COMMUNICATION TO THE EDITOR

Terpenoid Indole Alkaloid Production by *Catharanthus roseus* Hairy Roots Induced by *Agrobacterium tumefaciens* Harboring *rol ABC* Genes

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Abstract: We have established *Catharanthus roseus* hairy root cultures transgenic for the *rol ABC* genes from T_L-DNA of the agropine-type *Agrobacterium rhizogenes* strain A4. The *rol ABC* hairy root lines exhibit a wild-type hairy root syndrome in terms of growth and morphology on solid medium. However, they differ from wild-type hairy root lines in that they more frequently have excellent adaptability to liquid medium and do not appear to form calli during cultivation. Moreover, they do not produce detectable levels of mannopine and agropine which, in contrast, are often synthesized abundantly in wild-type hairy root lines. The absence of these opines does not appear to cause the *rol ABC* lines to have higher levels of terpenoid indole alkaloids than wild-type hairy root lines. Unlike wild-type lines, *rol ABC* lines produce very similar levels of total alkaloids despite wide variations in individual alkaloid contents. This work demonstrates that the three genes *rol ABC* are sufficient to induce high-quality hairy roots in *Catharanthus roseus*.

Keywords: *Catharanthus roseus*; hairy roots; rol ABC genes; terpenoid indole alkaloids

INTRODUCTION

Infection of stems or leaves of plants with *Agrobacterium rhizogenes* often results in the appearance of genetically transformed roots, known as hairy roots, at the wound site. This differentiated hairy root tissue provides an ideal culture system from the industrial point of view because it grows rapidly, requires only a basic medium without phytohormones for growth, and remains genetically stable and reproducible in metabolite production over a long period of time. Moreover, hairy root cultures are not affected by seasonal variations or the limited resources sometimes available to wild plants. Accordingly, they have been investigated for their potential for in vitro synthesis of many secondary metabolites of commercial interest (Hamill and Lidgett, 1997). For example, *Catharanthus roseus* hairy root cultures have been extensively studied by several groups in attempts to improve the yields of clinically valuable terpenoid indole alkaloids (Bhadra et al., 1993; Hughes et al., 2004; O'Keefe et al., 1997; Parr et al., 1988; Rodríguez et al., 2003; Shanks et al., 1998).

Hairy roots often produce low molecular weight compounds termed opines, which are not present in normal plant tissues. Based upon the opines synthesized by the resulting hairy roots, *A. rhizogenes* have been classified into three types mannopine, agropine, and cucumopine (Dessaux et al., 1992). Agropine-type strains (15834, A4, 1855, HR1) are most effective in transforming *C. roseus* (Batra et al., 2004). Agropine-type Ri (root-inducing) plasmids are almost identical to one another and carry two separate T-DNA segments, T_L and T_R (Huffman et al., 1984). The T_R region contains loci encoding biosynthesis of auxin, mannityl opines, and agrocinopine A/B (Cardarelli et al., 1985). The mannityl opine family, in which mannose and glutamine or glutamic acid are combined, includes mannopine, mannopinic acid, agropine, and agropinic acid (Dessaux et al., 1992). Agrocinopine A consists of sucrose and L-arabinose linked by a phosphodiester bond, whereas agrocinopine B

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lacks the glucose moiety present in agrocinopine A (Ryder et al., 1984). Mannityl opines were shown to accumulate to very high levels (about 10% dry weight) in transgenic tobacco plants and in hairy roots (Savka and Farrand, 1992; Saito et al., 1992). They cannot be readily metabolized by the plant and are released to the extracellular medium. Thus, opine biosynthetic pathways in the T_R region redirect primary metabolism to opine production and thus may potentially reduce the carbon and nitrogen sources available for the production of secondary metabolites.

The T_L region contains 18 open reading frames including several loci called *rol* (root loci), which are responsible for hairy root induction (Slightom et al., 1986). Among them, the products encoded by the three loci *rol A*, *B*, and *C* were found to have a synergistic effect on root induction and induce increased sensitivity to auxin. These three loci were shown to be sufficient to induce hairy roots in tobacco, kalanchoe, and *Atropa belladonna* plants (Bonhomme et al., 2000; Spena et al., 1987).

