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Metabolic, Biochemical and Molecular Profiling of *Catharanthus roseus* Flower Cultivars and Transformed Hairy Roots for Monoterpenoid Indole Alkaloid Accumulation

by

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Abstract

Catharanthus roseus (L.) G Don is a commercially significant flower species and in addition is the only source of the monoterpenoid indole alkaloids (MIA) vinblastine and vincristine, which are key pharmaceutical compounds that are used to combat a number of different cancers. Therefore, procurement of the antineoplastic agents is difficult but essential procedure. Alternatively, *Catharanthus* tissue cultures have been investigated as a source of these agents; however they do not produce vindoline, which is an obligate precursor to vinblastine and vincristine.

The interest in developing high MIA cultivars of *Catharanthus roseus* has prompted metabolic profiling studies to determine the variability of MIA accumulation of existing flowering cultivars, with particular focus on the vindoline component of the pathway. Metabolic profiling studies that used high performance liquid chromatography of MIAs from seedlings and young leaf extracts from 50 different flowering cultivars showed that, except for a single low vindoline cultivar (Vinca Mediterranean DP Orchid), they all accumulate similar levels of MIAs. Further enzymatic studies with extracts from young leaves and from developing seedlings showed that the low vindoline cultivar has a 10-fold lower tabersonine-16-hydroxylase activity than those of *Catharanthus roseus* cv Little Delicata. Additionally, studies aimed at metabolic engineering of vindoline biosynthesis in *Catharanthus roseus* hairy root cultures have been performed by expressing the last step in vindoline biosynthesis [Deacetylvindoline-4-*O*-acetyltransferase (DAT)]. Enzymatic profiling studies with transformed hairy roots have confirmed that over-expressing *DAT* leads to lines with high levels of *O*-acetyltransferase activity when compared to non-expressing hairy roots. One particular *DAT* over-

expressing hairy root culture (line 7) contained 200 times the DAT activity than leaves of control lines. Additional MIA analyses revealed that *DAT* over-expressing hairy roots have an altered alkaloid profile with significant variation in the accumulation of hörhammericine. Further analysis of transformed hairy root line 7 suggests a correlation between the expression of DAT activity and hörhammericine accumulation with root maturation. These studies show that metabolic and selective enzymatic profiling can enhance our ability to search for relevant MIA pathway mutants and that genetic engineering with appropriate pathway genes shows promise as a tool to modify the MIA profile of *Catharanthus roseus*.

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General Introduction

Plants produce a large number of chemical compounds that include essential primary metabolites that are directly vital to cell functions and secondary metabolites that contribute to the overall fitness of the organism. While secondary metabolites are not considered essential for survival of individual cells, they do play a significant role in plant environment interactions (Meijer *et al.*, 1993). Their formation may be controlled by stimuli like light, wounding, microbial attack or pathogenesis-related elicitation (Meijer *et al.*, 1993). Several of these compounds have considerable economic value and are used extensively as medicines, flavourants or odorants.

One of the most important group of secondary metabolites for humans are the alkaloids, which are nitrogen-containing, low molecular weight cyclic compounds (De Luca & St-Pierre, 2000). Many alkaloids are toxic, powerful hallucinogens and have important pharmacological activity as analgesics such as morphine and codeine (De Luca & Laflamme, 2001; Facchini, 2001). Of the various sub-classes of alkaloids, the monoterpenoid indole alkaloids (MIAs) that derived from tryptophan and terpenoid precursors are of particular interest (De Luca & St. Pierre, 2000). Several of the MIAs produced by the tropical under shrub *Catharanthus roseus* (L.)G.Don (Madagascar periwinkle) are important pharmaceuticals. For example, ajmalicine is used in the treatment of circulatory diseases and the antineoplastic agents vinblastine and vincristine are powerful anticancer drugs (van der Heijden *et al.*, 2004). These alkaloids are at limited concentrations in the plant tissue and as a result researchers have focused on the development of commercial production of the MIAs by *in vitro* plant cell and tissue cultures. Unfortunately, these alkaloids are not produced in the undifferentiated cells

found in plant tissue culture (van der Heijden *et al.*, 2004). In order to overcome these problems, the possibility of genetic manipulation of the secondary metabolism in plant and culture systems is under investigation.

This thesis describes metabolic profiling studies to determine the variability of MIA accumulation among existing cultivars of *Catharanthus roseus*. In addition, genetic engineering studies aimed at expressing the last step in vindoline biosynthesis in hairy root cultures are described. These studies show that metabolic and selective enzymatic profiling can help to find relevant MIA pathway mutants and that genetic engineering with appropriate pathway genes shows promise as a tool to modify the MIA profile of this plant. The literature review concerning the biosynthesis of *Catharanthus* alkaloids, product formation and the problems associated with cell and tissue cultures is presented in Chapter 2. Chapter 3 describes the use of high performance liquid chromatography for metabolic profiling of the MIAs in 50 different flowering cultivars of *C. roseus* and Chapter 4 reports the expression of a leaf-specific, deacetylvindoline-4-*O*-acetyltransferase (DAT) in hairy root cultures.

2. Literature Review

2.1 *Catharanthus roseus*

2.1.1 The Taxonomy and origin of *Catharanthus roseus*

Catharanthus roseus is a perennial plant belonging to the family Apocynaceae and is one of the eight species that comprise the genus *Catharanthus*; which includes *C. coriaceus* Markgr., *C. lynceus* (Bojer ex A.DC.) Pichon, *C. longifolius* (Pichon) Pichon, *C. ovalis* Markr., *C. roseus* (L.) G.Don, *C. scitulus* (Pichon) Pichon, *C. trichophyllus* (Bak.) Pichon, *C. pusillus* (Murray) G.Don (van der Heijden *et al.*, 2004). *Catharanthus roseus*, commonly known as the Madagascar periwinkle, was originally classified as *Vinca rosea* by Lineaus in 1759; however, when structural differences between *rosea* and other *Vinca* species were discovered, new classifications were made. The *rosea* species was named *Lochnera* (a synonym of *Catharanthus*) *rosea* by Ludwig Reichenback in 1828 and than *Catharanthus roseus* by George Don in 1835 (van der Heijden *et al.*, 2004).

The genus *Catharanthus* originated in Madagascar, except for the species *Catharanthus pusillus* which originated in India. *Catharanthus* is now naturalized pantropically and it is found in Africa, America, Asia, Australia, Southern Europe and on a few islands in the Pacific Ocean (van der Heijden *et al.*, 2004). This pan tropical distribution of *C. roseus* may be attributed to sailors who often transported and utilized the plant leaves to suppress the sensations of hunger and fatigue (van der Heijden *et al.*, 2004). Although the dispersal of *C. roseus* occurred centuries ago, both cultivated and wild *Catharanthus* plants are harvested for use in pharmaceutical preparations.

Apart from its medicinal properties, *C. roseus* is cultivated as an attractive ornamental shrub that has 30-100 cm dark green leafy stems and many pink or white blossoms that appear continuously through the growing season (van der Heijden *et al.*, 2004). Traditional cultivars have an upright growth habit, but a few years ago several trailing cultivars growth in hanging baskets were developed. All *Catharanthus* cultivars contain specialized laticifers and idioblasts that are enriched with white alkaloid rich latex that produces a strong smell when damaged (van der Heijden *et al.*, 2004). While seed stocks of *Catharanthus* were commercially available in the second half of the 19th century, the first flowering cultivars were only appeared in the 1960s. Apart from its ornamental appeal, the medicinal value of certain monoterpenoid indole alkaloids (MIAs) found in *C. roseus* has triggered mutation breeding efforts to increase the concentration of pharmaceuticals, vinblastine and vincristine (Dutta *et al.*, 2004).

2.1.2 The *Catharanthus* alkaloids

Catharanthus roseus synthesizes a large group of structurally diverse MIAs with more than 130 known MIAs derived from strictosidine identified some of which have been useful as medicinal compounds (van der Heijden *et al.*, 2004). The biosynthesis of the MIAs is complex and the pathway is variable in terms of the precursors required for their biosynthesis and the various products produced.

The formation of MIAs requires the conjunction of two metabolic pathways for the information of the central MIA intermediate strictosidine. It is formed by the conjugation of secologanin derived from the plastidic 2-C-methyl-D-erythritol 4-phospate (MEP) pathway and tryptamine, a product of the shikimate pathway (Burlat *et al.*, 2004; van der Heijden *et al.*, 2004) (Fig.1). The tryptamine precursor is derived from primary

metabolism by a single enzymatic conversion of the amino acid tryptophan, and it supplies the indole moiety of the MIAs (van der Heijden *et al.*, 2004). The terpenoid moiety of the MIAs is derived from secologanin and it is the arrangement of the secologanin moiety portion that determines the structural type of the alkaloid (van der Heijden *et al.*, 2004).

The condensation of tryptamine and secologanin is catalyzed by the enzyme strictosidine synthase (STR). The strictosidine product is regarded as the central building block for about 3000 different alkaloids. When the glucose moiety is removed from strictosidine, a highly reactive dialdehyde algycone is formed (van der Heijden *et al.*, 2004). The algycone is converted by molecular rearrangement to a number of different basic alkaloid skeletons that have been classified into three structural types. They are the Corynanthe, Aspidosperma and Iboga monoindole alkaloids (Fig. 2) (van der Heijden *et al.*, 2004).

Several major classes of *Catharanthus* alkaloids are of interest medicinally, including ajmalicine (Corynanthe), catharanthine (Iboga), vindoline (Aspidosperma) and the bisindole alkaloids vinblastine (VBL) and vincristine (VCR). Ajmalicine (Fig. 2) is used therapeutically as an antihypertensive. For commercial use, it is isolated from another member of the Apocynaceae family, *Rauvolfia serpentina*, where it is more abundant than in *C. roseus* (De Luca & Laflamme, 2001; van der Heijden *et al.*, 2004). The alkaloids VBL and VCR (Fig. 4) are used as antineoplastic agents and are dimeric derivatives of vindoline (Fig. 3) and catharanthine and this review will focus on them. Pharmaceutical uses of VBL and VCR will be discussed in Section 2.1.3. The biosynthesis of these alkaloids will be discussed in Section 2.2 with particular emphasis

on the vindoline pathway and enzymes associated with this pathway. The advantages of tissue culture systems and its application towards *C. roseus* will be discussed in Section 2.3.

