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Transgenic Plant Development in *Catharanthus roseus*: Limiting Factors and Scope

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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
Abbreviations: PMI, phosphomannose isomerase; **TDC**, tryptophan decarboxylase; **AS**, anthranilate synthase; **GFP**, green fluorescent protein; **2,4-D**, 2,4-dichloro phenoxyacetic acid; **BA**, 6-benzyl amino purine; **KT**, kinetin; **NAA**, α-naphthalene acetic acid; **TDZ**, thidiazuron; **MS**, Murashige and Skoog; **ZT**, zeatin

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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 pharmaceutically important compounds known as terpenoid indole alkaloids (TIA). Among more than 130 of them (Mishra and Kumar 2000; van der Heijden *et al*. 2004), vinblastine and vincristine are of the greatest clinical value for anti-cancer chemotheraphy (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-

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Table 1 Plant regeneration in *Catharanthus roseus*.

Explant type[Ref.]	Mode of regeneration	Medium	PGR
Anthers[1]	Somatic embryogenesis	MS, semi solid and suspension	2,4-D, KT
Immature zygotic embryo[2]	Somatic embryogenesis	MS, semi solid and suspension	2,4-D
Cut seedling[3]	Somatic embryogenesis	B5, semi-solid and suspension	2,4-D, KT, NAA
Mature embryo[4]	Somatic embryogenesis	MS, semi-solid	TDZ
Hypocotyl[5]	Somatic embryogenesis	MS, semi-solid	BA, NAA, GA_3
Leaf segment[6]	Organogenesis	MS, semi-solid	IAA, BA, ZT
Stem node[7]	Organogenesis	MS, semi-solid	BA, NAA
Shoot tip[8]	Organogenesis	MS, semi-solid	2,4-D, Kinetin
Hypocotyl[9]	Organogenesis	MS, semi-solid	BA, NAA, IBA
Petiole[10]	Organogenesis	MS, semi-solid	BA, NAA
(Mature embryo, cotyledon, hypocotyl, petiole, stem node, shoot tip)[11]	Organogenesis	MS, semi-solid	BA, NAA, TDZ

Ref. = References: 1, Kim *et al*. 1994; 2, Kim *et al*. 2004; 3, Piovan *et al*. 2000; 4, Dhandapani *et al*. 2007; 5, Junaid *et al*. 2006; 6, Constabel *et al*. 1982; 5, Junaid *et al*. ; 6, Constabel *et al*. 1982; 7, Mollers and Sarkar 1989; 8, Kaur *et al*. 1996; 9, Choi *et al*. 2003; 10, Lee *et al*. 2003; 11, Dhandapani *et al*. 2007.

pension culture. 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 laticifers 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 alkaloid 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 (Piovan *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 (Brillanceau *et al*. 1989). TIA content was examined in transgenic hairy root lines, but no

Table 2 Transformation of *C. roseus*.

Explant type[Ref.]	Mode of transformation	Medium	Selection marker	Light condition	Pre-culture period	Co-culture period	Reporter	Regeneration
Intact seedlings	*A. rhizogenes* (pRi15834)	B5, semi-solid	Hairy root	Light	No	No	No	Yes
Hypocotyl[2]	*A. rhizogenes* (pRI000)	MS, semi-solid	Hairy root	Light	No	No	No	Yes
Intact seedlings[3]	*A. tumefaciens* GV3101(pPZProlABC)	B5, semi-solid	Hairy root	Light	No	No	No	No
Nodular callus[4]	*A. tumefaciens* LBA1119(pMOG)	MS, semi-solid	hygromycin	Dark	No	72 hours	GUS	No
Nodular callus[5]	*A. tumefaciens* LBA4404(pMOG22BG:: *vir*GN54D)	B5, semi-solid	Hygromycin, kanamycin	Dark	No	72 hours	GUS	No
Mature embryo[6], hypocotyl[6], cotyledon[6]	*A. tumefaciens* EHA105(pBIN-mGFP5ER), GV3101(pBI121 & pBIHT)	MS, semi-solid	Hygromycin, kanamycin, G-415, mannose	Dark/light	24 hours	24 hours	GUS, GFP	No
Node with two auxillary buds[7]	Particle bombardment	MS, semi-solid	No	Light	No	No	GUS, GFP	Yes

