharma products. Genetic engineering of plant has evolved smarter for the transient expression with profound benefits and have been substantially fruitful in achieving its worth in biopharmaceutical sector. Therefore, in such emergencies, vaccine antigens can be produced transiently without stably integrating the transgenes into the plant genome [172]. The amazing speed of this system was very recently demonstrated by Medicago Inc, a pioneer of plant-based transient expression and manufacturing, by producing VLPs (Virus-Like Particles) in just 20 days after having access to SARS-nCoV-2's spike (S) protein sequence [173].

There are other concerns of transgene spread to weedy relatives through pollen. However, the chance of spreading transgenes become meager if the transgenes-coding for a fitness-enhancing trait under certain circumstances such as herbicide tolerance is not used. Any transgene offering no selective advantage under certain circumstances to the host plant would remain neutral and pose no threat to the population. Research shows that transgenes offering no selection advantage to the host would rather become a source of extra metabolic burden at the cellular level. Nevertheless, researchers have developed alternative approaches such as the manipulation of non-nuclear genomes to address such pollen-mediated gene transfer to weedy relatives from transgenic plants when cultivated in open fields. Non-nuclear genomes such as plastids and mitochondria are often inherited maternally, and therefore, provide a sort of natural gene compartment for the

transgene(s). Manipulation of the plastid genome has emerged as a successful alternative to nuclear transformation while the efforts to engineer the mitochondrial genome are underway. Another significant advantage of using plants as a host for the production of recombinant proteins lies in their suitability for the development of edible vaccines. Vaccine antigen production in edible parts of plants would offer a significant advance not only in the cost-effective production but also downstream application including purification, distribution as well as administration of the vaccines—the three major phases incurring high input costs. The seeds of plants expressing edible vaccine/recombinant protein can be stored at room temperature for quite long periods and can be distributed to remote distances without requiring special cold storage as well as special transportation facilities. Despite these advantages, plant-made pharmaceuticals have not yet reached the market or captured the market share. However, the situation is changing now. Many funding agencies are funding proposals revolving the recombinant protein productions using plant-based expression systems.

CONSENT FOR PUBLICATION

Not Applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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