FUNDAMENTALS FOR INTEGRATION OF SOMATIC HYBRIDIZATION IN ROSE BREEDING

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Abstract

With the intention to exploit new sources of disease resistance in rose breeding, the feasibility of introgression of genes by means of somatic hybridization is evaluated. Protocols have been established for (I) regeneration of protopasts in a variety of rose genotypes, (II) PEG mediated fusion, and (III) preferential regeneration of heterologous fusion products. Protoplasts isolated from non embryogenic source material gave rise to callus in *R. canina*, *R. caudata*, *R. corymbifera* 'Laxa', *R. multiflora* (two accessions), *R. roxburghii*, *R. spinosissima*, *R. wichuraiana* (two accessions), as well as in *R.* x *hybrida* 'Elina' and 'Pariser Charme'. Protoplasts isolated from embryogenic cell suspensions of 'Heckenzauber' and 'Pariser Charme', as well as from non embryogenic suspensions of the hybrid *R. persica* x *R. xanthina* were regenerated into plants. In order to suppress sustained cell divisions of non-fused protoplasts or of homologous fusion products, protoplasts were pretreated with either rhodamine 6G (0.1 mmol) or iodoacetate (0.5 – 1 mmol) for 15 minutes as well as with X-rays (300 Gy) at a dose rate of 3 Gy/min. Induced defects are mostly complementary, thus corresponding pretreatment of protoplasts prior to fusion allows preferential regeneration of the heterologous fusion products aimed at. Specific genotypes of different wild species within the genus *Rosa* were identified to carry resistance genes against *Diplocarpon rosae*, the causal agent of blackspot (von Malek-Podjaski, 1999). For introgression of resistance into cultivars by means of somatic hybridization, experiments concentrate at the time being on diploid accessions of *Rosa multiflora*, *Rosa wichuraiana* and *Rosa roxburghii*. Putative somatic hybrid callus lines were obtained from 'Heckenzauber' + *Rosa wichuraiana* or *Rosa multiflora* as well as from 'Pariser Charme' + *Rosa wichuraiana*, *Rosa multiflora* or *Rosa roxburghii*, respectively. Shoots were regenerated from the combination of 'Pariser Charme' and *Rosa wichuraiana*. The hybrid character of some selected regenerates was exemplarily confirmed by flow cytometry and AFLP-analysis.

1. Introduction

Somatic hybridization offers additional potentials in rose breeding in creating novel combinations of nuclear and extranuclear genetic materials, which cannot be achieved by conventional crossbreeding due to sexual incompatibility, maternal inheritance and meiosis preceding gametogenesis. Prerequisite for practical application of protoplast fusion techniques is the availability of a protoplast to plant regeneration system, which can be efficiently applied to different genotypes. Isolation of viable rose protoplasts has been described as early as 1973 (Pearce and Cocking), however only 18 years later the feasibility of plant regeneration was demonstrated for a *Rosa persica* x *R. xanthina* hybrid genotype (Matthews *et al.*, 1991). PEG (polyethyleneglycol) mediated protoplast fusion was successfully applied in attempts to obtain tetraploid *Rosa persica* x

R. xanthina genotypes (Mottley *et al.*, 1997). In addition, somatic hybridization has been performed between rose cultivars and cherry as well as blackberry, respectively. However, the regenerated plants were similar to the rose parent in morphology and RAPD analyses and their hybrid character could not be demonstrated (Mottley *et al.*, 1997). At the Institute for Ornamental Plant Breeding in Ahrensburg integration of protoplast based techniques in breeding of roses is evaluated with special emphasis on exploitation of new sources of resistance against fungal pathogens such as *Diplocarpon rosae* (blackspot). Protocols have been established for (I) regeneration of protopasts in a variety of rose genotypes, (II) PEG mediated fusion and (III) preferential regeneration of heterologous fusion products.

