ABSTRACT

This review deals with the factors limiting for the plant regeneration and genetic transformation of Catharanthus roseus (L.) G. Don. Despite its importance in producing pharmaceutically valuable terpenoid indole alkaloids, the lack of a reliable C. roseus regeneration system at a high frequency is currently a bottleneck step for the transgenic plant development. The efficiency of Agrobacterium-mediated transformation is dependent on the type of explant, explant age, pre-culture and co-culture period, *vir* genes and antioxidants supplementation and *Agrobacterium* strains. In addition, the disadvantages of negative selection markers, the utility of GFP as a visual selection marker, and the advantages of positive selection markers such as phosphomannose isomerase, tryptophan decarboxylase and feedback-resistant anthranilate synthase are discussed along with selection agents to obtain high frequency genetic transformation. To optimize the factors that are discussed in this review may successfully lead to transgenic *C. roseus* for the metabolic engineering of terpenoid indole alkaloids.

Keywords: Madagascar periwinkle, plant regeneration, secondary metabolism, terpenoid indole alkaloids, transformation

INTRODUCTION

Madagascar periwinkle (*Catharanthus roseus*) is an important medicinal plant growing in tropical and subtropical countries with attractive foliage and different flower colors. Most often, plants are grown in pots as well as in gardens for ornamental value. The leaves contain pharmacologically important compounds known as terpenoid indole alkaloids (TIA). Among more than 130 of them (Mishra and Kumar 2002; van der Heijden et al. 2004), vinblastine and vincristine are of the greatest clinical value for anti-cancer chemotherapy (van der Heijden et al. 2004). A complex chemical structure of vinblastine and vincristine makes *in vitro* chemical synthesis of this compound very difficult. Currently, periwinkle plant is the sole source of vinblastine and vincristine, but the yield of the two alkaloids from the plant is very low (1 g and 20 mg per 1000 kg of plant material, respectively), thus making the cost of these life-saving drugs very expensive (Tyler 1988). Current market value of vincristine is about 20 million dollars per kilogram (Kumar and Kumar 2002). Also ajmalicine and serpentine are used to treat hypertension and other circulatory disorders. The low yield and high market value of these valuable indole alkaloids are the major motivation of the research interest in periwinkle plant. Over the past several decades, *C. roseus* has received considerable interest from both academic and industrial scientists as a model plant to study TIA (reviewed extensively in Teixeira da Silva 2006). Many genes involved in TIA biosynthesis have been cloned and appear to be coordinately regulated by the same signal transduction pathway as they are up-regulated by methyl jasmonate and down-regulated by auxin (Misra et al. 1996; Rischer et al. 2006). A jasmonate-inducible AP2/ERF class of transcription factor ORCA3 was identified that increases metabolic fluxes from primary metabolism to TIA secondary metabolism (van der Fits and Memelink 2000). Over-expression of ORCA3 enhanced expression of multiple TIA biosynthetic genes but did not increase the production of TIA in cell sus-
pension strategy. Despite many literatures, overall regulation of TIA biosynthesis is not well understood yet.

T-DNA activation tagging or T-DNA insertional lines of transgenic *C. roseus* are not currently available. Absence of an efficient transformation and regeneration system is a major drawback in studying the gene function at the whole plant level. Two bisindole alkaloids, vinblastine and vincristine rarely accumulate on cell suspension or hairy root lines because of the absence of specialized cells called latifrons and idioblasts (Vazquez-Flota et al. 2002). Even the stably transformed cell culture lines of *C. roseus* gradually lost the ability to accumulate TIA over time (Whitmer et al. 2003). The TIA pathway is so complex that biosynthesis of metabolic intermediates takes place in different tissues and subcellular compartments (St-Pierre et al. 1999; Kutchan 2005; Mahroug et al. 2006). Without transgenic plants, it will be difficult to metabolically engineer the TIA pathways to increase alkaloid yield (Pasquali et al. 2006). Conventional breeding may offer a way to increase alkaloid production, but it is usually time-consuming and one has to screen a large number of genotypes with high alkaloid content. Screening mutants with economically competitive high yields of alkaloids have not been successful thus far despite the considerable efforts made in mutation breeding (Kulkarni et al. 1999, 2001). Consequently, it is important to establish a regeneration system to further our understanding for TIA gene expression as well as to metabolically engineer *C. roseus* alkaloid production. *C. roseus* is recalcitrant to regeneration following *Agrobacterium* infection. In this review, we discuss limiting factors and the ways to overcome the barrier to regeneration of transformed *C. roseus* explants.