2.1.3 Pharmacological uses of the *Catharanthus* alkaloids

The functions of the alkaloids in *C. roseus* may include insect their antifeedant activity and protection against pathogens or UV radiation (Meijer *et al.*, 1993). For humans, several of the alkaloids have important therapeutic applications. The medicinal properties of *Catharanthus* alkaloids were first investigated in the late 1950s when two independent research groups tested *C. roseus* extracts for antidiabetic activity (reviewed in Noble, 1990) which led to the discovery of their antineoplastic activity in experimental animals. Cutts *et al.* (1957), found a dramatic decrease in the white blood cell counts of rats when assayed with a highly active *Catharanthus* alkaloid extract and Svoboda *et al.* (1962) observed repressed growth of rapidly growing lymphoma in mice from an isolated *C. roseus* alkaloid (Noble, 1990). Further studies led to the isolation and characterization of VBL as the antineoplastic compound.

These *Catharanthus* alkaloids were subsequently tested for their effectiveness against cancer. VBL proved to be effective in the treatment of tumorous cancers and was introduced in 1960 for use against Hodgkin's disease, non-Hodgkin lymphomas, testis-carcinomas and sometimes against breast cancer and chorio-carcinomas (van der Heijden *et al.*, 2004). Vincristine was introduced in 1963 and used in the treatment of acute leukemia, Hodgkin's disease, non-Hodgkin lymphomas, breast cancer and Wilm's tumors in children (van der Heijden *et al.*, 2004). Anhydrovinblastine has also been introduced as an agent against cervical and lung cancer (van der Heijden *et al.*, 2004).

The ability of the bisindole alkaloids to act as antineoplastic agents is attributed to the disruption of microtubules (van der Heijden *et al.*, 2004). These agents form tubulin paracrystals, preventing tubulin polymerization and interfere with the mitotic spindle during cell division which causes metaphase arrest (Avila, 1997; Dumontet & Sikic, 1999). The dimeric alkaloids of *Catharanthus* are the earliest tubulin binding agents to be used as clinical antimitotics and they have been described as microtublule depolymerizing agents. However, their antineoplastic activity may actually arise from the combined disruption of multiple microtubule-dependent processes, inhibition of the cell cycle and induction of programmed cell death (Dumontet & Sikic, 1999, van der Heijden *et al.*, 2004).

There have been attempts to find a relationship between the chemical structure of these bisindole alkaloids and their antineoplastic activity but as yet it is still not well understood. The chemical structures of VBL and VCR were eventually shown to be very similar. VCR has a formyl group in the number one position where VBL posses a methyl group in that position (Noble, 1990) (Fig. 4). In spite of their chemical similarity, these two drugs differ markedly in the type of tumors they affect and in the types of toxic side effects that they display (Noble, 1990). VBL, as mentioned earlier, is particularly useful in treating tumorous cancers, but has toxic effects on bone marrow, while VCR is more effective against leukemia and it negativey effects the peripheral nervous system (Noble, 1990). That being said the dimeric structure of these alkaloids appears to be essential to their oncolytic activity.

Unfortunately, prolonged treatments with *Catharanthus* alkaloids cause severe side effects, including hair loss, vomiting and loss of reflexes (Avila, 1997). The search

for drugs with fewer side effects has led to the development and marketing of several semi-synthetic bisindole alkaloids with a lower toxicity and different pharmacological profiles. Vindesine was introduced in 1980 for the treatment of bronchus-carcinoma, mamma- carcinoma, myeloma and treatment of leukemia. Vinflunine showed superior antitumour activity in preclinical models and Vinglycinate, which was never marketed, also has significant antineoplastic activity (van der Heijden *et al.*, 2004). With continued research of this type, it is hoped that structural modifications of *Catharanthus* alkaloids will result in antineoplastic drugs with fewer side effects.

2.2 Biosynthesis of VBL and VCR

As described above the long and complex pathway involved in VBL and VCR biosynthesis has led to the characterization of numerous biosynthetic intermediates and enzymes from extracts of cell cultures, organized hairy root cultures or from whole plant organs. The characterization of MIA pathway enzymes has been useful to confirm pathway precursors and intermediates, as well as providing information about pathway regulation that could be useful when designing tissue cultures for alkaloid production.

2.2.1 Production of the Tryptamine Precursor

In plants, microorganisms and in some animals, there is a common Shikimate pathway for the biosynthesis of the aromatic amino acids, phenylalanine, tyrosine and tryptophan, (Herrmann & Weaver, 1999). The indole precursor tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC) (Facchini, 2001; Hashimoto & Yamada, 1994; De Luca *et al*. 1989).

The critical involvement of TDC at the interface between primary and secondary metabolism has led to extensive study of this enzyme in relation to a potential regulatory role in MIA biosynthesis (Meijer *et al.*, 1993). In *C. roseus*, this soluble cytosolic enzyme occurs as a dimer (molecular weight 115kDA) consisting of two 54kDa monomeric subunits with a pI of 5.9 (De Luca, 1993). A TDC cDNA clone was isolated by immunoscreening of a cDNA library (DeLuca *et al.*, 1989, Meijer *et al.*, 1993). Its identity was confirmed by expression in *Escherichia coli* (De Luca *et al.*, 1989) and *Nicotiana tabacum* (Songstad *et al.*, 1989; Meijer *et al.*, 1993). TDC is highly substrate specific and its activity increases in response to external factors, like treatments with fungal extracts and hydrolytic enzymes (Meijer *et al.*, 1993). TDC can also be regulated by tissue specific and developmental controls, and by abiotic stresses such as UV treatment (Meijer *et al.*, 1993).

The activation of TDC expression is not the sole prerequisite for MIA biosynthesis since overexpression of the *TDC* gene in *C. roseus* cell cultures did not result in an increased production of MIAs, in spite of an enhanced accumulation of TDC protein and enzyme activity (Meijer *et al.*, 1993). The studies showed that in spite of the enhanced availability of tryptamine in transformed cultures, it did not lead to increased MIA accumulation and reaffirmed earlier experiments that TDC alone is not the only rate-limiting enzyme involved in the synthesis of these metabolites (Meijer *et al.*, 1993). Further, dramatic reduction of TDC activity in hairy root cultures showed that a loss of TDC activity did not result in a reduction of MIA accumulation (Moreno-Valenzuela *et al.*, 1998). Similar results were also obtained by Whitmer *et al.* (2002) using transgenic *Catharanthus* cultures over-expressing the TDC gene.

2.2.2 Biosynthesis of Secologanin

Secologanin originates from isopentyl diphospate (IPP) through a number of steps that begin with the formation of the monoterpene 10-hydroxygeraniol from geraniol via the geraniol 10-hydroxylase (Collu *et al.*, 2001). While the mevalonate pathway was considered the sole biosynthetic route leading to IPP, an alternative mevalonate-independent pathway (MEP pathway) was recently discovered in bacteria and plants (Chahed *et al.*, 2000) (Fig. 1). Biosynthetic studies with *Catharanthus* cell cultures have suggested that only the MEP pathway is responsible for the biosynthesis of secologanin (Contin *et al.*, 1998). Geraniol 10-hydroxylase (G10H) has been purified to homogeneity from induced *C. roseus* cell cultures and this led to the successful cloning of a full-length cDNA (Collu *et al.*, 2001). This endoplasmic reticulum (ER) membrane associated cytochrome P450 dependent monooxygenase was dependent on NADPH, molecular oxygen and displayed a typical light-reversible inhibition by carbon monoxide (Facchini, 2001; Hashimoto & Yamada, 1994). Recombinant G10H hydroxylated the C-10 position of geraniol to generate 10-hydroxynerol or alternatively the pathway can proceed via nerol, the *cis*-isomer of geraniol (Meijer *et al.*, 1993).

The enzyme loganic acid methyltransferase (LAMT) has been purified from *C. roseus* etiolated seedlings by Madyastha *et al.* (1973), but the corresponding gene remains to be cloned. This enzyme catalyzes the methylation of loganic acid and secologanic acid to produce loganin and secologanin, respectively. The relative extents of conversion of both substrates were comparable, suggesting that both the acids and their products are part of the biosynthesis of indole alkaloids (Madyastha *et al.*, 1973).

The conversion of loganin to secologanin represents the final step in the pathway and is also catalyzed by a P450-dependent enzyme (Facchini, 2001, Yamamoto *et al.*, 2000) known as secologanin synthase (SCS). This enzyme was detected in the microsomal fraction of cell suspension cultures and was shown to be epidermis-specific in *C. roseus* leaves (Irmler *et al.*, 2000). SCS has also been observed in cell suspension cultures of *Lonicera japonica* (Yamamoto *et al.*, 2000).

In general, while both the tryptamine-producing segment of the pathway and the secologanin portion of the pathway are necessary for alkaloid production, very little is known about the exact mechanisms by which these precursor pathways are regulated. A better understanding of the coordinated induction of precursor formation is needed.

2.2.3 Biosynthesis of Strictosidine

The stereospecific condensation of the iridoid glucoside, secologanin, with tryptamine is catalyzed by strictosidine synthase (STR). This vacuole-specific enzyme is responsible for the formation of strictosidine, the common precursor of all MIAs (Meijer *et al.*, 1993).

The purification of extracts of cell suspension cultures and leaves revealed that 4 (Kutchan, 1993; Yamamoto *et al.*, 2000) and 6 (De Waal *et al.*, 1995) charge isoforms of STR could be identified in cell suspension cultures and/or leaves of *Catharanthus*. It remains to be determined if the occurrence of multiple isoforms of STR in *Catharanthus* has any significance in relation to cell-, development- or tissue-specific regulation of alkaloid biosynthesis (Meijer *et al.*, 1993). STR was purified to homogeneity and its related gene was cloned from *Rauvolfia serpentina* and *Catharanthus roseus* cell cultures, respectively (reviewed in Facchini, 2001). Recombinant STR expressed in *E.*

coli showed high substrate specificity for secologanin and tryptamine. While the biological roles of different isoforms of STR are unknown, STR is encoded by a single-copy gene, suggesting that the isoforms occur as a result of post-translational modifications (Kutchan, 1993; Meijer *et al.*, 1993).