Ref. = References: 1, Brillanceau *et al*. 1989; 2, Choi *et al*. 2004; 3, Hong *et al*. 2006; 4, Canel *et al*. 1998; 5, van der Fits *et al*. 2000; 6, unpublished results; 7, Zarate *et al*. 1999.

bisindole alkaloids were found (Bhadra *et al*. 1993; O'Keefe *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 hypocotyl 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 supervirulent *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 (Sunilkumar *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* hypocotyl (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 antimicrobial 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) (Olhoft *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, hypocotyl 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: EFFEICIENCY 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. These 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

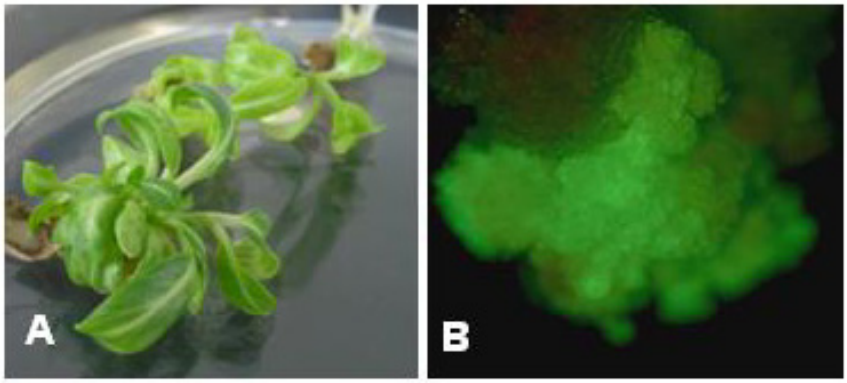

Fig. 1 ***In vitro*** **regeneration and transformation of** ***C. roseus*** (**A**) Plant regeneration from cotyledon of *C. roseus* cv. "Little Bright Eye" on MS medium supplemented with 1 mg/l NAA and 0.5 mg/l BA; (**B**) Stable GFP expression in 2 month-old *C. roseus* callus selected on kanamycin containing medium derived from hypocotyls infected with *Agrobacterium tumefaciens* strain EHA105 (pBIN-mGFP-5-ER).

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 benzyladenine (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 cytokinin glucuronide 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 analogues, 4- and 5-methyl tryptophan (4- or 5-mT) are toxic to plants and TDC is able to convert them into nontoxic 4- or 5-methyl tryptamine, it was successfully used as a selection marker in transgenic tobacco (Goddjin *et al*. 1993). A transcription factor (ORCA3), which regulates TIA biosynthetic pathway in *C. roseus*, was isolated from 4-mT resistant cell suspension lines by T-DNA activation approach (van der Fits and Memlink 2000; van der Fits *et al*. 2001). Endogenous activity of TDC can be overcome with an effective inhibitory concentration of 4- or 5-mT. A tryptophan feedback-resistant anthranilate synthase was also found to detoxify 5-mT and effectively used for rice and potato transformation with selection efficiency being similar to that of conventional selection markers (Yamada *et al*. 2004). Expression of a feedback-resistant anthranilate synthase of *Arabidopsis thaliana* in *C. roseus* hairy roots was reported (Hughes *et al*. 2004; Hong *et al*. 2006). So it is possible to use 5-mT as a selection agent and feedback-insensitive anthranilate synthase as a selection marker for *C. roseus* transformation.

