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Somatic Embryogenesis in Rose: Gene Expression and Genetic Transformation

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Abstract Induction of somatic embryogenesis in roses involves several critical steps requiring specific tissue culture media compositions and particular manipulations of explants. However, it is important to note that although there are various reports on successful induction of somatic embryogenesis in rose, these are often limited to particular genotypes. Therefore, to date, there is no single protocol for inducing somatic embryogenesis that can be used for multiple rose genotypes. Nevertheless, advances have been made in studying regulation of gene expression during somatic embryogenesis. Moreover, successful genetic transformation of rose has been achieved using embryogenic cultures. Transgenic rose lines with desirable traits have now been obtained. Further opportunities for exploiting somatic embryogenesis for genetic manipulation and improvement of roses will become available with all these current achievements and future efforts.

1 Introduction

Somatic cells of plant tissues have the capacity to undergo cellular dedifferentiation into a mass of unorganized cells, or callus, as well as the ability to generate differentiated cells. It is this latter ability to produce morphologically and developmentally normal organs from somatic plant cells that presents an intriguing and unique phenomenon in plants. In recent years, this observed phenomenon, referred to as totipotency of plant cells, has become critical for successful asexual propagation of plants. Moreover, it serves as a limiting step in the ever-expanding area of transgenic plant development. Therefore, this fascinating phenomenon is worthy of investigation to expand our fundamental knowledge of cellular behavior by elucidating the regulatory and morphogenetic events in plant cell growth and development.

Induction of in vitro embryogenesis from somatic plant tissues is an alternative developmental process that occurs in response to high concentrations of auxin or better yet to a functional analog of auxin, namely 2,4-D, added to the culture medium. This unique ability of vegetative plant cells to undergo cellular differentiation into somatic embryos has provided a valuable model system for fundamental studies on embryogenesis as the developmental process of somatic embryogenesis is considerably similar to that of

zygotic embryogenesis (Zimmernman 1993). Cell competence for embryogenesis is acquired in the presence of auxin in the medium as cells form proembryogenic masses (PEMs). Upon removal of auxin from the culture medium, these PEMs then undergo differentiation from the globular stage to the heart/torpedo stage, and into plantlets. It is believed that in the presence of auxin, the PEMs synthesize all gene products necessary to complete the globular stage of embryogenesis, but new gene products are needed for the transition to the heart stage which can only be synthesized when the exogenous auxin is removed from the medium (Zimmerman 1993). It is likely that there are other gene products that are synthesized in PEMs in the presence of auxin that prohibit globular embryos from further development into the heart stage. Therefore, these developmental switches are most likely regulated at the transcriptional level, and it is generally believed that somatic embryogenesis is mediated by a signal transduction pathway that is triggered by exogenous auxin.

Successful development of regeneration systems for a number of rose species has already been reported. Embryogenic callus has been initiated from in vitro-derived leaf or stem segments of *Rosa hybrida* cv. Carl Red and *R. canina* (Visessuwan et al. 1997), *R. hybrida* cv. Carefree Beauty, and R. chinensis minima cv. Baby Katie (Hsia and Korban 1996). Embryogenic callus has also been induced in leaves of *R. hybrida* cvs. Domingo and Vicky Brown (De Wit et al. 1990), petioles and roots of *R. hybrida* cvs. Trumpeter and Glad Tidings (Marchant et al. 1996), root explants of both *R. hybrida* cv. Moneyway (van der Salm et al. 1996) and R. Heritage × Alista Stella Gray (Sarasan et al. 2001), petals of *R. hybrida* cv. Arizona (Murali 1996), and immature seeds of R. rugosa (Kunitake et al. 1993). This has also been achieved using immature leaf or stem segments of *R. hybrida* cv. Landora (Rout et al. 1991), in vivo mature leaves of *R. hybrida* cv. Soraya (Kintzios et al. 1999), anther filaments of *R. hybrida* cv. Royalty (Noriega and Söndahl 1991), as well as anthers, petals, receptacles, and leaves of *R. hybrida* cv. Meirutal (Arene et al. 1993). The wide range of explants and experimental approaches that have been employed with different rose species and cultivars strongly suggest that it is difficult to develop a universal genotype-independent method for the production of embryogenic callus in rose (Marchant et al. 1996). Recent progress on rose regeneration has been reviewed by Rout et al. (1999). However, in this chapter we will provide detailed protocols for initiation of embryogenic cultures of rose as well as review some of the applications for these embryogenic cultures for genetic improvement and/or manipulation of roses.