2. Material and methods

2.1. Plant Material

Experiments were conducted with rose cultivars ('Pariser Charme', 'Heckenzauber', 'Elina'), the hybrid *R.persica x R. xanthina* (obtained from A. Roberts, UK) and selected genotypes of wild species, i.e. *R. canina*, *R. caudata*, *R. corymbifera* 'Laxa', *R. indica*, *R. multiflora* (2 genotypes), *R. roxburghii*, *R. spinosissima*, and *R. wichuraiana* (2 genotypes). For isolation of protoplasts cell suspensions of embryogenic or nonembryogenic character were used as source material. Initiation and maintenance of cultures was performed as described before (Schum *et al.*, 2001). Cell suspensions were cultured in 200 ml Erlenmeyer flasks in MS-media (Murashige and Skoog, 1962) with 30 g/l sucrose, 0.5 ppm 2.iP and 0.5 ppm or 2 ppm of either one of the auxins Picloram or 2.4.5-T. Batch cultures were placed on a gyratory shaker at 90 rpm and incubated at 25 °C in continuous darkness. Cultures were supplied with fresh medium every three to four days.

2.2. Protoplast isolation

For the isolation of protoplasts 8 ml of cell suspension in the state of the exponential growth phase were pelleted by centrifugation for 10 minutes at 1000 rpm. The supernatant culture medium was discarded and cell material transferred to 20 ml CPW-solution (cell and protoplast washing medium according to Cocking and Peberdy, 1974) with 1 mg/ml MES (2[N-morpholino] ethanesulfonic acid), 0.6 M mannitol, 1% Cellulysin (Calbiochem), 0,05% Driselase (Sigma) and 0,5% Macerase (Calbiochem). Enzyme incubation was carried out in the dark for 5 hours at 25 °C, followed by 15 hours at 10 °C. The resulting suspension was sieved through a set of gauze with 300, 150 and 100 µm meshsize, respectively. Protoplasts and cell debris were pelleted by centrifugation at 900 rpm. Upon discarding the supernatant enzyme solution the residues were resuspended in 1ml CPW-0.6 M mannitol solution and pipetted on top of a layer of 8ml CPW-solution with 20 % sucrose. After centrifugation at 2000 rpm for 10 minutes protoplasts were collected from the sucrose/mannitol interface and further washed with CPW-0.6 M mannitol solution.

2.3. Protoplast inactivation and fusion

Prior to PEG mediated fusion, protoplasts were pretreated with either iodoacetate (IA, 0.5 - 1 mM, 15 minutes), rhodamine 6G (R6G, 0.1 mM, 15 minutes) or X-rays (300 Gy, dose rate 3 Gy/min), respectively. After treatment with antimetabolites protoplasts were washed twice with a CPW-0.6 M mannitol solution. Symmetric and asymmetric fusions of protoplasts were carried out by application of 15% - 25% PEG 3350 or PEG 6000 following the procedures described by Galun and Aviv (1991). Protoplasts were finally washed with a 0.6 M mannitol solution without the CPW salts.

2.4. Protoplast culture

Protoplast density was adjusted to 100000 per ml and this solution was mixed with the same volume of alginate (2.1% in 0,58 M mannitol). The mixture was pipetted in 0.75 ml aliquots on $CaCl_2$-agar (2.94g/l $CaCl_2 \cdot 2\ H_2O$, 0,58 M mannitol, 15 g/l agar) in petridishes of 3.5 cm diameter. After one hour, solidified alginate films with immobilized protoplasts were transferred to 1.5 ml liquid culture media in plastic petridishes (Ø 3.5 cm). Protoplast culture media consisted of inorganic salts according to Pelletier et al. (1983) and organic supplements 8p according to Kao and Michayluk (1975) with 0.5 M glucose. Media were supplemented with either 3 ppm 2.4-D or 0.25 ppm 2.4-D, 1 ppm NAA and 1 ppm TDZ. Media were adjusted to pH 5.6 and filter sterilized. Protoplasts derived from fusion variants were incubated on top of feeder layers of protoplast derived rapid growing cell colonies. Nurse cultures and fusion products were separated by nylon gauzes of 30-300 µm meshsize. Cultures were incubated at 25 °C in the dark. In some experiments, manipulated protoplasts were allowed to rest at 6 °C overnight prior to immobilization in alginate for further culture.