Thus far all of the wild-type *C. roseus* hairy root lines cited in the literature have been obtained by infection with agropine-type *A. rhizogenes* strains harboring pRi plasmids. We now report the generation of high-quality *C. roseus* hairy roots named *rol*ABC hairy roots by infection with *A. tumefaciens* carrying the pPZPROL plasmid construct. The hairy root lines generated by the new method exhibit excellent liquid adaptability, and their growth rates and alkaloid contents are comparable to those of wild-type hairy root lines produced by the conventional method.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Constructs

An *E. coli* strain carrying pPCV002-ABC was kindly provided by Dr. Thomas Schmülling from the University of Tübingen, Germany. Plasmid pPCV002-ABC (Spena et al., 1987) contains the rol ABC loci of pRiA4. A 4.4-kb EcoRI fragment carrying the rol ABC genes was cloned into the pPZP200 *Agrobacterium* binary vector (Hajdukiwicz et al., 1994). This construct was named pPZPROL (Fig. 1).

Transformation and Culture Conditions

The construct pPZPROL was transformed into disarmed *Agrobacterium tumefaciens* strain GV3101 by electroporation. The transformants harboring pPZPROL were selected on LB plates containing spectinomycin (100 μg/mL) and then analyzed for the presence of the pPZPROL plasmid by digestion with restriction enzymes. *A. tumefaciens* GV3101 (pPZPROL) was grown to a cell density of 0.6–0.8 at A_{600} in LB-spectinomycin. The cells were harvested and resuspended in one-half initial volume of induction medium (½ MS medium, 3% sucrose, 50 μ*M* acetosyringone). The cultures were further grown at 28°C for approximately 3 h and were used to inoculate the stem tip and two leaf veins per 3-week-old plantlet of *C. roseus* (cv. Little Bright Eye). Infected

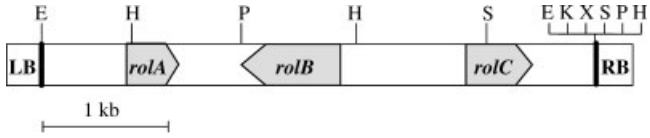

Figure 1. Map of the pPZPROL binary vector (11,110 bp). The vector backbone is pPZP200 that confers spectinomycin resistance and is able to replicate in both *Agrobacterium* and *E. coli* (Hajdukiwicz et al., 1994). The relative positions of *rol A*, *rol B*, and *rol C* open reading frames in a 4369-bp *Eco*RI fragment are shown according to Spena et al. (1987). The plasmid has the T-DNA left (LB) and right (RB) border sequences and two unique restriction enzyme sites, *Kpn*I and *Xba*I. The approximate location of restriction sites is indicated above the map which is drawn to scale except for the RB and LB. Abbreviations: E, *Eco*RI; K, *Kpn*I; X, *Xba*I; S, *Sal*I; P, *Pst*I; H, *Hind*III.

plantlets were placed overnight in a dark room and then grown under a 16 h light and 8 h dark photoperiod at 25°C. One to two cm-long roots that appeared on the infection sites were excised and transferred to solid medium (3% sucrose, 6% phytoagar, 0.25 g/L cefotaxime, 0.25 g/L carbenicillin, ½ Gamborg's B5 salts, and full strength Gamborg's vitamins, pH 5.7). Axenic hairy roots were adapted to liquid media and growth curves were determined as previously described (Bhadra et al., 1993).

Opine Analysis

Extraction and detection of opines from hairy roots was performed as previously described (Savka and Farrand, 1992). High voltage paper electrophoresis was done at 2,500 V for 30 min using a Savant LT-48 electrophoresis apparatus with EC-128 coolant (Savant).

PCR Analysis

Total genomic DNA was isolated from hairy root tissues using a DNA isolation kit (Sigma). The agropine synthase gene (*ags*) in the T_R-DNA region of *A. rhizogenes* pRiA4 was amplified and detected as previously described (Batra et al., 2004).

Alkaloid Analysis

Cultures in the mid-exponential phase (dry weight between 0.1 and 0.2 g) and in the late-exponential phase (dry weight between 0.2 and 0.3 g) were blotted onto paper towels and then immediately frozen at −80°C and lyophilized overnight. Lyophilized roots were pulverized and methanol extracts of 50 mg aliquots of tissue were analyzed for alkaloid quantification by HPLC as previously reported (Hughes et al., 2004).