2 Embryogenic Culture Initiation

2.1 Explant Preparation

Among all different tissues used for induction of somatic embryogenesis, it is apparent that in vitro-grown leaves provide the most reliable source of explants for induction of somatic embryogenic cultures from various genotypes of rose.

2.2 Establishing Proliferating Shoot Cultures

To begin with, proliferating shoot cultures of rose must be first established. On the basis of our own experience with various genotypes of *R. hybrida* and *R. chinenesis* minima, nodal stem segments (2 cm in length) that are closest to the apical meristem must be collected from healthy and vigorously growing greenhouse-grown plants. Once cut from actively growing donor plants, all leaves must be removed from stem segments, but retaining the apical meristem intact.

Stem segments (1.5 cm in length) are surface-sterilized with 0.525% sodium hypochlorite solution (10% Clorox commercial bleach) for 10 min, and rinsed three times with sterilized-distilled water (5 min per rinse). Nodal stem sections are then given a fresh cut (along the basal end), and placed in 25 × 150 mm culture tubes containing the medium listed in Table 1. It is important to point out that stem segments with relatively large diameter (0.6–0.8 mm) and long internodes (> 2 cm) are preferred. Cultures should be incubated under a 16 h photoperiod provided by cool-white fluorescent light (60 mmol m^{-2} s^{-1}).

Table 1 Composition of media for establishment and proliferation of shoot cultures of rose using nodal stem segments

Medium component	Culture establishment (per liter)	Shoot proliferation (per liter)
MS salts	4.30 g	4.43 g (salts + MS vitamins)
BA	4.44 mM	2.22 mM
NAA	0.54 mM	0.27 mM
Sucrose	30.00 g/l	30.00 g/l
Agar	7.00 g (Difco-bacto)	2.5 g (gelrite)
pH	5.7	5.7

Table 2 Composition of media for induction of callogenesis followed by embryogenesis from rose leaf explants

Medium component	Callus induction (per liter)	Induction of embryogenesis (per liter)
MS salts + MS vitamins	4.43 g	2.25 g (1/2 MS salts + full MS vitamins)
2,4-D	11.3 mM	0.00 mM
TDZ	0.00 mM	2.30 mM
GA3	0.00 mM	2.90 mM
Sucrose	30.00 g/l	30.00 g/l
Agar	2.50 g (gelrite)	2.5 g (gelrite)
pH	5.7	5.7

Within two weeks following culture establishment, shoots developing from buds should be excised and transferred to a fresh medium to promote shoot growth and proliferation. Proliferating shoot cultures should be periodically subcultured to fresh medium once every 4–5 weeks to maintain growth and proliferation of healthy and vigorous shoots.

2.3 Callus Induction

The top four vigorously growing leaves are excised from in vitro-grown proliferating shoots. Either whole leaves or leaflets should be used as explants for callus induction. All leaf explants should be placed with the abaxial surface in contact with the medium. The basal medium containing full-MS salts, MS vitamins, 30 g sucrose, is supplemented with 2,4-D, and solidified with 2.5 g gelrite. Concentrations of 2,4-D of either 11.3 or 45.2 mM are recommended. pH of the medium is adjusted to 5.7. However, the concentrations of 2,4-D may have to be amended depending on the rose genotype used. Cultures are then incubated in the dark for 4 weeks at a temperature of 23 ± 1 °C.

2.4 Induction of Somatic Embryogenesis

Explants with callus, previously incubated on medium containing 2,4-D, are transferred to a 1/2 MS basal medium, full-strength MS vitamins, 30 g sucrose, and containing either no PGRs, 2.9 mM gibberellic acid (GA_3) alone, or 2.9 mM GA_3 with either 2.2 mM BA or 2.3 mM thidiazuron (TDZ). The medium is solidified with 2.5 g gelrite gellan gum (PhytoTechnology), and pH is adjusted to 5.7. Cultures are grown under light conditions as described above.