In case of sustained cell divisions the concentration of the osmoticum was reduced by replacing ½ of the culture medium every 7 days by a medium with the glucose content decreased by 0.1 M until a final concentration of 0.2 M was reached. When regenerated microcalli had gained a size of approximately 1 mm, alginate films were dissolved in a solution consisting of 20 mM citric acid monohydrat and 0.2 M sucrose (pH 5.6). Calli were subcultured on agar solidified MS-media with 3 ppm 2.4-D (Matthews *et al.*, 1991) at 25 °C in an 16-h-photoperiod. After two subcultures of 4 weeks, calli were transferred to MS-medium with 1.5 ppm Zeatin, 0.25 ppm NAA and 1 ppm GA_3. Regenerated somatic embryos were transferred to the differentiation medium according to Noriega and Söndahl (1991) containing 1 ppm GA_3 and 2 ppm ABA and were incubated at 7 °C in the dark for 4 weeks. Shoot induction was obtained upon transfer to MS medium with 100 ppm FeEDDHA, 1 ppm BAP, 0.004 ppm NAA and 0.1 ppm GA_3. Protoplast derived shoots were cloned on MS-media supplemented with 0.5 - 2 ppm BAP, 0.004 – 0.1 ppm NAA and 0.1 ppm GA_3 according to their habitus. Rooting was induced in ½ concentrated MS liquid culture medium with 0.05 ppm NAA or IAA, respectively.

2.5. Analysis of fusion products

Regenerated putative hybrid calli and shoots were analyzed cytologically by application of the high resolution kit for plant ploidy analysis (Partec 05-4004) for flow cytometry and, so far in a limited number of cases, by application of AFLP marker technique (Debener and Mattiesch, 1999).

3. Results

3.1. Protoplast regeneration

By application of the protocol published by Schum et al. 2001 or the optimized procedure described here, protoplasts isolated from non embryogenic cell suspensions regularly regenerated callus. Protoplast derived callus lines of non embryogenic character were obtained from *R. canina*, *R. caudata*, *R. corymbifera* 'Laxa', *R. multiflora* (two accessions), *R. roxburghii*, *R. spinosissima*, *R. wichuraiana* (two accessions) as well as from cultivars 'Elina' and 'Pariser Charme'. Protoplasts isolated from embryogenic cell suspensions of 'Heckenzauber' and 'Pariser Charme', as well as from non embryogenic suspensions of the hybrid *R. persica* x *R. xanthina* gave rise to callus lines of embryogenic character and were regenerated into plants. These exhibited a high degree of somaclonal variation in characters such as plant habitus, flower morphology and flower colour.

3.2. Protoplast fusion

Specific genotypes of several wild rose species were identified to carry genes conferring resistance to *Diplocarpon rosae* (von Malek-Podjaski, 1999). For introgression of resistance into cultivars by means of somatic hybridization, experiments concentrated on diploid accessions of *Rosa multiflora*, *R. wichuraiana* and *R. roxburghii*. Fusion experiments were carried out between protoplasts derived from non embryogenic cell suspensions of the wild rose species and protoplasts isolated from embryogenic cell suspension cultures of cultivars 'Heckenzauber' and 'Pariser Charme'. In order to suppress subsequent development of non-fused protoplasts as well as of homologous fusion products, partners were complementarily pretreated with different antimetabolites or X-rays, respectively. Application of a dose of 300 Gy of X-rays regularly inhibited regeneration of callus in all genotypes tested so far. Increasing concentrations of iodoacetate decreased the percentage of deviding cells and callus regeneration was definitively inhibited at 1 mM. Protoplasts proved to be very sensitive to rhodamine 6G, which generally suppressed cell regeneration and division at a concentration of 0.1 mM. However, sensitivity of protoplasts to both chemicals was inconsistent between different experiments. In some cases the majority of protoplasts was severely damaged and no fusion products were obtained at the end of subsequent manipulative procedures. In other cases treatment of protoplasts with antimetabolites did not completely suppress their regeneration capacity, which became obvious in unfused control cultures. Routinely, concentrations between 0.5 and 1 mM were selected for treatment of protoplasts with iodoacetate and 0.1 mM for treatment with rhodamine 6G.