RESULTS AND DISCUSSION

Establishment of Axenic *rol ABC* and Wild-Type Hairy Root Lines

Wild-type hairy root cultures were established by transformation with *Agrobacterium rhizogenes* 15834 strain as

previously described (Bhadra et al., 1993). Plasmid pPCV002-ABC contains a kanamycin plant selection marker and *rol ABC* genes (Spena et al., 1987). To eliminate the antibiotic selection marker and to explore the possibility of utilizing the *rol ABC* genes as a direct selection marker that confers a hairy root phenotype, a DNA fragment encompassing the *rol ABC* loci was inserted into the pPZP200 binary vector to yield pPZPROL (Fig. 1). Approximately 50 *C. roseus* plantlets were infected with *A. tumefaciens* strain GV3101 (pPZPROL). A total of 11 hairy roots emerged from stems or leaves and were transferred to phytohormone-free solid media containing cefotaxime and carbenicillin. This rate of transformation is much lower as compared with a 60% transformation efficiency with *A. rhizogenes* 15834. Control inoculations with *A. tumefaciens* GV3101 harboring pPZP200 were also made. As predicted, no roots formed on the inoculation sites of the control plants. Six axenic hairy root lines were established after four passages of subculturing on solid media and then transferred to liquid media without antibiotics.

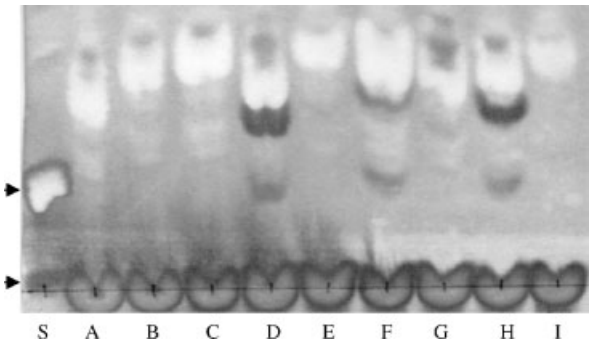

Figure 2. High voltage paper electrophoresis (HVPE) of extracts from wild-type and *rol ABC* hairy root lines. Extracts were prepared by crushing 0.2 g fresh weight of tissues in 120 µl of 70% ethanol followed by centrifugation. Twenty microliters of supernatants were spotted onto Whatman 3 mm paper, electrophoresed, and stained with silver nitrate. Agropine is a cyclized derivative of mannopine (Hong et al., 1997). Lanes: S, mannopine standard; A, B, and C, three independent *rol ABC* lines; D, E, F, G, H, and I, six independent wild-type 15834 hairy root lines. Neutral sugar compounds remain at the origin. Abbreviations: AGR, agropine; MOP, mannopine; O, origin of separation.

Growth Characteristics of the *rol ABC* Hairy Root Lines

All of the six *rol ABC* root cultures displayed a typical hairy root phenotype on solid medium, as characterized by thin, straight, highly branched adventitious roots. All *rol ABC* lines were immediately adapted to liquid media without fragility when transferred to agitated liquid media. This feature contrasts with wild-type 15834 hairy root lines whose fragility is a major problem. Typically, less than 7% of 15834 hairy roots emerged from the infected seedlings of *C. roseus* cv. Little Bright Eye are able to grow in liquid cultures and these lines often grow as a mixture of dissociated cells, cell aggregates and roots even when they are adapted to liquid media (Bhadra et al., 1993; our observation). Analysis of large numbers of *C. roseus* hairy roots induced by the agropine-type *A. rhizogenes* strain A4 suggested that the T_R-DNA may be responsible for these callus-forming and slow-growing phenotypes (Batra et al., 2004). Consistent with this assumption, no such phenotypes were noted in any of the *rol ABC* hairy roots. Moreover, no calli were produced at the cut ends of the roots in both solid and liquid media, as is often observed in wild-type 15834 hairy roots. These phenotypes have been stably maintained through successive subcultures over a period of 2 years.

Opine Synthesis

Wild-type and *rol ABC* hairy root lines were tested for the presence of opines by HVPE analysis (Fig. 2). No opines were detected in any of the five *rol ABC* hairy root lines tested. In contrast, some of the wild-type 15834 hairy roots produced opines although others did not. This variability in opine production between wild-type hairy roots is most likely due to the independent integration of the T_R- and T_L-DNA segments into the *C. roseus* genome. The absence of T_R-DNA in the *C. roseus* genome would result in no opine production in hairy roots. This interpretation is supported by PCR analysis (Fig. 3), which shows that an agropine synthase gene from the T_R-DNA is absent in *rol ABC* hairy roots but is present in two 15834 hairy root lines producing agropine (Table I). Previous reports (Batra et al., 2004; Brillanceau et al., 1989) showed that the T_L- and T_R-DNAs are integrated into the *C. roseus* genome either separately or continuously. This random nature of T-DNA integration would further increase clonal variation among hairy root lines. All 15834 hairy root lines capable of synthesizing opines contained more agropine than mannopine. Agropine was also detected in the culture medium of the 15834 hairy root lines producing opines (data not shown). The same observations were previously made in other transgenic plants (Saito et al.,