Studies using *in vitro* cultures showed *STR* gene expression is affected by a variety of signals including auxins, methyl jasmonate and fungal elicitors. Expression of *STR* may not be a rate-limiting step in the indole alkaloid biosynthesis since *C. roseus* cell suspension cultures transferred to an alkaloid induction medium, produced an increased accumulation of alkaloids which did not correlate with an increase in STR activity (Kutchan, 1993; Meijer *et al.*, 1993). However, transgenic *Catharanthus* callus expressing the STR gene, resulted in a positive correlation between the increase in enzymatic activity and alkaloid accumulation (Canel et al., 1998). Similar results have also been observed in developing *Catharanthus* seedlings (Aerts. et al., 1994).

2.2.4 Conversion of Strictosidine to other *Catharanthus* MIAs

Once the central intermediate, strictosidine is synthesized it can be converted to ajmalicine, catharanthine, vindoline and other alkaloids (Hashimoto & Yamada, 1994). Strictosidine is deglucosylated by strictosidine glucosidase (SGD) to form a highly reactive algycone that may be rearranged to generate a number of different basic alkaloid skeletons (van der Heijden *et al.*, 2004).

SGD is a putative glycoprotein that is localized to the ER and its activity is highly strictosidine specific (Geerlings *et al.*, 2000). SGD has been purified and characterized from cell suspension cultures of *C. roseus* (Luijendijk *et al.*, 1998) and is encoded by a single-copy gene (Geerlings *et al.*, 2000). *SGD* is expressed in flowers, stems, leaves and

roots in a developmentally regulated manner. The enzyme consists of a high molecular weight complex formed from several 63kDa subunits and the appearance of enzyme activity is proportional to the mRNA levels detected (Geerlings *et al.*, 2000; Luijendijk *et al.*, 1998).

The removal of glucose from strictosidine produces unstable derivatives that are converted to 4, 21-dehydrogeissoschizine (Meijer *et al.*, 1993; Stockigt *et al.*, 1977). This compound either undergoes cyclization to form cathenamine or is reduced enzymatically to geissoschizine (Meijer *et al.*, 1993, Stockigt *et al.*, 1977). Cathenamine is metabolized by cathenamine reductase to the Corynanthe alkaloid ajmalicine, or is converted to another iminium form, whereas geissoschizine serves as a precursor for more complex alkaloids such as catharanthine or vindoline (Meijer *et al.*, 1993, Stockigt *et al.*, 1977). Unfortunately the detailed steps involved in these interconversions have not been characterized.

2.2.5 Biosynthesis of Catharanthine and Vindoline

Catharanthine and vindoline are members of the Iboga and Aspidosperma classes of alkaloids, respectively. Catharanthine is thought to be derived from geissoschizine, through the intermediate stemmadenine (Moreno *et al*, 1995). Although these catharanthine intermediates have been identified, nothing is known about the enzymology of catharanthine biosynthesis (van der Heijden *et al.*, 2004).

Vindoline is derived from geissoschizine and stemmadenine precursors that are ultimately converted to tabersonine (Moreno *et al*, 1995; van der Heijden *et al.*, 2004). It is interesting that cell suspension cultures of *C. roseus* have the ability to synthesize catharanthine and tabersonine, but they do not express the enzymes necessary for

converting tabersonine into vindoline. As a result researchers have focused on the enzymes involved in vindoline biosynthesis and their regulation in whole plants rather than in cell culture systems.

The transformation of tabersonine to vindoline involves six strictly ordered reactions (Fig. 3) starting with the hydroxylation of tabersonine at position C-16 to 16-hydroxytabersonine by tabersonine-16-hydroxylase (T16H) **(1)** (Facchini, 2001; van der Heijden *et al.*, 2004). Enzyme extracts from young leaves of *C. roseus* (St. Pierre & De Luca, 1995) required NADPH and molecular oxygen as substrates in addition to tabersonine for activity that was inhibited by CO, cytochrome c, clotrimazole and miconazole. These studies and the localization of the enzyme to the ER (St. Pierre & De Luca, 1995) established T16H as a cytochrome P450-dependent monoxygenase. In contrast to enzymes involved in the later stages of vindoline biosynthesis T16H could be detected in *C. roseus* cell suspension cultures from which T16H was cloned by screening a cell culture based cDNA library (Schroder *et al.* 1999).

The next step involves the *O*-methylation of 16- hydroxytabersonine by 16-hydroxytabersonine *O*-methyltransferase (OMT) **(2)**, an enzyme that is highly substrate specific (van der Heijden *et al.*, 2004) and requires S-adenosyl-L-methionine (AdoMet) as a methyl group donor. Initially, two consecutive hydroxylations at the C-3 and C-4 positions were proposed to follow the 16-hydroxylation of tabersonine. The isolation of an AdoMet associated *N*-methyltransferase, 2, 3-dihydro-3-hydroxytabersonne-*N*-methyltransferase (NMT) **(4)**, highly specific for the indole-ring nitrogen of 16-methoxy-2, 3-dihydro-3-hydroxy-tabersonine indicated that the *O*-methylation preceeded *N*-methylation (Facchini, 2001). NMT was localized to cholorplasts thylakoids and has been

partially purified. The enzyme has an apparent molecular mass of 60 kDa (Dethier & De Luca, 1993). Further attempts to purify NMT were unsuccessful and resulted in large losses of enzyme activity (Dethier & De Luca, 1993). The reaction prior to *N*-methylation, involving the hydration of the 2, 3-double bond has not yet been characterized (van der Heijden *et al.*, 2004).

The penultimate step in vindoline biosynthesis is catalyzed by desacetoxyvindoline 4-hydoxylase (D4H) **(5)**. The enzyme exhibited an absolute requirement for 2-oxoglutarate and enzyme activity was enhanced by the addition of ascorbate, thus classifying it as a 2-oxoglutarate-dependent-dioxygenase (De Carlois *et al.*, 1990; De Carlois & De Luca, 1993). D4H is a cytosolic enzyme specific for the hydroxylation of desacetoxyvindoline at the C-4 position producing deacetylvindoline (Facchini, 2001). The native enzyme is a monomer with a molecular mass of 45 kDa, respectively. The hydroxylase was purified to apparent homogeneity from young leaves of *C. roseus* and was found to exist in three charged isoforms with pI values 4.6, 4.7, and 4.8 (De Carlois & De Luca, 1993). Further, substrate interaction kinetics and product inhibiton studies by De Carlois & De Luca (1993) suggested D4H follows an ordered Ter Ter mechanism where 2-oxoglutarate is the first substrate bound followed by the binding of molecular oxygen and the indole alkaloid substrate (De Carlois & De Luca, 1993).

The final step in vindoline biosynthesis is catalyzed by Acetyl-CoA 4-*O*-deacetylvindolne 4-*O*-acetyltransferase (DAT) **(6)** that transfers an acetyl group from acetyl-CoA to position 4 of deacetylvindoline. This enzyme was purified to homogeneity (Power *et al.*, 1990) as a heterodimer with an apparent molecular weight 32/21 kDa that occurred as 5 isoforms (pI= 4.3 to 5.4). Later studies with the cloned gene showed that

DAT is expressed as a single 50 kDa protein and that the isolated DAT heterodimer was in fact a proteolytic artefact of protein purification (St. Pierre *et al*, 1998). Inhibition of DAT was also observed with the addition of tabersonine, but it was not inhibited by vindoline until it reached a concentration of 2mM (Power *et al.*, 1990).

2.2.6 Formation of the bisindole Alkaloids

The bisindole alkaloids arise in the plant by an oxidative enzymatic coupling of catharanthine and vindoline to form 3, 4-anhydrovinblastine (AVLB), the precursor of vinblastine and vincristine. An H_2O_2-dependent enzyme capable of catalyzing the formation of AVLB was purified to homogeneity from *C. roseus* leaves (Sottomayor *et al.*, 1998; Sundberg & Smith, 2002). In addition to AVLB synthase activity, the enzyme, with a molecular weight of 45.4 kDa, had peroxidase activity (Sottomayor *et al.*, 1998). Feeding experiments have indicated AVLB as the direct coupling product of catharanthine and vindoline *in planta*, and that it may be further converted to VBL and VCR by cell-free extracts of *C. roseus*. Interestingly, this reaction seems to be carried out by vacuolar peroxidases, suggesting that the rate-limiting step in the formation of the dimeric alkaloids is the availability of the monomers (Sottomayor *et al.*, 1998; Sundberg & Smith, 2002).

2.3 Developmental Regulation of Vindoline Biosynthesis

Although not all of the enzymes leading to the *bis*-indole precursor vindoline have been fully characterized several initial observations indicated that the pathway would be strictly regulated. In addition, a number of early studies showed that alkaloid biosynthesis could be regulated by a number of biotic and abiotic stimuli and is activated at particular stages of plant development (De Luca & Laflamme, 2001).

2.3.1 Compartmentalization

Monoterpene indole alkaloid biosynthesis is highly compartmentalized. For example, the mRNA for STR has a signal peptide coding region that directs the transport of the enzyme to the vacoule, SGD and T16H are located in the ER and NMT is associated with chloroplast thylakoids, whereas D4H and DAT appear to be cytosolic enzymes. Additional studies using *in situ* RNA hybridization and immunocytochemistry establish the differential cellular distribution of the vindoline pathway in *Catharanthus* (St. Pierre *et al.*, 1999). While *TDC* and *STR* were localized to the epidermis of stems, leaves and flower buds in above ground plant parts, they also appeared in protoderm and cortical cells around the apical meristem of the root tips (De Luca & Laflamme, 2001; De Luca & St. Pierre, 2000; St. Pierre *et al.*, 1999; van der Heijden *et al.*, 2004). Alternately, the mRNAs for *D4H* and *DAT* were preferentially associated with laticifers and idioblast cells of leaves, stems and flower buds (De Luca & St. Pierre, 2000; St. Pierre *et al.*, 1999; van der Heijden *et al.*, 2004). In addition to intracellular specialization involving multiple cellular compartments, the results from this study showed that at least two cell types were involved in elaboration of vindoline biosynthesis in leaves that should require

the translocation of a pathway intermediate (De Luca & St. Pierre, 2000; St. Pierre *et al.*, 1999).