PEG treatment results in undirected fusion of protoplast partners. Homologous as well as multicellular fusion products will be formed in addition to the fusion between two different partners. However, such heterologous fusion products aimed at can be regularly observed upon addition of 15 – 25 % PEG (Fig. 1).

3.3. Selection of Somatic Hybrids

Regeneration has been obtained after fusion of protoplasts from different parental combinations (Tab. 1). Hundreds of independently regenerated putative somatic hybrid callus lines were obtained from 'Heckenzauber' + *Rosa wichuraiana* or *Rosa multiflora* as well as from 'Pariser Charme' + *Rosa wichuraiana*, *Rosa multiflora* or *Rosa roxburghii*, respectively. Shoots were regenerated upon asymmetric hybridization of 'Pariser Charme' and *Rosa wichuraiana*.

Pretreatment of protoplasts with antimetabolites inducing complementary defects or with X-rays, respectively, allows preferential regeneration of heterologous fusion products (Fig. 2). Irradiation with X-rays at a dose of 300 Gy regularly inhibits regeneration of callus in all genotypes tested so far. In contrast, sensitivity of protoplasts to chemicals is more inconsistent, resulting in regeneration of differing percentages of cell lines other than of hybrid character in some experiments. Therefore calli need to be further analyzed in order to distinguish between true somatic hybrids and escapes. Differential colour of regenerated callus lines may help to preselect putative hybrid cell lines. Further identification of somatic hybrids is being performed by application of flow cytometry and molecular marker techniques.

Flow cytometry analysis with PARTEC high resolution kit 05-4004 in combination with chopping material with a razor blade gives quick and satisfying results with in vitro plantlets, somatic embryos and callus of hard consistency. However, analysis of loose, friable types of callus was not possible. In such cases, signals were distributed over all channels and no peaks were obtained. Other methods for isolation of nuclei including enzyme treatment, immobilization in alginate prior to chopping, freezing, vortexing, use of mortar and pestle with addition of liquid nitrogen as well as use of various different buffer solutions did not lead to any better histograms either. Results of flow cytometric analysis performed so far are as follows. Callus, regenerated from control

protoplasts either displayed the original ploidy level of the corresponding genotype or was doubled. However, plantlets regenerated from such cultures, exclusively possessed the original chromosome number without any exception. Putative hybrid calli, obtained by symmetric fusion of protoplasts from a diploid and a tetraploid parent, displayed a wide range of ploidy levels between 2n = 6x and 2n = 18x, including different degrees of aneuploidy (Schum and Hofmann, 2001). The hybrid character of some selected calli was exemplarily confirmed by AFLP-analysis (Schum and Hofmann, 2001).

The majority of shoots which were regenerated upon asymmetric fusion between X-ray treated protoplasts of *Rosa wichuraiana* (2n = 2x) and iodoacetate treated protoplasts of 'Pariser Charme' (2n = 4x) initially proved to be tetraploid-aneuploid. However, within the course of subcultures many of these displayed a tendency to the loss of chromosomes. Six callus lines with shoot initials were aneuploid between 8x and 9x. Again, loss of chromosomes occured in the course of culture towards ploidy levels between 6x and 8x. Up to now, none of the regenerated shoots could be induced to form roots.

4. Discussion

Protoplast fusion has been performed between tetraploid rose cultivars 'Pariser Charme' or 'Heckenzauber' and specific diploid genotypes of *Rosa multiflora*, *Rosa wichuraiana* or *Rosa roxburghii* displaying resistance to *Diplocarpon rosae*. Somatic hybridization with *Rosa roxburghii* is of special interest, as sexual compatibility with cultivated roses is very limited (Debener, personal communication). Protoplasts of rose cultivars were isolated from embryogenic cell suspensions and could be regenerated to plants via somatic embryogenesis. In case of the wild species, non embryogenic cell suspensions were used as protoplast source. These regenerate callus of non embryogenic character. Suppression of plant regeneration of wild species was thought to be of advantage with respect to preferential regeneration of fusion products containing the genome of rose cultivars. However, shoot regeneration potential of putative hybrid calli proved to be very low. While hundreds of independently regenerated callus lines have been obtained from different parental combinations, so far shoots were recovered only from asymmetric fusions between 'Pariser Charme' and *Rosa wichuraiana*. In order to possibly increase the regenerative capacity of somatic hybrid cells, attempts were initiated to induce embryogenic callus from the wild species to be used as protoplast source in further experiments.