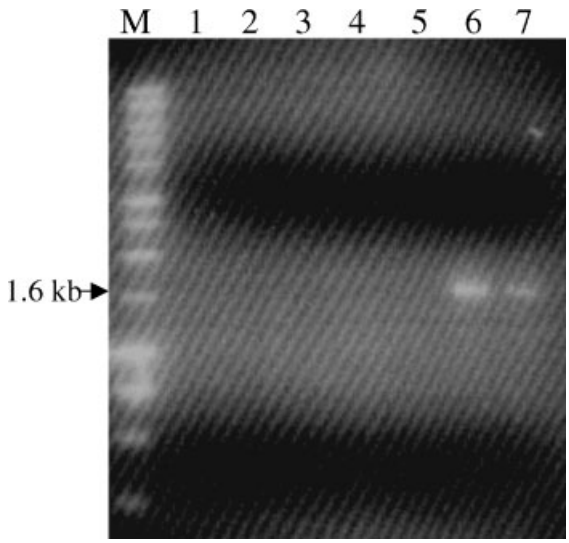

Figure 3. PCR analysis showing the presence or absence of the *ags* gene in hairy root lines. M, 1 kb DNA ladder;1, *rol* ABC1; 2, *rol* ABC4; 3, *rol* ABC6; 4,WT- RBL; 5, WT-297; 6, WT-AB1; 7, WT-256.

Table I. Comparison of total alkaloid and opine contents of *rol ABC* and wild-type 15834 hairy root lines.

Root lines	Total[a] alkaloids (mg/g DW)	Opines[b]	Day[c] of culture
rol ABC1	6.4	−	17
rol ABC4	6.0	−	17
rol ABC5	5.9	−	20
rol ABC6	5.9	−	20
rol ABC11	6.0	−	26
WT-256	6.4	+	28
WT-297	6.6	−	25
WT-RBL	4.6	−	18
WT-AB1	4.3	+	18
WT-AB2	6.0	−	30

[a]Total alkaloid contents represent the sum of tabersonine, lochnericine, hörhammericine, serpentine, ajmalicine, and catharanthine.
[b]The presence (+) and absence (−) of mannopine and agropine.
[c]Day of harvesting the mid-exponential phase cultures for the analysis of total alkaloids and opines.

1992; Savka and Farrand, 1992). Interestingly, the wild-type 15834 hairy root line WT-AB1 (lane D in Fig. 2) grew as fast as the *rol ABC* lines and faster than any other 15834 hairy root lines despite the production of very high levels of opines, suggesting that opine production may not severely affect growth rate under the culture conditions tested. However, this opine-producing line displayed brittleness and produced calli or callus-like material. Unlike the random phenotypes of wild-type hairy root lines, the minimal construct pPZPROL consistently yielded hairy root lines that do not produce opines and adapt very well to liquid medium.