Additional studies (Murata & De Luca 2005) used a new carborundum abrasion technique to localize MIA metabolites, enzyme activities and gene expression of vindoline biosynthetic enzymes at the cellular level. They preferentially detected G10H, SCS, TDC, STR, SGD and T16H in epidermal cells. The results of the study suggested that the whole pathway from primary metabolism to 16-methoxytabersonine may be expressed in leaf epidermal cells, whereas the final 3 or 4 steps in vindoline biosynthesis are expressed in separate cells (mesophyll/idioblast/laticifer) within the leaf (Murata & De Luca, 2005).

2.3.2 Regulation of MIA biosynthesis

2.3.2.1 Regulation of early stages in MIA biosynthesis

Studies involving TDC have shown that its expression is regulated by transcriptional, translational and post-translational controls (De Luca & Laflamme, 2001). The effects of precursor feeding of different transgenic lines of *C. roseus* over-expressing *TDC* and *STR* has been investigated. The addition of secologanin, in general, produced an important increase in the amount of MIAs, at the point of inoculation. However, in combination with tryptophan or tryptamine, feeding of loganin resulted in an even greater increase in alkaloid accumulation in the transgenic lines (Whitmer *et al.*, 2002).

2.3.2.1.1 Elicitor-responsive elements in the promoter of MIA biosynthetic genes

Regions in the promoter of the *TDC* gene have been identified that are responsive to UV-B light and to chemical elicitors (De Luca & Laflamme, 2001). Other studies

involving promoter analyses of the *STR* gene revealed that a GCC-box-like element was both necessary and sufficient to elicit a jasomonic acid (JA) responsive expression of *STR* in *Catharanthus* cell suspension cultures (Menke *et al.*, 1999). Yeast one-hybrid screening identified two ORCA (octadecanoid-derivative-responsive *Catharanthus* APETALA2-domain) proteins that bind in a sequence-specific manner the JA- and elicitor-responsive element. ORCA2 showed trans-activating capabilities of the *Str* promoter and its expression is rapidly induced in the presence of JA and fungal elicitors (Menke *et al.*, 1999). The cloning of *ORCA3* showed it was closely related to ORCA2 (van der Fits & Memelink, 2000).

2.3.2.2 Regulation of late stages in MIA biosynthesis

The later stages of vindoline biosynthesis appears to be regulated differently, since both vindoline and the *bis*-indole alkaloids accumulate in green tissues, but are not found in roots or in cell suspension cultures (Moreno *et al.*, 1995). During seedling development, the enzyme activities of TDC and STR in seedlings are expressed 36-48h prior to D4H and DAT. The activation of D4H and DAT coincides with the conversion of tabersonine into vindoline and requires treatment with light (Vasquez-Flota *et al.*, 2000). However light is not necessary for the development of the specialized cells associated with these two enzymes (Vasquez-Flota *et al.*, 2000). Further, it was determined that a basipetal gradient of expression of TDC, STR, D4H and DAT exists in maturing leaves, suggesting that the expression of the vindoline pathway occurs transiently during development of leaves, stem and roots (St. Pierre *et al.*, 1999). The expression of D4H appears to be partially regulated by phytochrome, since treatment of etiolated seedlings with red-light caused an increase in D4H transcripts, D4H protein and D4H enzyme

activity, whereas the process was reversed by far-red-light treatment (Vazquez-Flota & De Luca, 1998).

In *C. roseus* leaves, the concentration of vindoline, catharanthine and AVLB are age-dependent (Moreno *et al.*, 1995). During leaf maturation, the concentrations of catharanthine and vindoline decreases while the concentration of AVLB increases.

2.4 *Catharanthus roseus* tissue cultures as a source of MIAs

The use of VBL and VCR for the treatment of cancer has created a demand for these compounds. However, the concentration of VBL and VCR in leaves represent only 0.0005% and 0.0002% of the dry weight of the plant, respectively. This means it takes about 200 kg of dried periwinkle leaves to obtain one gram of VBL, and 500kg to produce one gram of VCR. Consequently the isolation of VBL and VCR is laborious and costly (van der Heijden *et al.*, 2004).

Plant cell cultures have proved to be useful in the production of some pharmaceuticals of plant origin. Tissue culture systems have several advantages over the use of cultivated plants, for example continuous rather than seasonal production of compounds is possible, and conditions can be optimized and metabolic pathways can be manipulated for maximum MIA yields (Moreno *et al.*, 1995). The potential to genetically manipulate cell cultures to favour the accumulation of specific metabolites, for example VBL and VCR also exists.

Much early work in MIA production focused on screening for culture conditions that promoted secondary metabolism and accumulation alkaloids. This work was based on the assumption that cell lines possessed the inherent biosynthetic capabilities of the parent plant and just needed induction conditions. Thus cultures derived from the leaves

would synthesize leaf alkaloids while those derived from roots would then synthesize root alkaloids (Moreno *et al.*, 1995; van der Heijden *et al.*, 2004). While this was partially true in some cases, most cell lines tended to lose their biosynthetic capabilities or they lacked pathways that were constitutively expressed in particular tissues or cell types (Moreno *et al.*, 1995; van der Heijden *et al.*, 2004).

2.4.1 Development of *Catharanthus* cell suspension cultures

From early studies in the late 1940s, *Catharanthus roseus* was one of the first species to be used in the development of cell culture techniques using crown-gall tumours (Moreno *et al.*, 1995; van der Heijden *et al.*, 2004). Subsequently, by the beginning of the1980s it was shown that plant cells, as a suspension of single cells in a liquid medium could be grown in bioreactors (Moreno *et al.*, 1995; van der Heijden *et al.*, 2004) and MIA producing *C. roseus* cultures began to be used as a model system for large scale production of alkaloids. Success was limited by the low MIA productivity and to long-term instability of the cultures (Whitmer *et al.*, 2003). It was hoped that genetic engineering could be used to establish highly productive cell lines of *C. roseus* by constitutive over-expression of the *Str* and/or *Tdc* genes (Canal *et al.*, 1998), but these lines only showed high MIA productivity during the first few months of cultivation (Whitmer *et al.*, 2003) and declined over time as a result of instability of the cell lines.

Phytohormones influence secondary metabolism by affecting both culture growth and secondary metabolite production (Whitmer *et al.*, 1998). The influence of various growth regulators on alkaloid production by *C .roseus* cultures has been studied. Auxins have been shown to inhibit alkaloid accumulation and 2, 4-D in the culture medium

rapidly reduced culture aggregation and repressed secondary metabolism (Whitmer *et al.*, 1998).

2.4.1.1. Vindoline production in C. roseus suspension cultures

Suspension cultures of *Catharanthus* do not produce the dimeric indole alkaloids VBL and VCR (Moreno *et al.*, 1995), due to their inability to make vindoline. However, high yielding catharanthine cultures that also accumulate small amounts of tabersonine, have been successfully produced (O'Keefe *et al.*, 1997; Sundberg & Smith, 2002). The lack of vindoline production was attributed to the inability of these cultures to express post-tabersonine enzymes and in particular the last 3 steps in vindoline biosynthesis (NMT, D4H and DAT) (Moreno *et al.*, 1995). Surprisingly, stable low level vindoline production was reported in an undifferentiated *C. roseus* suspension cell line (O'Keefe *et al.*, 1997), but these results have yet to be reproduced by other laboratories. In addition, transformation of *C. roseus* explants with *A. tumefaciens*, resulted in the development of transformed, hormone-independent cultures that contained DAT activity in cell-free extracts (Moreno *et al.*, 1995). Other studies however also showed that a separate root-specific acetyltransferase (Minovincinine-19-*O*-acetyltransferase; Laflamme *et al.*, 2001) that is responsible for the biosynthesis of echitovenine (Fig. 5) with weak DAT activity might have been detected in these studies.

Due to this inability to accumulate vindoline in cell-suspension cultures, various types of differentiated tissue cultures have been used, including shoot cultures via different combinations of auxins and/or cytokinins (van der Heijden *et al.*, 2004). VBL was detected in callus derived from explants. The amount of VBL was increased rapidly following the induction of shoot formation (van der Heijden *et al.*, 2004).

2.4.2 Biosynthesis of MIAs in *Catharanthus* seedlings

The regulation of vindoline biosynthesis lies in the coordinate expression of the enzymes involved in the early and late stages of the process (Vasquez-Flota et al., 2000).

The events that activate the vindoline pathway have been partially elucidated with germinating *C. roseus* seedlings of growing in the dark or in the light (De Luca & St. Pierre, 2000). Etiolated seedlings accumulate high levels of tabersonine, together with small amounts of four tabersonine intermediates involved in vindoline biosynthesis. The addition of light stimulated the large-scale turnover of tabersonine and intermediates into vindoline which suggests that the enzymes from later stages of synthesis are induced in the presence of light (De Luca & St. Pierre, 2000).

2.4.2.1 Jasmonate Elicitation of C. roseus seedlings

Catharanthus seedlings were used to study the light-dependent regulation of D4H expression and the effects of methyl jasmonate (MeJa) treatment. The induction of plant defense mechanisms, for instance by wounding of pathogens, is mediated by jasmonic acid or its volatile methyl ester, MeJa (Aerts *et al.*, 1994). Methyl jasmonate vapor was found to significantly enhance alkaloid biosynthesis during germination, resulting in a doubling of alkaloid content in seedlings, while exogenous application of MeJa resulted in increased allocation of alkaloid precursors and enhanced enzyme activities in alkaloid biosynthesis (Aerts *et al.*, 1994). Interestingly, susceptibility to MeJA was found to be strictly dependent on the developmental stage of the seedlings.