Pretreatment of protoplasts with antimetabolites inducing complementary defects or with X-rays, respectively, allows preferential regeneration of heterologous fusion products. However, in spite of standardized incubation conditions for source material sensitivity of protoplasts to chemicals proved to be inconsistent, resulting in regeneration of differing percentages of cell lines other than of hybrid character. In order to reduce work with subculture of non heterologus fusion products, selection of somatic hybrids at the callus level would be of advantage. In some cases colour of regenerated calli differs from that of both parental lines and may help to preselect putative hybrid cell lines. Ploidy analyses allows identification of calli with increased DNA levels, which are to be expected upon somatic hybridization. Nuclei from in vitro plantlets, somatic embryos and callus of hard consistency can be readily isolated and their DNA content measured by flow cytometry. However, analyses of callus of loose and friable character proved to be impossible either due to insufficient disintegration of cells or due to predominance of debris. Therefore further efforts on identification of somatic hybrids will concentrate on application of molecular marker techniques.

Protoplast derived plantlets display a high degree of somaclonal variation. As long as fertility of somatic hybrids is not negatively affected, this should be tolerable as somatic hybridization with wild rose species requires further backcrosses with cultivated roses in any case.

Fundamentals for integration of protoplast culture in rose breeding have been

established by developing protocols for (I) regeneration of protopasts in a variety of rose genotypes, (II) PEG mediated fusion and (III) preferential regeneration of heterologous fusion products. However, for practical use of somatic hybridization in rose breeding many problems remain to be solved, such as optimization of hybrid plant regeneration and characterization, elimination of undesirable traits and selection of stable disease resistance.

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References

Cocking E.C., and Peberdy J.F., 1974: The use of protoplasts from fungi and higher plants as genetic systems – a practical handbook. Univerity of Nottingham, University Park, Nottingham.

Debener T., and Mattiesch L., 1999. Construction of a linkage map for roses using RAPD and AFLP markers. Theor. Appl. Genet. 99: 891-899.

Galun E., and Aviv D., 1991. Cybrid production and selection. In: Lindsey, K. (Ed.), Plant Tissue Culture Manual. Kluwer Academic Publishers, Dordrecht, Boston, London. D4: 1-17.

Kao K.H., and Michayluk M.R., 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126: 105-110.

Matthews D., Mottley J., Horan I., and Roberts A.V., 1991. A protoplast to plant system in roses. Plant Cell Tissue and Organ Culture 24: 173-180.

Mottley J., Yokoya K., Matthews D., Squirrell J., and Wentworth J.E., 1997. Protoplast fusion and ist potential role in the genetic improvement of roses. Acta Hort. 424: 393-397.

Murashige T., and Skoog F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Noriega C., and Söndahl M.R., 1991. Somatic embryogenesis in hybrid tea roses. Bio/Technology 9: 991-993.

Pearce R.S., and Cocking E.C., 1973. Behaviour in culture of isolated protoplasts from 'Paul's Scarlet' rose suspension culture cells. Protoplasma 77: 165-180.

Pelletier G.C., Primard C., Vedel P., Chetrit P., Remy R., Rousselle P., and Renard M. 1983. Intergeneric cytoplasmic hybridization in cruciferae by protoplast fusion. Mol. Gen. Genet. 191: 244-250.

Schum A., and Hofmann K., 2001. Use of isolated protoplasts in rose breeding. Acta Horticulturae 547: 35-44.

Schum A., Hofmann K., Ghalib N., and Tawfik A., 2001. Factors affecting protoplast isolation and plant regeneration in *Rosa* spec. Gartenbauwiss. 66 (3): 115-122.

von Malek-Podjaski B., 1999. Erschließung von Resistenzquellen gegenüber dem Erreger des Sternrußtaus (*Diplocarpon rosae* Wolf) an Rosen unter Verwendung molekularer Marker. PhD-Thesis, University of Hannover.