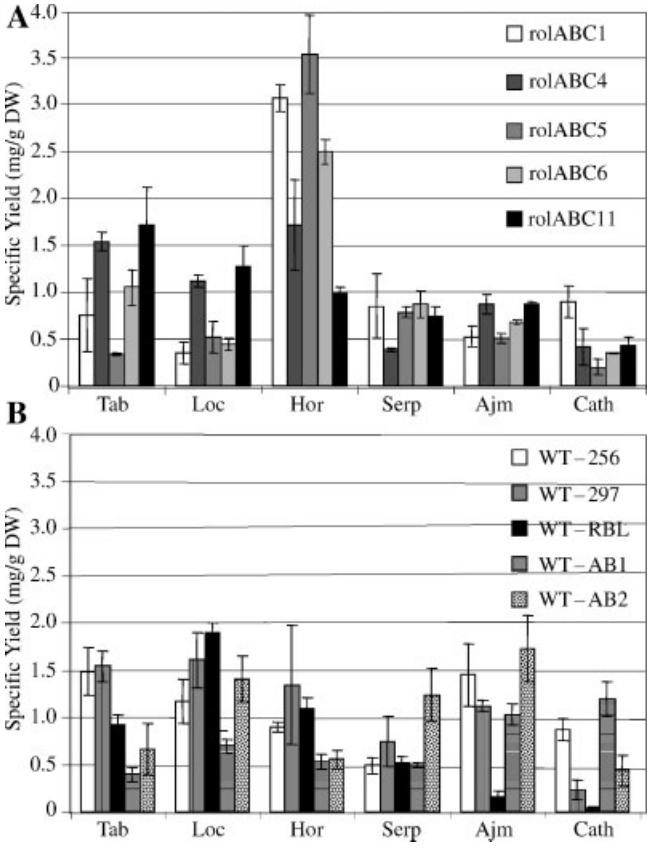

Figure 4. (A) Levels of terpenoid indole alkaloids of the five *rol ABC* hairy root lines in the mid-exponential phase. (B) Levels of terpenoid indole alkaloids of five wild-type 15834 hairy root lines in the mid-exponential phase. Abbreviations: Tab, tabersonine; Loc, lochnericine; Hor, hörhammericine; Serp, serpentine; Ajm, ajmalicine; Cath, catharanthine. Data represents mean and standard deviation from three cultures.

Alkaloid Profiles

In order to determine if there are any differences in alkaloid accumulation between the *rol ABC* and wild-type hairy root lines, six terpenoid indole alkaloids were quantified during the mid-exponential (Fig. 4) and late-exponential stages (data not shown). Both groups of lines exhibit significant variability in individual alkaloid contents. However, the only statistically significant difference in alkaloid contents between *rol ABC* and wild-type lines was detected in hörhammericine levels. In cultures harvested during the mid-exponential phase, *rol ABC* lines differed in hörhammericine contents with a *P*-value of 0.012 from wild-type lines. A similar observation was made in hörhammericine contents with a *P*-value of 0.016 during the late-exponential phase. No other statistically significant differences were noted in specific yields of the other five alkaloids between the mid- and late-exponential phase cultures, except for the *rol ABC*11 line. All of the lines except the *rol ABC11* line exhibited increases in total yields of all alkaloids as hairy root biomass transitioned from the mid-exponential phase (average biomass 0.15 g DW/50 mL) to the late-exponential phase (average biomass 0.25 g DW/50 mL). This linear relationship between total alkaloid levels and growth stage suggests that the total flux to alkaloids is constant during this period, confirming the previous observation that hairy root biomass and total alkaloid yields are linear with time (Morgan and Shanks, 2002). However, the *rol ABC*11 line was exceptional as the tabersonine and lochnericine specific yields of the late-exponential phase cultures were about one half of those of the mid-exponential phase cultures. It is notable that individual levels of tabersonine or hörhammericine significantly ($P < 0.05$) differ from each other in mid-exponential phase *rol*ABC1, 4, 5, and 6 lines despite the similar total contents of tabersonine plus hörhammericine (Fig. 4). Therefore, when tabersonine versus hörhammericine levels were plotted, accumulation levels of tabersonine and hörhammericine were found to be inversely correlated in *rol ABC* and WT-297 lines, whereas no such correlation exists in other wild-type lines. This correlation in *rol ABC* lines reflects very well the conversion of tabersonine into hörhammericine in the indole alkaloid pathways of *C. roseus* (Shanks et al., 1998). Notably, total alkaloid contents of *rol ABC* lines are remarkably similar (∼6.0 mg/g DW) in contrast with those of wild-type hairy root lines which were relatively variable (Table I). However, the average total alkaloid levels of *rol ABC* lines are not statistically higher than those of some of the wild-type lines, suggesting that total alkaloid contents are not necessarily significantly different in

rol ABC than in wild-type lines. It was reported that the presence of mannopine reduced the contents of tropane alkaloids in hairy root cultures (Ford et al., 2000; Sauerwein and Wink, 1993). In contrast, no correlation was observed between the presence of opines and terpenoid alkaloid contents. The fastest growth was not always associated with the highest alkaloid accumulation. Thus, alkaloid productivity could not be determined solely based on the phenotypes of hairy root lines.