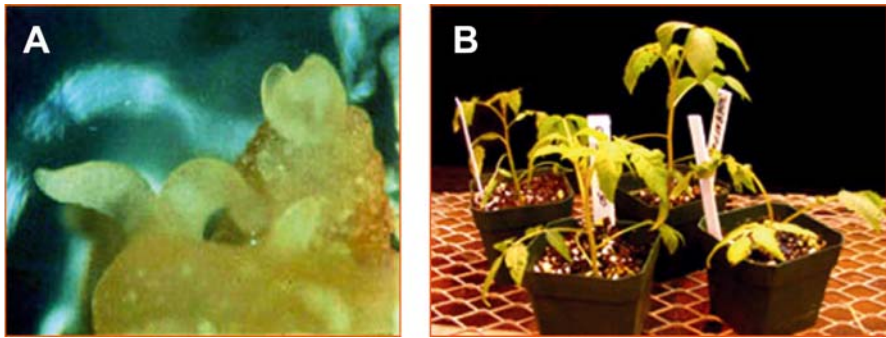

Fig. 1 Somatic embryogenesis and plantlet regeneration in rose. **a** Primary somatic embryos induced from embryogenic callus; **b** whole plantlets regenerated from somatic embryos

Callus explants are incubated for a period of two months on the above media and then subcultured to a PGR-free medium for an additional two months. The development of embryogenic callus should be observed throughout this incubation regime. An asynchronous development of embryogenic callus with globular, heart-shaped, and cotyledonary stages are observed throughout this period (Fig. 1a). Embryogenic callus is soft, friable, and opaque-white in color. At times, explants might turn brownish in color (especially those continuously incubated on PGR-free medium), but this callus can still produce somatic embryos. However, if hard, compact, and green-colored callus is observed, it is most likely to be either a nondifferentiating callus or organogenic callus.

2.5
Induction and Proliferation of Secondary Somatic Embryogenesis

Induction of secondary embryogenesis is highly desirable for both micropropagation and genetic improvement (e.g., via transformation) efforts. Inducing secondary embryogenesis from primary somatic embryos can be accomplished by transferring primary embryogenic callus onto petri plates containing 1/2 MS basal salts, full-strength MS vitamins, and solidified with 2.5 g gelrite gellan gum for a period of one month. These are then transferred onto a PGR-free medium with monthly subcultures. All cultures are maintained under light conditions as described above. Proliferation of somatic embryos can be maintained for at least 1 year.

2.6
Maturation and Germination of Somatic Embryos

Maturation and germination of somatic embryos is achieved by transferring individual clumps of somatic embryos onto a similar medium as described

above, but with a slight modification. Essentially, the medium consists of 1/2 MS basal salts, full-strength MS vitamins, 30 g sucrose, 3.8 mM abscisic acid (ABA), and solidified with 2.5 g gelrite gellan gum. Bipolar plantlets are then excised, and individually transferred to a PGR-free shoot elongation medium consisting of 1/2 MS medium, full-strength vitamins, and 30 g sucrose for a period of one month. This medium also promotes shoot elongation and root development.

2.7 Plantlet Development, Acclimatization, and Transfer to the Greenhouse or Field

Rooted plantlets are transferred to a soil mix (1 : 1 : 1 of soil, peat, and perlite) in 4 cm plastic pots for a period of two weeks, and covered with a clear plastic bag. If the plantlets are in flats, then a clear plastic cover can be used instead. The top of the plastic bag/cover is gradually removed/opened to allow for plantlets to be acclimatized. This process can take anywhere from two to three weeks.

Acclimatized plants are then transferred to the greenhouse and grown at 23 °C. Plants are watered daily using a drip-irrigation system, and fertilized once every 2 weeks with 250 ppm of a 20-20-20 NPK fertilizer solution. Once the plants are well established in the greenhouse (Fig. 1b), then these can be transferred to the field.