EXPLOITING GFP IN *C. ROSEUS* TRANSFORMATION: EFFECTIVE REPORTER, VISUAL SELECTION MARKER AND DUAL SELECTION MARKER

As *Agrobacterium*-mediated transformation involves various steps, optimum conditions for each step have to be standardized in order to increase the transformation efficiency. Monitoring of transformation event in each step is also necessary for effective selection of transformed cells. Visual inspection of such processes is aided by reporter genes and is helpful in standardizing optimum conditions and screening of transformation. Various reporter genes are available for plant transformation. Conventional reporter genes such as GUS (Jefferson *et al*. 1987), LUC (Ow *et al*. 1986) and LacZ (Helmer *et al*. 1984) have been routinely used in transformation experiments. Such reporter systems require addition of substrate for their functioning. In addition, they are destructive as they lose a stably transformed callus which has a potential to regenerate into whole plant. On the other hand, green fluorescent protein (GFP) is a non-destructive, *in vivo* reporter system that is widely used in transformation (Stewart Jr. 2001). GFP expression does not require any exogenous substrate for its activity, and real time observation is possible from the very early stage of transformation event. Using GFP as reporter system, various steps involved in *Agrobacterium*-mediated transformation can be monitored to explore suitable *Agrobacterium* strains, acetosyringone concentration, pre- and co-culture period (Zhou *et al*. 2004; Tang and Newton 2005; Wang and Ge 2005). We were successful in transformation of *C. roseus* hypocotyl explant by monitoring GFP expression in calli (**Fig. 1B**). We also made numerous attempts to regenerate the callus that stably expressed GUS or GFP along with antibiotics kanamycin, G-418 and hygromycin but consistently failed (unpublished results). Visual selection marker alone was not enough to get transgenic *C. roseus* in our case. Dual selection combining a positive selection marker with GFP would be more effective to select and regenerate the transformed *C. roseus* callus. GFP is a useful tool to evaluate parameters determining the high efficiency of transformation in *C. roseus*. Positive selection may allow the transformed cells to grow, while GFP expression is monitored to determine the efficiency. Such a dual selection marker was successfully used in producing transgenic bentgrass and sorghum (Fu *et al*. 2005; Gao *et al*. 2005). The same strategy could be applied for generating transgenic *C. roseus*.

CONCLUDING REMARKS

Transgenic *C. roseus* plant development is still an ongoing process. Systematic selection of suitable explants with high regeneration capacity viz., mature embryo, hypocotyl and cotyledon, suitable *Agrobacterium* strains, optimum culture conditions viz., pre- and co-culture period, addition of *vir* gene inducer and antioxidant supplements, exploitation of GFP visual and positive selection marker should be taken into consideration for the experiments to standardize *Agro-*

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.

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REFERENCES

Bhadra R, Vani S, Shanks JV (1993) Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus*. *Biotechnology and Bioengineering* **41**, 581-592

Brillanceau MH, David C, Tempé J (1989) Genetic transformation of *Catharanthus roseus* G. Don by *Agrobacterium rhizogenes*. *Plant Cell Reports* **8**, 63-66

Canel C, Lopes-Cardoso I, Whitmer S, van der Fits L, Pasquali G, van der Heijden R, Hoge JHC, Verpoorte R (1998) Effects of over-expression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. *Planta* **205**, 414-419

Choi PS, Lee SY, Chung HJ, In DS, Choi, DW, Liu JR (2003) Assessment of competence for adventitious shoot formation in hypocotyl explant cultures of 22 cultivars of *Catharanthus roseus*. *Journal of Plant Biology* **46**, 90-94

Choi PS, Kim YD, Choi KM, Chung HJ, Choi DW, Liu JR (2004) Plant regeneration from hairy-root cultures transformed by *Agrobacterium rhizogenes* in *Catharanthus roseus*. *Plant Cell Reports* **22**, 828-831

Constable F, Gaudet-Laprairie P, Kurz KGW, Kutney JP (1982) Alkaloid production in *Catharanthus roseus* (L.) G. Don IV: variation in alkaloid spectra of cell lines derived from one single leaf. *Plant Cell Reports* **1**, 139-142