In conclusion, *rol ABC* genes are able to induce high-quality hairy roots with improved liquid adaptability and can be used as a direct selection marker for transformation of *C. roseus*. The use of the pPZPROL vector will be useful not only to generate hairy roots that are metabolically more similar to untransformed roots by eliminating the other ancillary T-DNA genes such as biosynthetic genes for opines and auxin, but also to enhance the capacity of introducing foreign genes into hairy roots for metabolic engineering of secondary metabolites. The system allows for marker-free transgenic hairy roots that can be phenotypically selected in the absence of exogenous phytohormones.

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References

Batra J, Dutta A, Singh D, Kumar S, Sen J. 2004. Growth and terpenoid alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep 23:148–154.

Bhadra R, Vani S, Shanks JV. 1993. Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus*. Biotechnol Bioeng 41:581–592.

Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux M, Jacquin-Dubreuil A. 2000. Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes*15834 and *Agrobacterium tumefaciens* containing*rol A*, *B*, *C* genes only. J Biotechnol 81:151–158.

Brillanceau MH, David C, Tempé J. 1989. Genetic transformation of *Catharanthus roseus* G. Don by *Agrobacterium rhizogenes*. Plant Cell Rep 8:63–66.

Cardarelli M, Spano L, De Paolis A, Mauro ML, Vitali G, Constantino P. 1985. Identification of the genetic locus responsible for non-polar root induction by*Agrobacterium rhizogenes 1855*. Plant Mol Biol 5:385–391.

Dessaux Y, Petit A, Tempé J. 1992. Opines in *Agrobacterium* biology. In: Verma DPS, editor. Molecular signals in plant-microbe communications. Boca Raton, FL: CRC Press. pp 109–136.

Ford YY, Ratcliffe RG, Robins RJ. 2000. An*in vivo* ^{15}N NMR study of agropine synthesis in transformed root cultures of *Nicotiana tabacum*. Physiol Plant 109:123–128.

Hajdukiewicz P, Svab Z, Maliga P. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25:989–994.

Hamill JD, Lidgett AJ. 1997. Hairy root cultures–opportunities and key protocols for studies in metabolic engineering. In: Dora P, editor. Hairy root cultures and applications. Netherlands: Harwood Academic Publishers.

Hong SB, Hwang I, Dessaux Y, Guyon P, Kim KS, Farrand SK. 1997. A *T*-DNA gene responsible for agropine biosynthesis by transformed plants is structurally, functionally, and evolutionally related to a Ti plasmid gene required for catabolism of agropine by *Agrobacterium*. J Bacteriol 179:4831–4840.

Huffman GA, White FF, Gordon MP, Nester EW. 1984. Hairy-root inducing plasmid: Physical map and homology to tumor-inducing plasmids. J Bacteriol 157:269–276.

Hughes EE, Hong SB, Gibson SI, Shanks JV, San KY. 2004. Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. Biotechnol Bioeng 86:718–727.

Morgan JA, Shanks JV. 2002. Quantification of metabolic flux in plant secondary metabolism by a biogenetic organizational approach. Metabolic Eng 4:257–262.

O'Keefe BR, Mahady GB, Gills JJ, Beecher CWW. 1997. Stable vindoline production in transformed cell cultures of *Catharanthus roseus*. J Nat Prod 60:261–264.

Parr AJ, Peerless ACJ, Hamill JD, Walton NJ, Robins RJ, Rhodes MJC. 1988. Alkaloid production by transformed root cultures of *Catharanthus roseus*. Plant Cell Rep 7:309–312.

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*. Phytochem 64:401–409.

Ryder MH, Tate ME, Jones GP. 1984. Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose. J Biol Chem 259:9704–9710.

Saito K, Yamazaki M, Murakoshi I. 1992. Transgenic medicinal plants: *Agrobacterium*-mediated foreign gene transfer and production of secondary metabolites. J Nat Prod 55:149–162.

Sauerwein M, Wink M. 1993. On the role of opines in plants transformed with *Agrobacterium rhizogenes*: Tropane alkaloid metabolism, insect-toxicity and allelopathic properties. J Plant Physiol 142:446–451.

Savka MA, Farrand SK. 1992. Mannityl opine accumulation and exudation by transgenic tobacco. Plant Physiol 98:784–789.

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. Biotechnol Bioeng 58:333–338.

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D. 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid: Identification of open reading frames. J Biol Chem 261:108–121.

Spena A, Schmülling T, Koncz C, Schell JS. 1987. Independent and synergistic activity of *rol A*, *B*, and *C* loci in stimulating abnormal growth in plants. EMBO J 6:3891–3899.