Stable Vindoline Production in Transformed Cell Cultures of *Catharanthus roseus*

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Catharanthus roseus L. (G. Don) leaf disks were transformed by cocultivation with seven strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Four distinct transformed cell lines were obtained with *A. tumefaciens* strains A281, BO542, and *A. rhizogenes* strain K599, and selected on hormone-free medium. Transformation of the cell lines was confirmed by Southern blot analysis, and preliminary data on the alkaloid profiles was determined by 2D TLC and HPLC/MS. Stable vindoline production was demonstrated in the undifferentiated suspension cell lines strains, CR-BO542, CR-K599, and strain CR-A281S (shooty teratomas), by HPLC/MS analysis of the alkaloid fractions. Active vindoline biosynthesis in the suspension-cell lines was further confirmed by measuring the activity of acetyl-coenzyme-A-deacetylvindoline-*O*-acetyltransferase, one of the final enzymes in the biosynthetic pathway of vindoline, in cell-free extracts.

The monoterpene indole alkaloids number about 1000 members with a wide variety of different structural types and biological activities.[1] Within this group of compounds, the alkaloids produced by the plant species *Catharanthus roseus* (L.) G. Don (Apocynaceae) have been of particular interest to natural product researchers due to their structural complexity and their clinical usefulness in the treatment of cancer. Amid the more than 90 alkaloids that have been isolated from *C. roseus*,[2] of greatest clinical value are the antimitotic bisindole alkaloids vinblastine (VBL) and vincristine (VCR). *C. roseus* is the sole source of VBL and VCR, and the yield of the two alkaloids from the plant is very low (0.001%), thereby making the cost of these lifesaving drugs very expensive ($1,000,000/kg for VCR[3]).

Over the past 30 years, many attempts have been made to produce these clinically useful compounds using classical plant cell culture techniques.[4−12] Although the biosynthesis of many of the monomeric indole alkaloids including ajmalicine and catharanthine, has been observed in plant cell cultures,[4] the production of the bisindole alkaloids vincristine and vinblastine has not been reproducibly demonstrated in undifferentiated suspension-cell cultures.

The major obstacle in reaching this goal has been a persistent lack of vindoline biosynthesis in undifferentiated tissue cultures. It has been hypothesized that vindoline biosynthesis in *C. roseus* is developmentally regulated and may require chloroplast formation.[13] However, photoautotrophic suspension cultures did not produce vindoline,[14] indicating that the presence of chloroplasts alone is not sufficient to result in vindoline biosynthesis. Further investigations of heterotrophic *C. roseus* cell lines[15] and "hairy root" cultures[16] have reported the occurrence of vindoline, although only after four passages of the cell cultures. Up to this point, the stable production of vindoline by plant tissue cultures has been reported only in differentiated shoot cultures grown in the presence of the phytohormone benzyladenine.[17]

This report describes the transformation of *C. roseus* explants with several different strains of *A. tumefaciens* and *A. rhizogenes*, which resulted in the development of transformed, hormone-independent suspension-cell and shoot cultures. Using 2D TLC and HPLC−MS, we report the stable production of a variety of monoterpene indole alkaloids including ajmalicine, catharanthine, and vindoline for a period of six years. Furthermore, acetyl coenzyme A-deacetylvindoline-17-*O*-acetyltransferase (DAT), the final enzyme in the biosynthetic pathway of vindoline, was active in two strains of the suspension-cell cultures and the shooty teratomas.

Results and Discussion

Incubation of *C. roseus* explants with seven separate strains of *A. tumefaciens* and *A. rhizogenes* resulted in the generation of three strains of hormone-independent suspension-cell lines and one line of shooty teratomas (Table 1). Specifically, these cell lines represent the following crosses: *C. roseus* × *A. rhizogenes* strain K599 (CR-K599), *C. roseus* × *A. tumefaciens* strain A281 (CR-A281), and *C. roseus* × *A. tumefaciens* strain BO542 (CR-BO542). Transformation of the suspension-cell lines and shooty teratomas was verified by Southern blotting analysis. All transformants were maintained as callus and suspension-cell cultures, although CR-A281 also grew as hormone-independent shooty teratomas (CR-A281S). Each of these cultures have been maintained in our laboratory for a period of more than 6 years on hormone-free Gamborg B5 media. Callus cultures of *C. roseus* not transformed by *Agrobacterium* spp. were unable to survive on hormone-free media and died within two subcultures (6 weeks).