Table 1. Regeneration upon rose protoplast fusions of different parental combinations (IA = iodoacetate, R6G = rhodamine 6G)

Rose Cultivar	Treatment	Wild Species	Treatment	Regeneration
'Pariser Charme'	IA	*R. multiflora*	R6G	callus
	R6G		IA	callus
'Pariser Charme'	IA	*R. wichuraiana*	R6G	callus
	R6G		IA	callus
	IA		X-rays	shoots
'Pariser Charme'	IA	*R. roxburghii*	none	callus
'Heckenzauber'	IA	*R. wichuraiana*	X-rays	callus
'Heckenzauber'	IA	*R. multiflora*	X-rays	callus

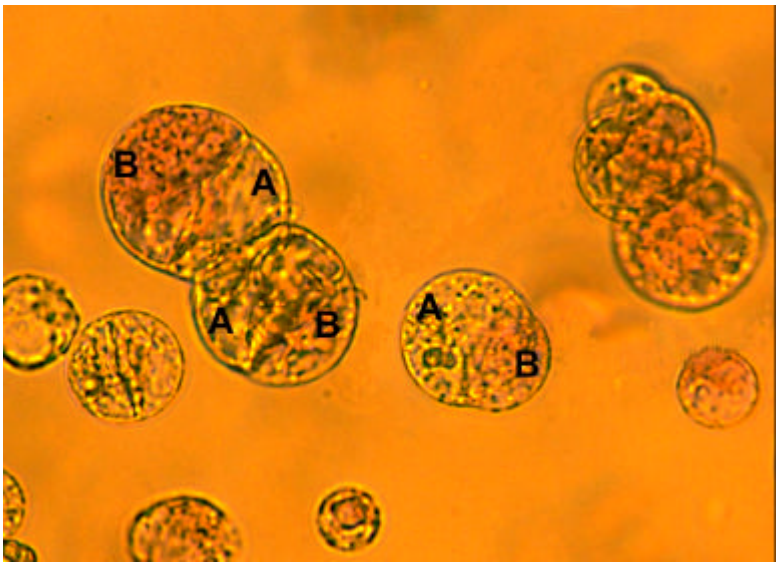

Figure 1. Fusion of rose protoplasts after pretreatment of cells with either iodoacetate (A) or rhodamine 6G (B) and addition of 20 % PEG

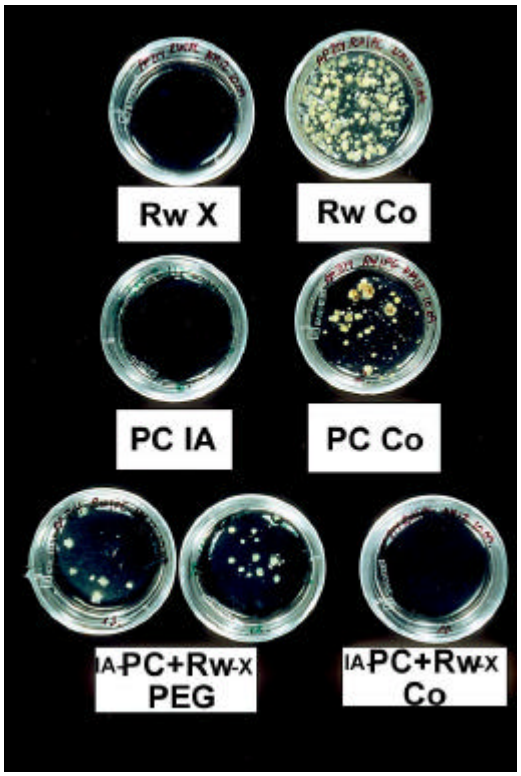

Figure 2. Regeneration of callus after fusion of protoplasts from 'Pariser Charme' (PC) and *Rosa wichuraiana* (Rw), (Co = control, X = treatment with X-rays, IA = treatment with iodoacetate, PEG = polyethyleneglycol)