2.4.3 MIA accumulation in *Catharanthus* hairy root cultures

Agrobacterium rhizogenes is a gram-negative soil bacterium, known for its ability to transfer T-DNA from the root-inducing (Ri) plasmid to the plant host genome and thereby causing hairy root disease of plants (Batra *et al.*, 2004). With their apparent amenability to genetic transformation by *Agrobacterium rhizogenes*, genetically stable hairy root cultures of *C. roseus* have been developed and investigated for their ability to produce root-specific MIAs investigated (Bhadra *et al.*, 1993; Rijhwani & Shanks, 1998). Ajmalicine, serpentine, catharanthine and tabersonine are readily synthesized in hairy root cultures of *C. roseus*; however is not (Rijhwani & Shanks, 1998).

Detailed studies that examined the timing of hairy root subculture showed that it significantly affected both growth and alkaloid production (van der Heijden *et al.*, 2004). A two-week cycle yielded the fastest growth rate and the highest lochnericine yields, compared to the slower growth and yield found when a four-week cycle was used (Rijhwani & Shanks, 1998; van der Heijden *et al.*, 2004). In contrast, to lochnericine the accumulation of hörhammericine and tabersonine were independent of growth rates (Rijhwani & Shanks, 1998; van der Heijden *et al.*, 2004). Further studies also revealed enormous variability in the morphology and growth patterns among individual hairy root clones. Based on growth pattern, type of branching and number of lateral roots, hairy root clones were grouped into four categories: less-branched, slow-growing type; highly branched, moderately growing type; profusely branched, fast-growing type; callusing and slow-growing type. While it is not clear why this variation exists, the authors speculated that there was a correlation with the integration sites of specific genes in hairy root cultures of *C. roseus* (Batra *et al.*, 2004).

2.4.3.1 Effects of elicitors on MIA accumulation in hairy roots

Hairy root cultures were treated in late exponential growth phase with elicitors, such as pectinase and jasmonic acid, to examine their effect on MIA accumulation (Rijhwani & Shanks, 1998; van der Heijden *et al.*, 2004). Addition of JA caused an increase in the yields of ajmalicine, serpentine, and induced oxidation of tabersonine to form lochnericine and hörhammericine (Fig. 5) (Rijhwani & Shanks, 1998). Tabersonine derivatives accumulated in a dose-dependent manner, increasing with the level of JA treatment. (Rijhwani & Shanks, 1998). Pectinase treatment of hairy roots increased tabersonine levels by 150% after 48 h, while catabolism of serpentine, lochnericine and tabersonine was observed immediately following treatment (Rijhwani & Shanks, 1998; van der Heijden *et al.*, 2004).

2.4.3.2 MIA accumulation in hairy roots transformed with TDC and ASα

Transgenic hairy roots of *C. roseus* expressing an inducible TDC gene, together with a tryptophan feedback-resistant form anthranilate synthase alpha subunit (ASα) from *Arabidopsis* accumulated tryptamine and serpentine to higher concentration than untransformed hairy root cultures (Hughes *et al.*, 2004). Despite this, increases in overall alkaloid yield however were not observed. A more recent study showed that combined constitutive expression of wild-type *Arabidopsis* ASβ subunit and *Catharanthus* TDC in *C. roseus* hairy roots (Hong *et al.*, 2006) produced lines that accumulated 14 times more tryptamine and had significant changes in MIA production than controls (Hong *et al.*, 2006).

2.4.3.3 Lochnericine and hörhammericine biosynthesis in hairy roots

Lochnericine and hörhammericine (Fig. 5), two oxygenated derivatives of tabersonine, containing an epoxide function at positions 6 and 7, have been detected in root cultures. The presence of these compounds in hairy roots suggests that enzyme-mediated epoxide formation from tabersonine could represent a possible competitive pathway that could divert flux away from the tabersonine to vindoline branch (Rijhwani & Shanks, 1998; Rodriguez *et al.*, 2003).

To investigate the epoxidation and hydroxylation of tabersonine, a dioxygenase and two P450 dependent monoxygenase inhibitors were selected. These inhibitor studies revealed that the accumulation of tabersonine, lochnericine and hörhammericine were associated with growth of hairy roots. Details of tabersonine epoxide formation in hairy roots were obtained by growing roots in the presence of jasmonic acid and various inhibitors. The results suggested that an inducible cytochrome P450 enzyme might be responsible for the formation of hörhammericine (Morgan & Shanks, 1999). The inhibitor study further revealed the ability of lochnericine and hörhammericine to be 'turned over' in hairy root cultures (Morgan & Shanks, 1999). In 2003, a tabersonine 6,7-epoxidase, the enzyme responsible for the conversion of tabersonine to lochnericine, was detected in protein extracts of jasmonate-induced hairy root cultures (Rodriguez *et al.*, 2003). In other studies, a homolog of the DAT gene, minovincinine-*O*-acteyltransferase (MAT), was cloned. MAT is only expressed in cortical cells of the root tip and catalyzes the 19-*O*-acetylation of minovincine and hörhammericine, tabersonine derivatives that have been detected in roots; (Laflamme *et al.*, 2001) (Fig. 5). Northern blot analyses from

hairy root sections showed MAT expression is restricted to root tips and is highly regulated during root development (Laflamme *et al.*, 2001).

2.5 Concluding remarks

Catharanthus roseus and its secondary metabolites have become an important model system in plant biotechnology research. Although large-scale production of *Catharanthus* alkaloids in culture has been an area of significant research interest no commercial production of these alkaloids by tissue culture means has yet been achieved. The technology for large-scale cultivation is available, but the levels of alkaloid production remain too low for commercial use (Moreno *et al*, 1995; van der Heijden *et al*, 2004). Metabolic engineering of specific enzymes and manipulation of *in vitro* culture studies show promise for the improvement of alkaloid production (reviewed in van der Heijden *et al.*, 2004). However, the complexity of the indole alkaloid biosynthetic pathway has presented a major challenge for the enhancement of secondary metabolite production and it appears that a combination of approaches will be essential for enhanced production (Rijhwani & Shanks, 1998). However, for MIA production to be improved it is clear that a better understanding of the regulation of secondary metabolism in *C. roseus* is needed.

Several enzymes involved in the biosynthesis of indole alkaloids have been isolated and characterized, allowing a more detailed study of MIA regulation. Over-expression of key genes and their related enzymes in the MIA biosynthetic pathway may represent a method for improving alkaloid production. Genetic modification of specific genes may also lead to modifications in MIA profile. Potentially, a metabolically

engineered system in which the enzymes between tabersonine and vindoline are expressed may provide the solution to overcome this obstacle, improve alkaloid accumulation and possibly lead to production of the dimeric alkaloids in tissue-culture systems.

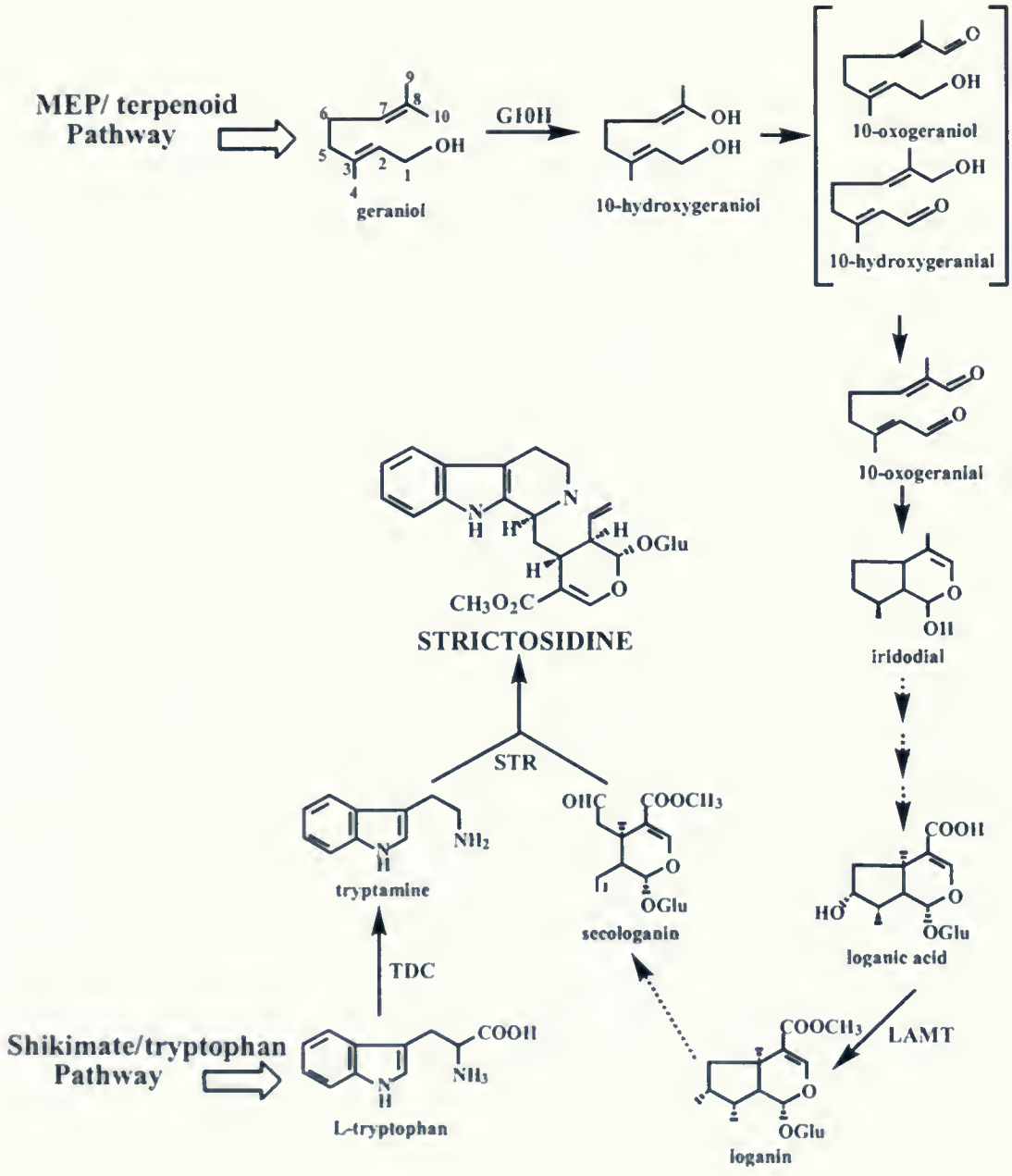

Figure 1. The formation of strictosidine from tryptophan and geraniol. Dotted lines represent uncharacterized enzyme reactions. **TDC**= tryptophan decarboxylase; **G10H**= geraniol –10- hydroxylase; **LAMT**= loganic acid methyltransferase; **STR**= strictosidine synthase. [adapted from van der Heijden *et al.*, 2004]