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Table 1. Summary of the Transformed Cell and Organ Cultures of *Catharanthus roseus* Generated Using Specific *Agrobacterium tumefaciens* and *rhizogenes* Strains as Vectors

Agrobacterium Strain	resultant cell line	culture type
C. roseus × *A. tumefaciens* C58	explants not transformed	none
C. roseus × *A. tumefaciens* BO542	CR-BO542	callus, suspension-cells
C. roseus × *A. tumefaciens* 15955	explants not transformed	none
C. roseus × *A. tumefaciens* A281	CR-A281, CR-A281S	callus, suspension, shooty teratomas
C. roseus × *A. rhizogenes* K599	CR-K599	callus, suspension-cells
C. roseus × *A. rhizogenes* A4	explants not transformed	none
C. roseus × *A. rhizogenes* 8196	explants not transformed	none

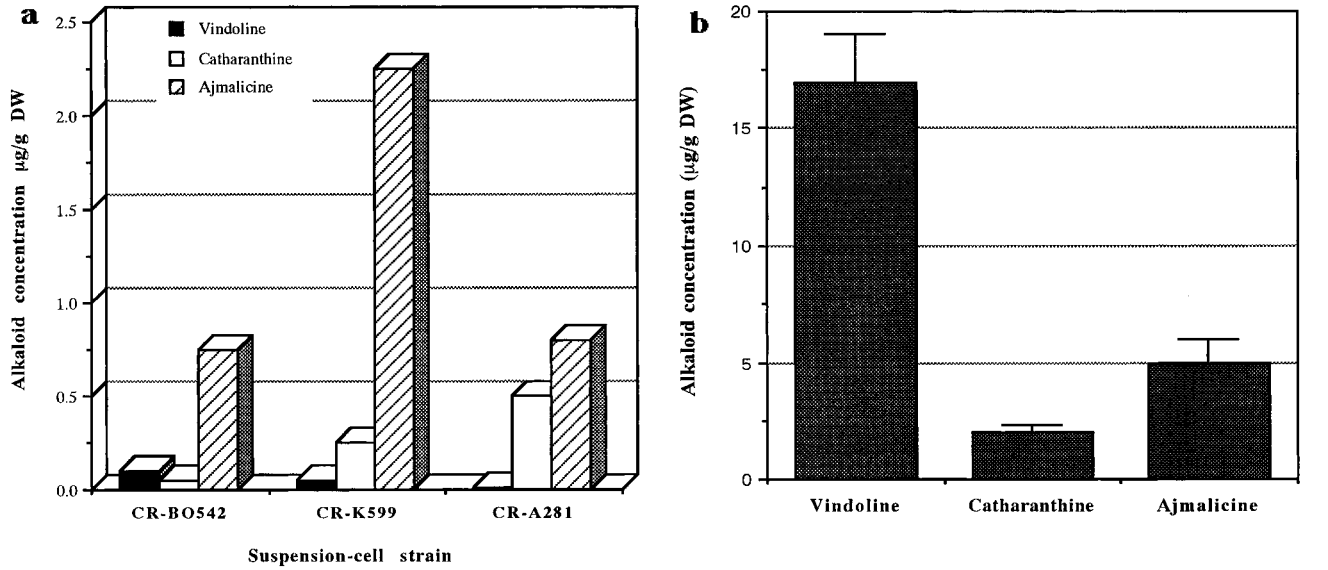

Figure 1. (a) Concentration of vindoline, catharanthine, and ajmalicine in the transformed suspension-cell lines: CR-BO542, CR-K599, and CR-A281. Indole alkaloid levels are defined in μg/g dry weight (DW). (b) Concentration of vindoline, catharanthine, and ajmalicine in the transformed shooty teratomas: CR-A281S. Indole alkaloid levels are defined in μg/g dry weight (DW).