### OPTIMIZATION OF PLANT REGENERATION AND SELECTION OF EXPLANTS FOR PLANT TRANSFORMATION OF *C. ROSEUS*

High frequency regeneration from explants is prerequisite for standardizing optimum transformation conditions to generate transgenic plants in any plant species. To date, there are only a few published reports on plant regeneration in *C. roseus* (Table 1). Low frequency of plant regeneration via organogenesis of callus induced from leaf segments of *C. roseus* was reported by Constabel et al. (1982). Plant regeneration was obtained from anther-derived cell suspension cultures via somatic embryogenesis (Kim et al. 1994). Although somatic embryo was induced in the same way as anther, plant was regenerated at a higher frequency (20%) via somatic embryogenesis when immature zygotic embryo of *C. roseus* cv. ‘Little Bright Eye’ was cultured in MS media containing 1 mg/l 2,4-D (Kim et al. 2004). Healthy plants were regenerated from stem node or shoot tip of *C. roseus* infected with mycoplasma-like organism and mosaic virus (Mollers and Sarkar 1989; Kaur et al. 1996). Segments of seedlings were cultured to obtain calli from which cell suspension culture was initiated to induce embryogenic calli, and later plantlets were regenerated from somatic embryos upon transfer to solid media (Pivwan et al. 2000). The efficiency of plant regeneration from leaf petiole and hypocotyl of *C. roseus* was found to be dependent on the genotype and combination of plant growth regulators (Lee et al. 2003; Choi et al. 2003). Somatic embryos were obtained from embryogenic calli induced from hypocotyls of *C. roseus* on MS medium supplemented with 1 mg/l NAA. Somatic embryos converted into plantlets when cultured on MS medium containing 0.5 mg/l BAP following treatment with 1 mg/l gibberellic acid (Junaid et al. 2006). All these protocols, however, are not practical in transformation that should be accompanied by high frequency plant regeneration. Being a low percentage in plant regeneration (below 20%) or lacking regeneration data, they took a long time to regenerate plant by passing suspension culture. The type of explants along with various combinations of growth regulators has not been extensively tested for high frequency regeneration.

We used TDZ as it frequently induces plant regeneration via somatic embryogenesis and organogenesis in many plant species (Li et al. 2000; Mithila et al. 2003; Liu et al. 2003). We found the conditions for the high frequency of *C. roseus* cv. ‘Little Bright Eye’ regeneration from mature embryo, hypocotyl and cotyledon via somatic embryogenesis and organogenesis by varying the TDZ and BA:NAA ratios (Dhandapani et al. 2007). Those explants are more easily available and prepared than other explants used in the published reports. Moreover, they are actively growing tissues that can be effectively transformed by *Agrobacterium tumefaciens*. Transformation of petioles obtained from two-month old seedlings was not successful (unpublished results). The reason may be a high alkaloid content in green tissue which reduces the infection frequency. It applies to other explants of *C. roseus* such as green leaf segment, stem node and shoot tip. In contrast, mature embryo, hypocotyl and cotyledon showed a better infection rate (unpublished results). It would be necessary to screen a large number of genotypes with these three explants to identify regeneration competent genotype which can be utilized in transformation.

### AGROBACTERIUM-MEDIATED TRANSFORMATION: SUITABLE STRAINS, VIR GENES SUPPLEMENTATION AND CULTURE CONDITIONS

Gene transfer through *A. tumefaciens* continues to be a popular technique. *Agrobacterium*-mediated transformation is simple and cost-effective, with the less chance of transgene recombination after integration. Co-transformation offers a way to introduce multiple genes to engineer the metabolic pathway (Gelvin 2003). In addition, T-DNA activation tagging or T-DNA insertional mutagenesis has been extensively utilized in functional genomic analysis (van der Fits et al. 2001).