3 Regulation of Gene Expression During Somatic Embryogenesis

As plant cells grown in vitro undergo the process of somatic embryogenesis, these are accompanied by changes in DNA methylation that are associated with regulation of gene expression (Finnegan 2001). In higher plants, the 5-methylcytosine (5 mC) is predominantly modified, and among all CpG sequences in a plant genome, 60–90% of those are methylated, while unmethylated CpG sequences are clustered as CpG islands (Ng and Bird 1999). DNA methylation can inhibit transcription by modifying target sites of transcriptional factors thus blocking their binding to these sites, but also changes occurring in the chromatin of a methylated template also contribute to the observed inhibition of transcription (Finnegan 2001). In plant genomes, methylation is not only restricted to CpG sequences as significant levels of cytosine methylation are also observed in nonCG sequences, which include symmetrical CNG and asymmetrical CNN sequences (Tariq and Paszkowski 2004).

The presence of 5 mC is a feature of transcriptionally silenced chromatin, and provides a plant genome with a mechanism to defend itself against transposable elements and retroviruses (Martinesen and Colot 2001; Bird 2002). Genetic alterations that reduce methylation levels result in various pleiotropic

phenotypes in plants (Bird 2002). The *Arabidopsis thaliana* genome contains at least 10 genes encoding DNA methyltransferases (Finnegan and Kovac 2000; Kankel et al. 2003). Among those, the *Arabidopsis* MET1 has been extensively investigated, and found to have a complex role in various developmental processes (Finnegan and Kovac 2000). Screening plants with reduced methylation of repetitive sequences, *MET1* missense mutations (*met1-1* and *met1-2*) have been isolated exhibiting delayed flowering and loss of gene silencing (Kankel et al. 2003). Methylation in nonCG sequences, which is a common modification in plant DNA, is also catalyzed by a domain containing plant-specific methyltransferase *CHROMOMETHYLASE3* (*CMT3*) (Bartee et al. 2001). Moreover, *CMT3*, is a key determinant in CpXpG methylation (Bartee et al. 2001).

Recently, Xu et al. (2004) have conducted a detailed investigation of DNA methylation alterations during reprogramming events in somatic tissues of *R. hybrida* using the amplified fragment-length polymophism (AFLP) technique. On the basis of banding patterns, it has been observed that the highest numbers of AFLP bands are observed in embryogenic callus and in regenerants from embryogenic callus. This indicates that a number of internal cytosines are methylated during the processes of somatic embryogenesis and subsequent regeneration of somatic embryos into whole plantlets. Moreover, methylation alterations during somatic embryogenesis have been found to be characterized by extensive demethylation of outer cytosines in $5'$-$_{\mathrm{m}}$CCGG-$3'$ sequences, and these are passed along to their regenerants. These findings provide support to the hypothesis that modified cytosines are likely essential for the acquisition of embryogenic potential in somatic cells of rose, and that these are then passed on to subsequent regenerants from somatic embryos (Xu et al. 2004).

Among methylation-related bands that have been sequenced, some have been found to be tissue-specific, and more specifically these are associated with embryogenic callus and regenerants of somatic embryos (Xu et al. 2004). The amino acid sequence of one such embryogenesis-specific band appears to be derived from the Deetiolated 1 (DET1) protein in rose. Although the function of this protein is not clearly identified in rose, it has been reported to be a regulatory gene that represses several signaling pathways controlled by light (Schafer and Bowler 2002). Moreover, some clues as to the function of this gene can be discerned from extensive studies in tomato. It has been reported that mutations in this gene are responsible for *high pigment-2* (*hp-2*) phenotypes in tomato that are characterized by exaggerated photo-responsiveness (Mustilli et al. 1999). Light-grown *hp-2* mutants display high levels of anthocyanins, are short, and more deeply-pigmented than wild-type plants. The higher pigmentation of mature fruits from these mutants is due to elevated levels of both flavonoids and carotenoids (Mustilli et al. 1999; Levin et al. 2003). Therefore, it is likely to expect that the DET1 in rose is also associated with anthocyanin content as well.

4 Genetic Transformation of Somatic Embryos

One of the most successful applications of somatic embryogenesis in rose has been the use of this cellular differentiation pathway for developing a genetic transformation system for roses. The ability to introduce and express diverse foreign genes into plants has long been employed for genetic improvement of various plant species, and it has become an important strategy for genetic improvement of roses as well. The promise of genetic transformation of roses is slowly being realized with opportunities for developing genotypes with enhanced and desirable traits coming along as recent advances are made in both somatic embryogenesis and genetic transformation protocols of rose.