The spectrum of alkaloids produced by each of the hormone-independent cell lines was determined by HPLC−positive ion thermospray and electrospray MS. LC−MS methods have played an integral role in the analysis of plant cell cultures by allowing the identification of very small quantities of the target alkaloids by their molecular ion.[18] Alkaloid identification was performed by matching the retention times and molecular ions of standard alkaloids in the HPLC−MS system. The production of ajmalicine, catharanthine, and vindoline in *C. roseus* cell-lines, strains BO542, K599, A281, and shooty teratomas A281S, is shown (Figures 1a and 1b), respectively. Quantification of the alkaloid accumulation was determined using a calibration of the molecular ion intensity at the corresponding retention time for the various standard alkaloids. Of particular importance is the accumulation of vindoline in the suspension-cell lines, CR-K599 and CR-BO542. Stable production of vindoline has not previously been reported in undifferentiated suspension cultures. To further demonstrate that vindoline biosynthesis is a stable aspect of the secondary metabolism in transformed cell lines, the activity of DAT, the final enzyme in the biosynthetic pathway of vindoline (Figure 2), was assayed in cell-free extracts. DAT activity was observed in each of the *C. roseus* cell-lines strains BO542, K599, and A281 (suspension-cells and shooty teratomas) (Figure 3). The activity of DAT in the shooty teratomas and the suspension-cell line CR-BO542 was similar to that of the intact plant; whereas DAT activity in the suspension-cell lines CR-K599 and CR-A281 was considerably lower (Figure 3). The difference in the DAT activities of the suspension-cell lines may explain the differences in the concentration of vindoline observed in the transformed cell lines, as both the suspension-cell line CR-BO542 and CR-A281S accumulated substantially more vindoline than either *C. roseus* strains K599 or A281 (Figure 1a and b). Furthermore, in some preliminary experiments, treatment of the suspension-cell cultures with an elicitor derived from a cell-wall homogenate of the fungus *Penicillium expansum* enhanced the activity of DAT by approximately 100% (Figure 3, CR-BO542 E). These observations suggest that the activity of DAT in the suspension cells may be inducible; and therefore, it may be possible to enhance the production of vindoline by optimization of culture conditions and elicitation protocols.

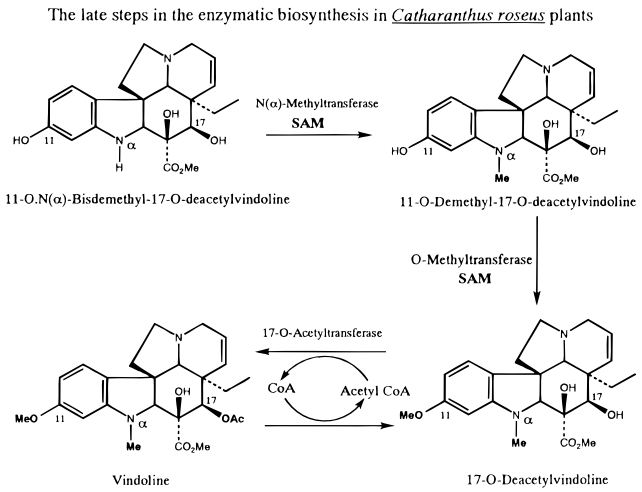

Figure 2. The final enzymatic steps in the biosynthesis of vindoline in *Catharanthus roseus*. Acetyl CoA: DAT is the final enzyme in the biosynthetic scheme.

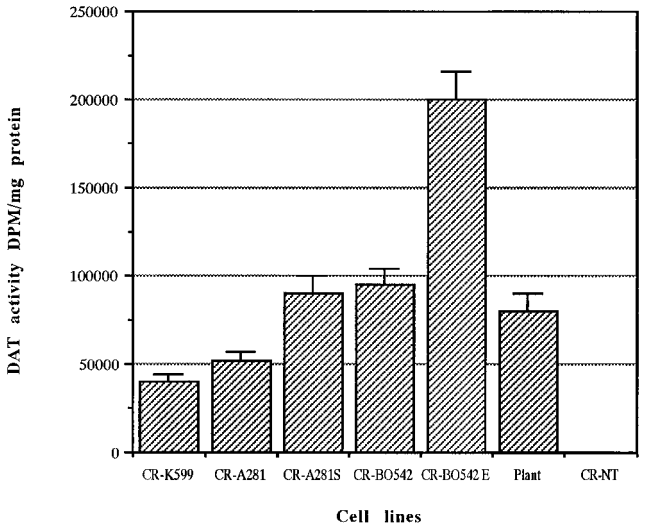

Figure 3. Comparison of the activity of DAT in the transformed suspension-cell lines (CR-BO542, CR-K599, CR-A281) and shooty teratomas (CR-A281S) of *Catharanthus roseus*. Enzyme isolated from shoot tips of the intact plant was used as a positive control, and cell free extracts of untransformed *C. roseus* suspension-cells (CR) were used as the negative control.

The fact that the suspension-cell lines and shooty teratomas are now more than 6 years old and still producing vindoline demonstrates that vindoline biosynthesis is a stable aspect of their secondary metabolism. Perhaps more interesting is the fact that both suspension-cell lines CR-BO542 and CR-K599 biosynthesize the production of catharanthine and vindoline, simultaneously (Figure 1a). These two monomer units form the antineoplastic bisindole alkaloids VCR and VBL, and their simultaneous production in undifferentiated plant cell lines has been sought for some time.