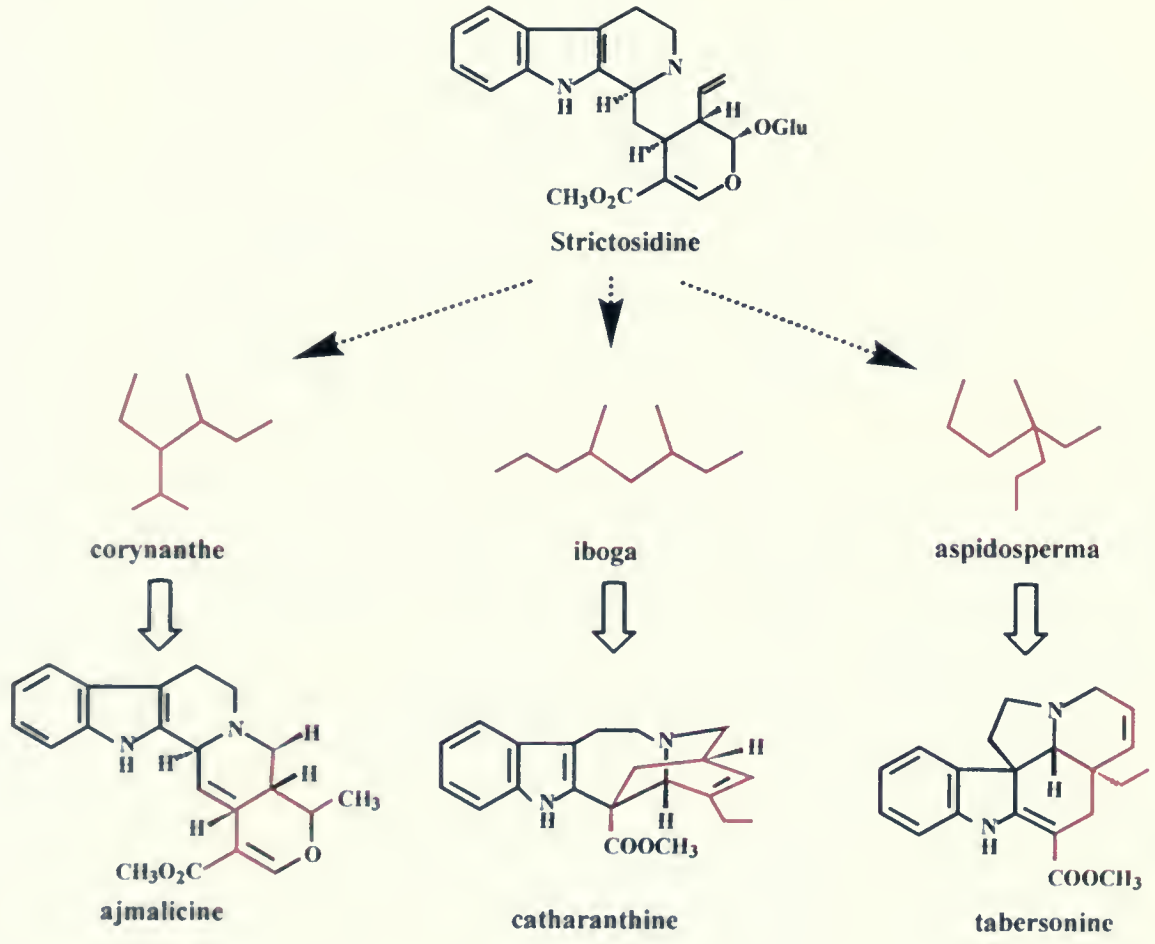

Figure 2. Formation of the Corynanthe, Iboga and Aspidosperma major classes of *Catharanthus* alkaloids derived from the central intermediate, strictosidine. Basic alkaloid skeletons illustrated in red.

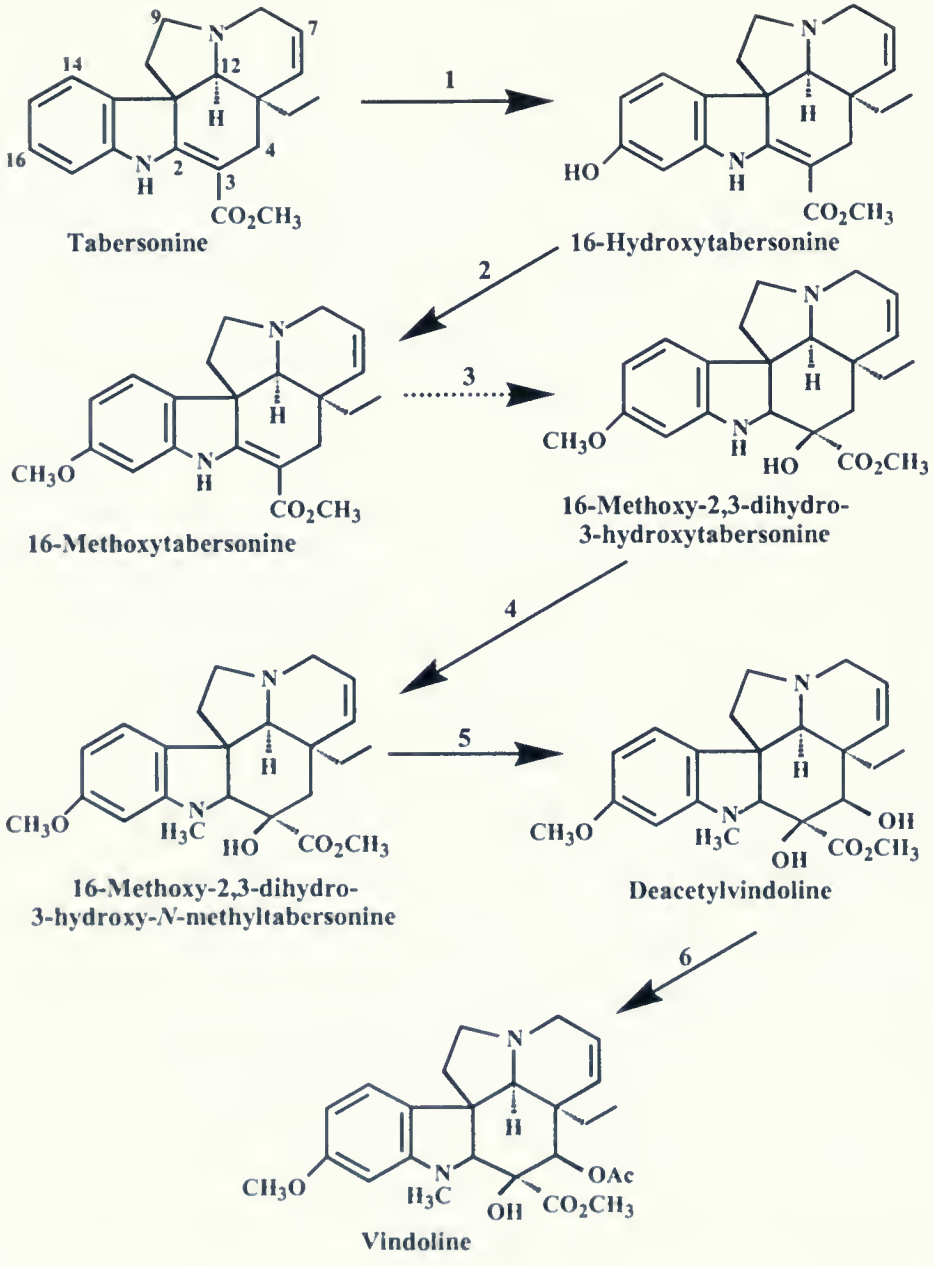

Figure 3. The six steps in the transformation of tabersonine to vindoline. (**1**) tabersonine-16-hydroxylase; (**2**) 16-hydroxytabersonine-*O*-methyltransferase; (**3**) an uncharacterized hydroxylase; (**4**) 2,3-dihydro-3-hydroxytabersonine *N*-methyl-transferase; (**5**) desacetoxyvindoline 4-hydroxylase, D4H; (**6**) acetyl CoA :deactyl-vindoline 4-*O*-acetyltransferase, DAT. [adapted from St-Pierre & De Luca, 1995]

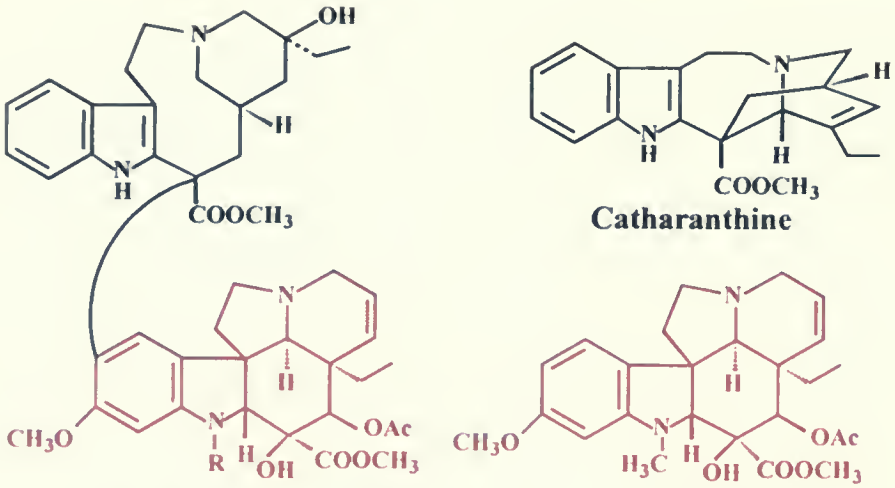

Figure 4. The dimeric antineoplastic agents, vinblastine (VBL) and vincristine (VCR) and their monomeric constituents' vindoline and catharanthine.