Several reports showing *Agrobacterium*-mediated transformation of *C. roseus* are made using hairy root and cell suspension culture (Table 2). *C. roseus* hairy root was reported to be regenerated into whole plant, though phenotypic alterations were noted in leaves and roots without data on regeneration frequency (Brillancceau et al. 1989). TIA content was examined in transgenic hairy root lines, but no
bisdindole alkaloids were found (Bhadra et al. 1993; O’Keeffe et al. 1997; Shanks et al. 1998; Rodriguez et al. 2003; Hughes et al. 2004). Recently a whole plant was regenerated from *C. roseus* hairy root line derived from hypicotyl infected by *A. rhizogenes* (Choi et al. 2004). Hairy roots were also generated by infecting intact seedlings with *A. tumefaciens* GV3101 containing rol ABC genes (Hong et al. 2006). To use hairy root system to regenerate into whole plant has certain disadvantages. Regeneration frequency is low, and phenotypes of plants generated from hairy roots are often abnormal (Choi et al. 2004). *A. tumefaciens*-mediated transformation of *C. roseus* cell suspension culture was reported to study the effects of overexpression of strictosidine synthase and tryptophan decarboxylase, but the transformation frequency was low (Canel et al. 1998). When *A. tumefaciens* strain LBA4404 was supplemented with a plasmid construct containing a constitutive virG mutant gene (virGN54D), transformation frequency of *C. roseus* suspension-cultured cells increased significantly, as judged by GUS assay and visual counting of blue spots (van der Fits et al. 2000). EHA101, EHA105 or GV3101 strains were successfully used for *C. roseus* transformation. Supplementation of extra vir genes through VirGN54D (independent of AS), or supervir vir genes derived from pTiBO542 may increase transformation efficiency (Hiei et al. 1994; van der Fits et al. 2000; Subha and Veluthambi 2003). Despite the success in transformation, whole plant has not been regenerated from cell suspension culture yet.

In plant transformation, culture conditions such as pre- and co-culture time are known to be important for increasing the transformation and regeneration efficiency. Pre-culturing explants activates virE gene which favors *Agrobacterium* to infect more effectively in tobacco (Sumikumar et al. 1999). In monocots such as rice, wheat and maize, pre-culturing embryogenic calli prior to infection was found to increase the transformation efficiency (Hiei et al. 1994; Ishida et al. 1996). Pre-culturing for 24 hours with 24-h co-cultivation at 25±1°C under light (10-h dark/14-h light photoperiod with cool-white fluorescent irradiance at an intensity of 60 mol m⁻² per second) gave higher stable transformation efficiency in *C. roseus* hypicotyl (unpublished results).

**ADDITION OF ANTIOXIDANT SUPPLEMENTS TO FACILITATE T-DNA DELIVERY AND SURVIVAL OF TRANSFORMED CELLS**

*Agrobacterium* infection at the wound sites of recalcitrant plant cells often induces necrotic hypersensitive response (HR) (Kuta and Tripathi 2005). HR response is due to the elicitation of cascades of reactions initiated by releasing oxygen free radicals such as superoxides and hydrogen peroxide. These molecules lead to a rapid, localized cell death around the infection site followed by the induction of pathogenesis-related proteins and the accumulation of antifungal microbial compounds, thereby reducing the efficiency of plant transformation and regeneration. To quench the *Agrobacterium*-induced oxidative burst, antioxidants such as L-cysteine, polyvinylpyrrolidone, dithiothreitol, and sodium thiosulphate are applied to the target plant tissues. Addition of thiol compounds during co-cultivation drastically increased the transformation efficiency of soybean cotyledonary-node explant by inhibiting the activities of wound response enzymes, such as peroxidases (PODs) and polyphenol oxidases (PPOs) (Ollhoft et al. 2001, 2003). Addition of L-cysteine to the co-cultivation media also increased the transformation efficiency of immature maize embryos (Frame et al. 2002). Necrotic spots and tissue browning during and after co-cultivation were observed in all the explants we infected viz., mature embryo, hypicotyl and cotyledon. As HR response may cause poor T-DNA delivery as well as poor survival and regeneration of transformed cells imbedded in necrotic tissue, addition of thiol compounds could alleviate the problems of HR response during *C. roseus* transformation.