Finally, the ability to regenerate intact plants from *C. roseus* cultures has been seen as a major obstacle to the production of transgenic plants of this species. When the shooty teratomas (CR-A281S), which display biosynthetic capacities similar to those of intact plants, are grown under high-intensity light conditions, they spontaneously produce roots, without the use of hormones, and the resulting plantlets have been able to grow in a nonsterile greenhouse environment. Currently, a detailed quantitative examination of these cell lines for the production of the bisindole alkaloids is currently underway.

Experimental Section

General Experimental Procedures. E. Merck 100 mm × 100 mm aluminum-backed plates coated with 0.2 mm of Si gel were washed with Me_2CO and dried overnight prior to use. Each plate was spotted with one of the extracts, and a 2D development scheme was used to separate the compounds.[19] The first dimension solvent system was $CHCl_3$−MeOH (95:5), and the second dimension system was EtOAc−EtOH (3:1). Each plate was developed to a distance of approximately 80 mm in each direction and allowed to dry for 30 min. Following development, each plate was sprayed with ceric ammonium sulfate, and the color and position of each spot was compared with those of standards; or, when standards were not available, by matching the spots to published color reactions.[20]

HPLC−MS. The HPLC−MS conditions were a modification of those published by Auriola *et al.*[18]

Chromatographic Conditions. The HPLC system consisted of a Hewlett Packard model 1090 ternary pump system equipped with a diode array detector in tandem with the mass spectrometer and using a Rheodyne injector with a 250-μL sample loop. The HPLC column was a Supelco LC-18DB C-18 (250 × 4.6 mm) reversed-phase column. The two solvent systems used were (A) [0.1 M ammonium acetate (pH 7.2)−MeCN (75:25)], and (B) [0.1 M ammonium acetate (pH 7.2)−MeCN (42:58)]. The column was run on a linear gradient from [70% (A): 30% (B)] to [0% (A): 100% (B)] at a rate of 1.0 mL min^{-1} over a period of 40 min.

Mass Spectrometry. The LC−MS system consisted of an HP thermospray interface coupled to a Hewlett-Packard 5989 quadropole mass spectrometer. The instrument was used in the positive ion thermospray mode with filament. The ion source temperature was 250 °C, the fragmenter voltage was 40 V, and the stem temperature program was as follows: 96 °C (0−10 min), 92 °C (10−20 min), 90 °C (20−40 min).

Explant Transformation. Shoot tips from a field-grown specimen of *Catharanthus roseus* (L.) G. Don were excised, and the leaves were removed and surface sterilized with 5% sodium hypochlorite solution and 70% EtOH according to standard procedures. After washing with sterile distilled H_2O, the leaves were cut into small pieces (2 mm) and placed into 1.5-mL solutions of 24-h-old, rapidly dividing cultures of *A. tumefaciens* (strains C58, BO542, 15955, or A281) or *A. rhizogenes* (strains K599, A4, or 8196). The explants were allowed to incubate for 24 h, after which the *Agrobacterium* solutions were removed and replaced with liquid Gamborg B5 media[21] containing 2.0% sucrose, 0.5 mM 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg mL^{-1} carbenicillin. After 24 h, the *C. roseus* explants were transferred to petri dishes containing the same media solidified with 7.0 g L^{-1} agar. After three weeks calli began to form and were transferred to plates containing a hormone-free Gamborg B5 media (2.0% sucrose, no 2,4-D, no carbenicillin). Upon the reccurrence of *Agrobacterium* contamination, cultures were transferred to Gamborg B5 media (hormone-free) containing 1 mg mL^{-1} carbenicillin, until no further signs of contamination appeared.

Plant Cell Culture. Cultures were maintained on hormone-free Gamborg B5 media and subcultured at four-week intervals for a period of one year. Suspension cultures were initiated from callus and grown in 1-L bottles containing 100 mL of media on a roller apparatus at 40 rpm. Suspension cultures were split into three and subcultured at three-week intervals. In the case of CR-A281S (shoot) cultures, tissue cultures were pruned and transferred to Magenta culture vessels containing 50 mL of hormone-free solid Gamborg B5 every 5 weeks. Untransformed *C. roseus* cell cultures were initiated from the same plant leaves and were maintained on Gamborg B5 media (2% sucrose and 0.5 mg/L 2,4-D) as both callus and suspension cultures.