Dhandapani M, Kim DH, Hong SB (2007) Efficient plant regeneration via somatic embryogenesis and organogenesis from the explants of *Catharanthus roseus*. *In Vitro Cellular and Developmental Biology – Plant*, in press

Flavell RB, Dart E, Fuchs RL, Frakey RT (1992) Selectable marker genes: safe for plants? *Biotechnology* **10**, 141-144

Frame B, Shou H, Chikwamba R, Zhang Z, Xiang C, Fonger T, Pegg SE, Li B, Nettleton D, Pei D, Wang K (2002) *Agrobacterium*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiology* **129**, 13-22

Fu D, Xiao Y, Muthukrishnan S, Liang GH (2005) *In vivo* performance of a dual genetic marker, *manA-gfp*, in transgenic bentgrass. *Genome* **48**, 722-730

Gao Z, Xie X, Ling Y, Muthukrishnan S, Liang GH (2005) *Agrobacterium tumefaciens*-mediated transformation using a mannose selection system. *Plant Biotechnology Journal* **3**, 591

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockying" tool. *Microbiology and Molecular Biology Review* **67**, 16-37

Goddjin OJM, van der Duyun PMS, Schilperoot RA, Hoge JHC (1993) A chimaeric tryptophan decarboxylase gene as novel selectable marker in plant cells. *Plant Molecular Biology* **22**, 907-912

Haldrup A, Petersen SG, Okkels FT (1998) Positive selection: a plant selection principle based on xylose isomerase, an enzyme used in the food industry. *Plant Cell Reports* **18**, 76-81

Helmer G, Casdaban M, Bevan M, Kayers L, Chilton MD (1984) A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* β-galacotosidase in sunflower and tobacco cells. *Bio-Technology* (*NY*) **2**, 520-527

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant Journal* **6**, 271-282

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Molecular Biology* **35**, 205-218

Hong SB, Peebles CAM, Shanks JV, San KY, Gibson SI (2006a) Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. *Journal of Biotechnology* **122**, 28-38

Hong SB, Peebles CAM, Shanks JV, San KY, Gibson SI (2006b) Terpenoid indole alkaloid production by *Catharanthus roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring *rol ABC* genes. *Biotechnology and Bioengineering* **93**, 385-290

Hughes EE, Hong SB, Gibson SI, Shanks JV, San KY (2004) Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides tight regulation of terpenoid indole alkaloid levels. *Biotechnology and Bioengineering* **86**, 718-727

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **14**, 745-750

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901-3907

Joersbo M, Okkels FT (1996) A novel principle for selection of transgenic plant cells: Positive selection. *Plant Cell Reports* **16**, 219-221

Joersbo M, Danaldson I, Kreiberg J, Petersen SG, Brunstedt J, Okkels FT (1998) Analysis of mannose selection used for transformation of sugar beet. *Molecular Breeding* **4**, 111-117

Junaid A, Mujib A, Bhat MA, Sharma MP (2006) Somatic embryo proliferation, maturation and germination in *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* **84**, 325-332

Kaur K, Lodha P, Kant U (1996) *In vitro* regeneration of mosaic virus free *Catharanthus roseus* (L.) G. Don. plants through callus culture. *Journal of Phytology Research* **9**, 25-28

Kim SW, Jung KH, Song NH, Kwak SS, Liu JR (1994) High frequency plant regeneration from anther-derived cell suspension cultures via somatic embryogenesis in *Catharanthus roseus*. *Plant Cell Reports* **13**, 319-322

Kim SW, In DS, Choi PS, Liu JR (2004) Plant regeneration from immature zygotic embryo-derived embryogenic calluses and cell suspension cultures of *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* **76**, 131-135

Kulkarni RN, Baskaran K, Chandrashekara RS, Kumar S (1999) Inheritance of morphological traits of periwinkle mutants with modified contents and yields of leaf and root alkaloids. *Plant Breeding* **118**, 71-74

Kulkarni RN, Sreevalli Y, Baskaran K, Kumar S (2001) The mechanism and inheritance of intraflower self-pollination in self-pollinating variants of periwinkle. *Plant Breeding* **120**, 247-250