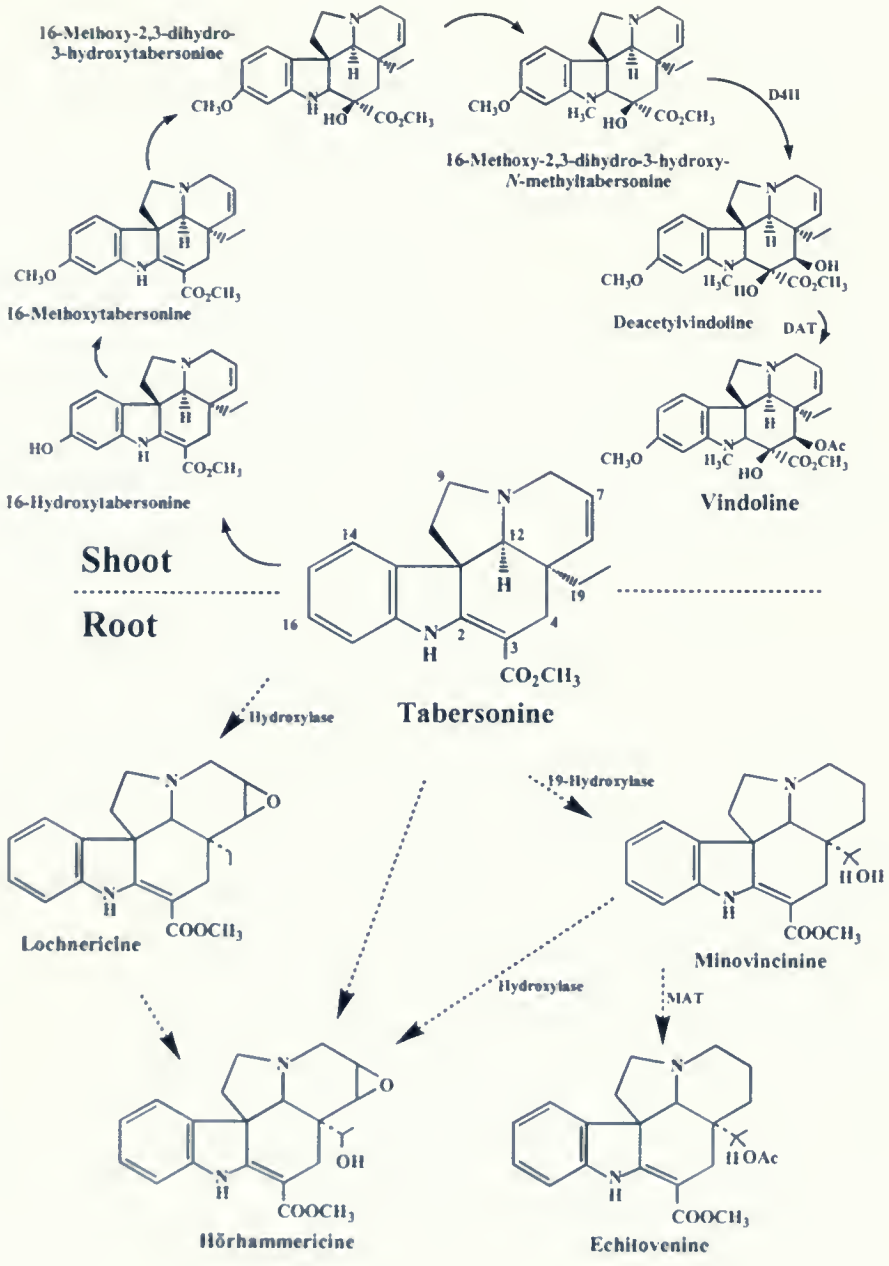

Figure 5. Biosynthesis of tabersonine in *Catharanthus roseus* shoot and root tissues. Tabersonine is converted into vindoline via six enzymatic steps. Tabersonine is converted into lochnericine and hörhammericine via uncharacterized hydroxylations and 19-hydroxy-indole alkaloids are converted into their respective products by *O*-acetylation (MAT). [Laflamme *et al.*, 2001]

3. Identification of a low vindoline accumulating cultivar of *Catharanthus roseus* (L.) G. Don by monoterpenoid indole alkaloid and enzymatic profiling

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Identification of a low vindoline accumulating cultivar of *Catharanthus roseus* (L.) G. Don by monoterpenoid indole alkaloid and enzymatic profiling

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ABSTRACT

The Madagascar periwinkle [*Catharanthus roseus* (L.) G. Don] is a commercially important horticultural flower species and is the only source of the monoterpenoid indole alkaloids (MIAs), vinblastine and vincristine, which are used to combat a number of different cancers. The present study uses high performance liquid chromatography for metabolic profiling of the MIAs extracted from seedlings and young leaves of 50 different flowering cultivars of *Catharanthus roseus* to show that, except for a single low vindoline cultivar (Vinca Mediterranean DP Orchid), they accumulate similar levels of MIAs. Further enzymatic studies with extracts from young leaves and from developing seedlings show that the low vindoline cultivar has a 10-fold lower tabersonine-16-hydroxylase activity than those of *Catharanthus roseus* cv Little Delicata. It is concluded that rapid metabolic and more selective enzymatic profiling of *Catharanthus* mutants could be useful for the identification of a range of altered MIA biosynthesis lines.

INTRODUCTION

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is a perennial tropical plant belonging to the family Apocynaceae that produces more than 100 monoterpenoid indole alkaloids (MIAs) including two commercially important cytotoxic dimeric alkaloids used in cancer chemotherapy. These two *bis*-indole alkaloids, vinblastine (**9**) (Fig. 1) and vincristine (**10**), accumulate in trace amounts in leaves and are formed from the oxidative coupling of catharanthine (**8**) and vindoline (**7**) (Van der Heijden *et al.*, 2005). The pharmacological and therapeutic value of these low yield indole alkaloids has prompted efforts to improve their production and accumulation in plants and cell suspension cultures. Intensive research efforts have highlighted the complexity and strict regulation of the MIA biosynthetic pathway (St. Pierre & De Luca, 1995; St. Pierre *et al.*, 1999; Van der Heijden *et al.*, 2005).

The pathway leading to the production of vindoline (**7**) (Fig. 1) in particular is even more complex since different cell types appear to participate in regulation in addition to developmental, environmental and tissue specific cues (St. Pierre *et al*, 1999; De Luca & St. Pierre, 2000; Murata & De Luca, 2005). Studies with *Catharanthus roseus* cv Little Delicata have shown that transformation of tabersonine (**1**) to vindoline **7** (Fig. 1) requires six strictly ordered enzymatic reactions, involving hydroxylation of tabersonine by a cytochrome P450-dependent monooxygenase, tabersonine 16-hydroxylase (T16H), O-methylation by a cytosolic S-adenosyl-L-methionine (AdoMet)-16-hydroxytabersonine *O*-methyltransferase (16-OMT), an uncharacterized hydration of the 2,3-double bond, a thylakoid associated AdoMet: 2,3-dihydro-16-hydroxytabersonine-*N*-methyltransferase (NMT), a cytosolic 2-oxoglutarate dependent

dioxygenase, desacetoxyvindoline 4-hydroxlase (D4H), and a cytosolic deactylvindoline-4-*O*-acetyltransferase (DAT) (De Luca & Laflamme, 2001; Vasquez-Flota & De Luca, 1998 St. Pierre & De Luca, 1995; Van der Heijden *et al.*, 2004) (Fig. 1). The complexity of this branch of the MIA biosynthetic pathway may explain the inability of cell culture systems to manufacture vindoline (**7**).

While *Catharanthus roseus* is believed to have originated on the island of Madagascar, it is found growing naturally in many tropical countries and is presently grown mainly as an ornamental throughout the world. The medicinal value of MIAs has triggered efforts to increase the levels of these pharmaceuticals by mutation and conventional breeding techniques (Dutta *et al.*, 2005; Pandey-Rai *et al.*, 2003). In addition, the versatility and continuous flowering habit of *Catharanthus roseus* have enhanced breeding efforts to expand the availability of flower colors and their sizes as well as to produce plants with different types of growth (Van der Heijden *et al.*, 2004). Among the numerous ornamental *Catharanthus roseus* available today, little is known about the effects of breeding for flower color or growth habit on the levels of MIAs.

The present study analyzed the MIA alkaloid profiles of 50 different cultivars of flowering *Catharanthus roseus* to show that breeding for changes in flower color or growth habit does not change, with a single exception, the alkaloid patterns or alkaloid contents in young plant shoots compared to our reference standard, *Catharanthus roseus* cv. Little Delicata. A single cultivar, Vinca Mediterranean Dp Orchid (line 49), accumulated 10 times less vindoline (**7**) compared to *Catharanthus roseus* cv. Little Delicata. Enzyme assays of different selected steps in the vindoline pathway showed this line to have 10 times lower tabersonine-16-hydroxylase T16H activity compared to those

of *Catharanthus roseus* cv. Little Delicata. Results obtained with plants could be duplicated with germinating seedlings and provide possible new biochemical and molecular markers for selection of altered alkaloid profiles in *Catharanthus*.