DNA Isolation and Analysis. *C. roseus* genomic DNA was isolated from transformed suspension-cells grown on hormone-free medium for 60 months, and from untransformed control cells using the procedure described by Murray and Thompson.[22] The concentration of DNA was determined by measuring the absorbance

at 260 nm. For Southern blot analysis, genomic DNA was digested with *Bam* HI, separated electrophoretically on a 1% agarose gel, and transferred to a Zeta-Probe GT membrane (Bio-Rad Laboratories, Hercules, CA) according to the protocols of the manufacturer. After 24 h the membrane was rinsed in 2× SSC air dried, and the DNA was UV cross-linked to the membrane. Plasmids pEHB107 and pEHB136 were isolated from two strains of *E. coli* (LE392), strain 107 and 136 (supplied by Dr. E. Hood, Pioneer Hi-Bred International, Inc.), using a plasmid isolation kit (Quiagen Inc., Chatsworth, CA). The isolated plasmids were digested with *Bam* HI and separated electrophoretically on a 1% agarose gel. The appropriate restriction fragments were excised from the gel and eluted from the agarose using a GenElute Agarose spin column (Supelco, Inc., Bellefonte, PA). The resulting fragments were labeled with ^{32}-P-dCTP using a DNA random priming kit (Sigma Chemical Co., St. Louis, MO). Prehybridization and hybridization of the Zeta-Probe GT membrane was performed as described according to the instructions of the manufacturer.

Alkaloid Extraction. Suspension cultures were filtered and the cell mass ground in a mortar, triturated with MeOH, and placed in stoppered flasks on a rotary shaker overnight. Shoot cultures were homogenized with MeOH in a blender and also placed on the shaker. The MeOH extracts were filtered and dried at room temperature. The dried extracts were resuspended in 0.25 M HCl (100 mL) and extracted with 100 mL $CHCl_3$ (3×). The acidified aqueous extract was then made basic (pH = 9−10) by the addition of Na_2CO_3 and KOH followed by extraction with $CHCl_3$ (100 mL, 3×) after which the resulting $CHCl_3$ extract was dried *in vacuo*.

Isolation and Assay of DAT. The enzyme DAT catalyzes the acetyl coenzyme A-dependent acetylation of 17-*O*-deacetylvindoline and is the final step in the biosynthetic pathway of vindoline.[23] Young leaves, fully opened (approximately 35 days old), shoot tips, or transformed suspension-cell cultures were fast frozen in liquid N_2 and then ground in a buffer containing 100 mM Hepes (pH 7.6), 1 mM DTT, and 5 mM EDTA as described.[23,24] The plant material was homogenized using a Polytron homogenizer (Beckman); the slurry was transferred to microcentrifuge tubes and centrifuged (20 000 g, 10 min). The supernatant was stirred with Dowex 1 × 2 (10% w/v) and then filtered. The filtrate was precipitated with ammonium sulfate, and the 40−70% fractions were collected by centrifugation. The protein pellet was resuspended in 20 mL of buffer (0.1 M imidazole, 1 mM dithiothreitol) and chromatographed on a Sephadex G 100 column, developed with the same buffer at a rate of 1 mL/min, and resulting fractions were assayed.

DAT activity was assayed as previously described.[25] Briefly, the assay mixture contained 5 μM deacetylvindoline, 4.4 μM [1-^{14}C]acetyl coenzyme A (0.045 μCi), and enzyme in a total volume of 100 μL 0.1 M Tris-HCl (pH 8). The mixture was incubated for 5 min at 30 °C, and the reaction was terminated by the addition of 50 μL of 1 N NaOH. The acetylated alkaloid was extracted by adding 250 μL of EtOAc and shaking for 2 min. The organic and aqueous phases were separated by centrifugation, 100 μL of the organic phase was transferred to scintillation vials, and the radioactivity in DPM was measured. Recovery of acetylated products by this method was approximately 95% under these conditions. Negative control tubes contained the standard assay mixture minus either enzyme or substrate.

Protein Assay. Protein concentrations were determined according to the procedure described by Bradford,[26] using bovine serum albumin as the standard.

Substrate Synthesis. 17-*O*-Deacetylvindoline was synthesized from vindoline using sodium methoxide in MeOH.[24] The identity of the compound was confirmed by mass spectrometry after purification by preparative-TLC.

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Supporting Information Available: Selected ion chromatograms of extracts from suspension-cells and shooty teratomas of transformed *C. roseus* (5 pages). Ordering information is given on any current masthead page.

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