**EFFECTIVE SELECTION MARKER FOR *C. ROSEUS* TRANSFORMATION: EFFICIENCY OF NEGATIVE SELECTION AND POSITIVE SELECTION**

Gene transfer to plants is a rare process. Usually *Agrobacterium* transfers its T-DNA to a single cell out of cell mass. Selection requires faster proliferation of transformed cells than that of non-transformed cells. This process is carried out by applying chemicals called selective agents. Antibiotics such as kanamycin, hygromycin, and geneticin or herbicides like basta are often applied to select transformed cells. These agents selectively kill untransformed cells, whereas resistance marker gene products will detoxify these agents, making transformed cells grow. Negative selection markers are certainly helpful in transformation of most plant species. However, presence of such negative selection markers may be undesirable by forming detoxified substances in some recalcitrant species. Though they are not toxic, the byproducts may not be metabolized by transformed cells, leading to interference with the morphogenetic potential of the recalcitrant species such as *C. roseus* (Flavell et al. 1992). Release of toxic metabolites from adjacent cells inhibited regeneration of transgenic sugar beet (Lindsey and Gallois 1990). We have consistently failed to regenerate stably transformed calli of *C. roseus* expressing GFP in presence of kanamycin in spite of following different selection schemes (unpublished results). Apart from regeneration point of view, negative selection is being questioned for controversial bacterial resistance genes in GMO plants which are concerned for environment limitations and scope. Dhandapani et al.
due to the transgene flow (Flavell et al. 1992; Nap et al. 1992). As an alternative, positive selection is effectively used to select transformed cells with high regeneration efficiency. Positive selection offers a metabolic advantage to the transformed cells thereby surpassing the non-transformants in growth. For example, β-glucuronidase hydrolyzes BA-3-glucuronide, a derivative of benzyladene (BA) and releases BA which leads to regeneration of transformed cells (Joersbo and Okkels 1996; Okkels et al. 1997). Plant regeneration of C. roseus via organogenesis can be standardized using BA as plant growth regulator. Such improved plant regeneration protocol would be utilized to generate transgenic C. roseus using β-glucuronidase and cytoxin glucuronidase as selection agent. Another example of a positive selection marker is a phosphomannose isomerase (PMI). When mannose is added to the media as sole carbon source, it is converted into mannose 6-phosphate by hexokinase. This leads to depletion of the inorganic phosphate reserve resulting in cell death. In transgenic cells, PMI from E. coli converts mannose 6-phosphate into fructose 6-phosphate which is an intermediate in glycolytic pathway. Mannose selection was found to be superior to kanamycin selection in many recalcitrant plant species such as sugar beet and tapioca (Joersbo et al. 1998; Negrotto et al. 2000; Wang et al. 2000; Zhang et al. 2000; Zhang and Puonti-Kaerlas 2000; Lucca et al. 2001). As compared to other negative selection agents, mannose is cheaper and more amenable to use in the field. Since C. roseus is a recalcitrant species, mannose selection may ease the regeneration following transformation. In addition to mannose, xylose was also used as a selection marker. Xylose isomerase converts xylose into xylulose which can be used as carbon source by plants (Haldrup et al. 1998). The plant enzyme tryptophan decarboxylase (TDC) converts tryptophan into tryptamine. Since tryptophan ana-mers (PMI). When mannose is added to the media as sole carbon source, it is converted into mannose 6-phosphate by hexokinase. This leads to depletion of the inorganic phosphate reserve resulting in cell death. In transgenic cells, PMI from E. coli converts mannose 6-phosphate into fructose 6-phosphate which is an intermediate in glycolytic pathway. Mannose selection was found to be superior to kanamycin selection in many recalcitrant plant species such as sugar beet and tapioca (Joersbo et al. 1998; Negrotto et al. 2000; Wang et al. 2000; Zhang et al. 2000; Zhang and Puonti-Kaerlas 2000; Lucca et al. 2001). As compared to other negative selection agents, mannose is cheaper and more amenable to use in the field. Since C. roseus is a recalcitrant species, mannose selection may ease the regeneration following transformation. In addition to mannose, xylose was also used as a selection marker. Xylose isomerase converts xylose into xylulose which can be used as carbon source by plants (Haldrup et al. 1998). The plant enzyme tryptophan decarboxylase (TDC) converts tryptophan into tryptamine. Since tryptophan ana-mers (PMI). When mannose is added to the media as sole carbon source, it is converted into mannose 6-phosphate by hexokinase. This leads to depletio
bacterium-mediated transformation of *C. roseus*. Although the high quality protocol is yet to be established, critical conditions to be examined are available based on the current success of several plant species that were previously presumed to be recalcitrant. Optimization of all the factors considered in this review will eventually lead to the development of *C. roseus* transgenic plant.

**ACKNOWLEDGEMENTS**

The authors wish to thank Dr. J. R. Liu (KRIIB, South Korea) for providing his published off-prints and his useful discussions. Dhandanapi is greatly thankful to the Konkuk University for the pre-doctoral research fellowship grant.

**REFERENCES**