Generally, plant transformation is achieved either via *Agrobacterium*-mediated transformation or via microprojectile bombardment. However, a small number of target cells typically receive the foreign DNA during these transformation events, and even a smaller number of these cells survive selection and subsequent regeneration of stable transformants. Therefore, efforts have been made to develop transformation protocols for rose using *Agrobacterium*-mediated transformation, and to a lesser extent via microprojectile bombardment.

Over a decade ago, Firoozabady et al. (1991) published the first report on successful *Agrobacterium*-mediated transformation of *R. hybrida* cv. Royalty. Later, transgenic rose plants were obtained by transforming friable embryogenic tissues of rose, recovered from filament cultures, with either *Agrobacterium tumefaciens* or *A. rhizogenes* (Firoozabady et al. 1994). Mathews et al. (1994) regenerated transgenic rose from protoplasts of embryogenic cell lines.

Van der Salm et al. (1997) obtained transgenic plants from roots derived from stem slices of the rootstock *R. hybrida* cv. Moneyway following co-cultivation with A. tumefaciens strain GV3101 containing an *nptII* gene and individual *rol* genes from *A. rhizogenes*. Grafting the transformed rootstock resulted in stimulation of both root development of the rootstock and axillary-bud break of the untransformed scion (Van der Salm et al. 1998). Marchant et al. (1998a) regenerated transgenic plants from embryogenic callus of *R. hybrida* following microprojectile bombardment with the biolistic gene gun. Subsequently, Marchant et al. (1998b) successfully introduced a chitinase gene into *R. hybrida* cv. Glad Tiding, and found that expression of the chitinase transgene reduced the severity of black spot (*Diplocarpon rosae* Wolf.) development by 13–43%.

Recently, Li et al. (2002b) have reported on an enhanced efficiency of *Agrobacterium*-mediated transformation of embryogenic cultures of *R. hybrida* cv. Carefree Beauty by taking advantage of induced secondary somatic embryogenesis (Li et al. 2002a). As transformed embryogenic cells act independently from neighboring cells, these develop into somatic embryos that further undergo secondary embryogenesis. It is observed that transgenic

lines with similar Southern hybridization profiles exhibit the same level of transcription as demonstrated by similar band intensities in Northern blots. Therefore, the transformation efficiency is estimated to be at least 9%. As the number of transgenic plants developing from the same transformation event is high (having undergone secondary somatic embryogenesis), this approach avoids the recovery of chimeric transgenic plants. This finding is especially important for plant species that rely on vegetative propagation.

In a later study (Li et al. 2003), this transformation protocol was used to introduce an antimicrobial protein encoding gene, *Ace-AMP1*, into *R. hybrida* cv. Carefree Beauty. Some of the recovered transgenic plants exhibited enhanced resistance to the fungal pathogen powdery mildew [*Sphaerotheca pannosa* (Wallr.: Fr.) Lev. var. *rosae*]. This was demonstrated in both a detached leaf assay and an in vivo greenhouse assay of whole plants. These promising findings offer new opportunities for developing roses with resistance to various economic diseases, among other useful and desirable traits such as flowering habit, growth habit, and flower quality and longevity.

5 Conclusions

Somatic embryogenesis has been successfully achieved in a number of rose genotypes. Various efforts have been made to induce somatic embryos from different tissues of rose plants as well. Recent efforts to induce secondary somatic embryogenesis have been quite promising and encouraging. However, it is important to note that plant cells may undergo some genetic changes while they undergo cellular differentiation, such as somatic embryogenesis, in vitro. As a result, it is important to monitor those changes in gene regulation that are often attributed to changes in DNA methylation. These changes in DNA methylation may contribute to tissue culture-induced mutagenesis, and can also lead to chromatin structure alternations, and changes in gene expression.

However, it is important to point out that the success in inducing somatic embryogenesis in roses has been critical for the successful development of transformation systems for roses. So far, these transformation protocols have resulted in the recovery of transgenic rose lines either with enhanced rooting, bud break, or disease resistance. Further opportunities for developing transgenic roses with other desirable horticultural traits will certainly arise in the near future.

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