Kumar GR, Kumar R (2002) Bridging traditional medicines with modern biotechnology. *Chemtracts* **15**, 693-705

Kuta DD, Tripathi L (2005) *Agrobacteirum*-induced hypersensitive necrotic reaction in plant cells: a resistance response against *Agrobacterium*-mediated DNA transfer. *African Journal of Biotechnology* **4**, 752-757

Kutchan TM (2005) A role for intra- and intercellular translocation in natural product biosynthesis. *Current Opinion in Plant Biology* **8**, 292-300

Lee SY, Choi PS, Chung HJ, In DS, Choi DW, Liu JR (2003) Comparison of adventitious shoot formation in petiole explant cultures of 20 cultivars of *Catharanthus roseus*. *Journal of Plant Biotechnology* **5**, 59-61

Li H, Murch SJ, Saxena PK (2000) Thidiazuron-induced *de novo* shoot organogenesis on seedlings, etiolated hypocotyls and stem segments of Huangqin. *Plant Cell, Tissue and Organ Culture* **62**, 169-173

Lindsey K, Gallois P (1990) Transformation of sugar beet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. *Journal of Experimental Botany* **41**, 529-536

Liu CZ, Murch SJ, EL-Demerdash M, Saxena PK (2003) Regeneration of the Egyptian medicinal plant *Artemisia judaica* L. *Plant Cell Reports* **21**, 525-530

Lucca P, Ye XD, Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Molecular Breeding* **7**, 43-49

Mahroug S, Courdavault V, Thiersault M, St-Pierre B, Burlat V (2006) Epidermis is a pivotal site of at least four secondary metabolic pathways in *Catharanthus roseus* aerial organs. *Planta* **223**, 1191-1200

Misra NR, Luthra R, Kumar S (1996) Enzymology of indole alkaloid biosynthesis in *Catharanthus roseus*. *Indian Journal of Biochemistry and Biophysics* **33**, 261-273

Mishra P, Kumar S (2000) Emergence of perwinkle *Catharanthus roseus* as a model system for molecular biology of alkaloids: phytochemistry, pharmacology, plant biology and *in vivo* and *in vitro* cultivation. *Journal of Medicinal and Aromatic Plant Sciences* **22**, 306-337

Mithila J, Hall JC, Victor JMR, Saxena PK (2003) Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). *Plant Cell Reports* **21**, 408-414

Mollers C, Sarkar S (1989) Regeneration of healthy plants from *Catharanthus roseus* infected with mycoplasma-like organisms through callus culture. *Plant Science* **60**, 83-89

Nap JP, Bijvoet J, Stiekema WJ (1992) Biosafety of kanamycin resistant transgenic plants. *Transgenic Research* **1**, 239-249

Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Reports* **19**, 798-803

O'Keefe BR, Mahady GB, Gills JJ, Beecher CWW (1997) Stable vindoline production in transformed cell cultures of *Catharanthus roseus*. *Journal of Natural Products* **60**, 261-264

Okkels FT, Ward JL, Joersbo M (1997) Synthesis of cytokinin glucuronides for the selection of transgenic plants. *Phytochemistry* **46**, 801-804

Olhoft PM, Somers DA (2001) L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Reports* **20**, 706-711

Olhoft PM, Lin K, Galbraith J, Nielsen NC, Somers DA (2001) The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. *Plant Cell Reports* **20**, 731-737

Ow DW, Wood KV, Deluca M, Dewet JR, Helinski DR, Howell SH (1986) Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859

Pasquali G, Porto DD, Fett-Neto AG (2006) Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma. *Journal of Bioscience and Bioengineering* **101**, 287-296

Piovan A, Filippini R, Caniato R, Dalla Vecchia F, Innocenti G, Cappelletti EM, Puricelli L (2000) Somatic embryogenesis and indole alkaloid production in *Catharanthus roseus*. *Plant Biosystems* **134**, 179-184

Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, van Montagu MCE, Inzé D, Oksman-Caldentey KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proceedings of the National Academy of Sciences USA* **103**, 56154-5619

Rodriguez S, Compagnon V, Crouch NP, St-Pierre B, de Luca V (2003) Jasmonate induced epoxidation of tabersonine by a cytochrome P-450 in hairy root cultures of *Catharanthus roseus*. *Phytochemistry* **64**, 401-409

Shanks JV, Bhadra R, Morgan J, Rijhwani S, Vani S (1998) Quantification of metabolites in the indole alkaloid pathways of *Catharanthus roseus*: Implications for metabolic engineering. *Biotechnology and Bioengineering* **58**, 333-338

St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. *Plant Cell* **11**, 887-900

Stewart Jr. CN (2001) The utility of green fluorescent protein in transgenic plants. *Plant Cell Reports* **20**, 376-382

Subha SJ, Veluthambi K (2003) A cointegrate Ti plasmid vector for *Agrobacterium tumefaciens*-mediated transformation of indica rice cv. Pusa Basmati1. *Journal of Plant Biochemistry and Biotechnology* **12**, 1-9

Sunilkumar G, Vijayachandra K, Veluthambi K (1999) Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. *Plant Science* **141**, 51-58

Tang W, Newton RJ (2005) Transgenic Christmas trees regenerated from *Agrobacterium tumefaciens* mediated transformation of zygotic embryos using the green fluorescence protein as a reporter. *Molecular Breeding* **16**, 235-246

Teixeira da Silva JA (2006) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vols I-IV), Global Science Books, London, 2511 pp

Tyler VE (1988) Medicinal plant research. *Planta Medica* **54**, 95-100

van der Fits L, Deakin EA, Hogel JHC, Memlink J (2000) The ternary transformation system: constitutive *virG* on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **43**, 495-502

van der Fits L, Memelink J (2000) ORCA3, a jasmoate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* **289**, 295-297

van der Fits L, Hilliou F, Memelink J (2001) T-DNA activation tagging as a tool to isolate regulators of a metabolic pathway from a genetically non-tractable plant species. *Transgenic Research* **10**, 513-521

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. *Current Medicinal Chemistry* **11**, 607-628

Vazquez-Flota F, de Luca V, Carrillo-Pech M, Canto-Flick A, de Lourdes Miranda-Ham M (2002) Vindoline biosynthesis is transcriptionally blocked in *Catharanthus roseus* cell suspension cultures. *Molecular Biotechnology* **22**, 1-8

Wang Z-Y, Ge Y (2005) *Agrobacterium*-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). *Journal of Plant Physiology* **162**, 103-113

Wang AS, Evans RA, Altendorf PR, Hanten JA, Doyle MC, Rosichan JL (2000) A mannose selection system for production of fertile transgenic maize plants from protoplasts. *Plant Cell Reports* **19**, 654-660

Whitmer S, Canel C, van der Heijden R, Verpoorte R (2003) Long-term instability of alkaloid production by stably transformed cell lines of *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* **74**, 73-80

Yamada T, Tozawa Y, Hasegawa H, Terakawa T, Ohkawa Y, Wakasa K (2004) Use of a feedback-insensitive α subunit of anthranilate synthase selectable marker for transformation of rice and potato. *Molecular Breeding* **14**, 363-373

Zárate R, Memelink J, van der Heijden R, Verpoorte R (1999) Genetic transformation via particle bombardment of *Catharanthus roseus* plants through adventitious organogenesis of buds. *Biotechnology Letters* **21**, 997-1002

Zhang P, Potrykus I, Puonti-Kaerlas J (2000) Efficient production of transgenic cassava using negative and positive selection. *Transgenic Research* **9**, 405-415

Zhang P, Puonti-Kaerlas J (2000) PEG-mediated cassava transformation using positive and negative selection. *Plant Cell Reports* **19**, 1041-1048

Zhou X, Chandrasekharan MB, Hall TC (2004) High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. *New Phytology* **162**, 813-822