RESULTS AND DISCUSSION

Accumulation of vindoline and catharanthine in seedlings and young leaves

Seedlings from 50 ornamental cultivars of *Catharanthus roseus* were germinated in the dark for 5 d and then exposed to light for a 72 h period before being submitted to alkaloid analysis. Light treatment was required since this activates the terminal stages of vindoline (**7**) biosynthesis (De Luca *et al.*, 1986) and triggers the quantitative conversion of tabersonine (**1**) into vindoline (**7**) (Balsevich *et al.*, 1986). Ten seedlings from each cultivar were separated into cotyledons with hypocotyls and into root tissues before extraction for MIAs in MeOH-H_2O (1:1) and preparation for HPLC analyses. Root tissues from the each cultivar accumulated tabersonine (**1**), hörhammericine and catharanthine (**8**) to highly variable extents, but none were shown to accumulate vindoline (**7**) (data not shown). These data are consistent with what is known about the alkaloid profiles of roots and about the sites of vindoline (**7**) biosynthesis and accumulation in above ground parts of *Catharanthus roseus* (van der Heijden *et al.*, 2004). When extracts of cotyledons with hypocotyls were screened for vindoline (**7**) and catharanthine (**8**) content, significant variation in the accumulation of these alkaloids was observed (Fig. 2A and B). For example lines 48 and 49 did not contain any detectable vindoline (**7**) compared to *C. roseus* cv. Little Delicata (Fig. 2A, line 50) whereas other lines (Fig. 2A, lines 1 to 21) accumulated 2 to 3 times higher levels of vindoline (**7**) compared to *C. roseus* cv. Little Delicata. Similarly, line 24 (Fig. 2B) had 5 times less catharanthine (**8**), whereas many other lines accumulated 2 to 3 times more catharanthine than *C. roseus* cv. Little Delicata.

Based on these results, each flowering cultivar was grown in the field over several months during the summer in order to establish if this variation was maintained by measuring MIAs in young leaves of 15 week old plants. Young leaves were harvested in triplicate from mature plants of each cultivar for alkaloid extraction and preparation for HPLC. The vindoline (**7**) content of all the *Catharanthus roseus* lines, except for line 49, was similar to that found in *Catharanthus roseus* cv Little Delicata (Fig. 2C), varying between 0.4 to 1.0 µg/mg fresh weight of tissue. In contrast the vindoline (**7**) level in line 49 was only 0.1 µg/mg fresh weight of tissue compared to that of *Catharanthus roseus* cv Little Delicata which contained 5 times more vindoline (**7**). The catharanthine (**8**) content in all lines was similar to that found in *Catharanthus roseus* cv Little Delicata (Fig. 2D), varying between 1.5 to 4 µg/mg fresh weight of tissue. Although the variation in vindoline content observed in different seedlings (Fig. 2A) was not maintained in mature plants (Fig. 2C), it is relevant that this combined analysis has produced a single line whose vindoline content was also low in young leaves of mature plants. The results suggest that with the exception of line 49, breeding for modified flower color or for growth habit does not also lead to significant selection for modifications in MIA patterns.

The low vindoline (7) phenotype of line 49 is caused by a decreased tabersonine-16-hydroxylase activity (T16H)

In order to determine if the low vindoline (**7**) phenotype of line 49 was due to changes in specific enzyme activities in the vindoline pathway, the activities of TDC, T16H, NMT, 16-OMT and DAT measured in comparison to those found in *Catharanthus roseus* cv Little Delicata (Fig. 3). The results obtained show that while the specific activities of TDC, NMT, 16-OMT and DAT were very similar between these two lines,

those of T16H were at least 10 times lower in line 49 than those of *Catharanthus roseus* cv Little Delicata (Fig. 3). This low T16H activity of line 49 suggested that this reaction was rate limiting in the conversion of tabersonine (**1**) to 16-hydroxytabersonine (**2**) leading to the lower levels of vindoline (**7**) found in this cultivar.

Developing seedlings of line 49 accumulate low levels of vindoline (7) as a result of reduced T16H activity

The results obtained with mature plants suggested that the lack of vindoline (**7**) production observed with 8 day old light-treated seedlings of line 49 (Fig. 2A) might also be due to low T16H enzyme activity. To test this, five day old etiolated seedlings were grown for a further 48 h in the presence or absence of light and whole seedlings were harvested at different time points in triplicate to perform MIA analysis and T16H enzyme assays. Alkaloid analyses clearly showed that no vindoline (**7**) was detected in etiolated or light-treated line 49, whereas tabersonine (**1**) levels dropped as vindoline (**7**) accumulated in *Catharanthus roseus* cv Little Delicata upon light treatment (Table 1, T = 24 and 48 h). The tabersonine (**1**) levels also dropped in light-treated seedlings of line 49 suggesting that these were converted to other products, but not into vindoline (**7**) (Table 1). Enzyme assays for T16H clearly showed the importance of light on the induction of this activity and on the appearance of vindoline (**7**) in light-treated developing seedlings of *Catharanthus roseus* cv Little Delicata (Fig. 4). These results corresponded with previous studies describing the light induction of T16H activity (Schröder *et al.*, 1999; St Pierre & De Luca, 1995) in this cultivar. In contrast, light treatment of line 49 resulted in virtually no increases in T16H activity in either etiolated or light treated seedlings as compared to those of *Catharanthus roseus* cv Little Delicata. Enzyme activity in 48 h

light treated seedlings of *Catharanthus roseus* cv Little Delicata was 10 fold higher than those of line 49. The similar results obtained with light treated seedlings and with young leaves of mature plants, suggest that the low vindoline (**7**) levels found in line 49 are caused by a decreased T16H activity.

In summary, the present report describes the value of seedlings and young leaves of mature plants to screen for altered MIA production and related pathway enzymes in flowering *Catharanthus roseus* cultivars. The technique can lead to rapid identification of candidate lines modified for alkaloid content and have been successfully used here to identify the biochemical modification that generates the low vindoline line # 49 (cultivar Vinca Mediterranean DP Orchid). These techniques could easily be scaled up for extensive screening of mutant populations of *Catharanthus roseus* and for the rapid identification of a range of altered MIA biosynthesis lines. Such mutant populations could then lead to high yielding lines that accumulate higher levels of commercially useful MIAs. Previous studies with several salt tolerant *Catharanthus roseus* cultivars (Pandey-Rai *et al.*, 2003) and related mutants (Dutta *et al.*, 2004) showed that it is possible to select for increased accumulation particular MIAs, including catharanthine (**8**), vindoline (**7**), vinblastine (**9**), vincristine (**10**) and ajmalicine. This report showed that there might be a correlation between salt tolerance and increased alkaloid production since these lines accumulate high levels of alkaloids compared to a reference flower cultivar known as Pacifica Blush (Fig. 2, line 21). Our results clearly suggest that all the *Catharanthus* flower cultivars described here accumulate low levels of MIAs compared to those of salt tolerant lines found in India (Dutta *et al.*, 2004). It is unfortunate that, perhaps due to the commercial value of these lines, it has not been possible to obtain high

MIA salt tolerant lines for more direct comparative analysis. The seedling and young leaf based MIA and enzymatic screening described in the present report may be very useful in early screening of large populations of mutants that could then be analyzed in greater detail for basic or commercial reasons.

EXPERIMENTAL

Plant materials

Catharanthus roseus seeds of the Vinca Sun series were kindly donated by S&G Seeds (St. Catharines, Ontario). Seeds from other flowering cultivars of *Catharanthus roseus* were purchased from S & G Seeds. The 50 cultivars that were obtained were numbered for experimental identification as follows: 1. Vinca Cooler Coconut; 2. Vinca Blue Pearl; 3. Vinca Pacifica Peach; 4. Vinca Pacific Red; 5, Vinca Cooler Red; 6. Vinca Pacifica Blush; 7. Vinca Cooler Grapes; 8. Vinca Cooler Rose; 9. Vinca Pacifica Apricot; 10. Vinca Pacifica Pink; 11. Vinca Pacifica Cherry Red; 12. Vinca Pacifica Lilac; 13. Vinca Pacifica Burguoy; 14. Vinca Cooler Apricot; 15. Vinca Stardust Orchid; 16. Vinca Cooler Orchid; 17. Vinca Pacifica White; 18. Vinca Cooler Raspberry Red; 19. Vinca Pacifica Polka Dot; 20. Vinca Sunglow ScarletW/Eye; 21. Vinca Sunstorm Bright Red; 22. Vinca Sunshower Pink; 23. Vinca Cooler Icy Pink; 24. Vinca Cooler Peppermint; 25. Vinca Sunshower Orchid W/Eye; 26. Vinca Sunstorm Blush; 27. Vinca Sunstorm Pink; 28. Vinca Sunshower White W/Eye; 29. Vinca Pacifica Coral; 30. Vinca Sunshower Lilac; 31. Vinca Cooler Pink; 32. Vinca Sunstorm Orchid; 33. Vinca Mediterranean Polka DT; 34. Vinca Cooler Strawberry; 35. Vinca Sunstorm Violet W/Eye; 36. Vinca Pacifica Deep Orchid; 37. Vinca Sunstorm White W/Eye; 38. Vinca Pacifica Icy Pink; 39. Vinca Sunstorm Apricot; 40. Vinca Pacifica Orchid Halo; 41. Vinca Apricot Delight; 42. Vinca Mediterranean Lilac; 43. Vinca Pacifica Punch; 44. Vinca Sunstorm Rose W/Eye; 45. Vinca Suoress White W/Eye; 46 Vinca Sunstorm Lilac; 47. Vinca Mediterranean Deep Rose; 48. Vinca Mediterranean WH Broad; 49. Vinca Mediterranean DP Orchid; 50. *Catharanthus roseus* cv. Little Delicata.

Culture medium and culture conditions

Cultivation of Seedlings for alkaloid analysis and enzyme assay

Seeds from different cultivars were surface-sterilized by treatment with a solution of 5% (w/v) NaOCl (commercial bleach, 4% active chlorine) containing a few drops of detergent and shaken for 20 min. Seeds were rinsed 3x with sterile distilled H_2O. Sterilized seeds from each cultivar were germinated on solidified nutrient medium composed of MS basal salts (Murashige & Skoog), 3% sucrose, 3g/L Gelrite. The pH of all media was adjusted to 5.8 before autoclaving. Medium (25ml) was dispensed into each plastic Petri dish (100mm^2). Approximately 25 seeds were used per plate and care was taken to avoid any contact between seeds. The plates were sealed with laboratory film and were grown for 5 d in the dark after which they were transferred to the light using a 16 h photoperiod at 25°C for 3 d. Eight day old seedlings were separated into cotyledons with their hypocotyls and into roots before being extracted in MeOH-H_2O (1:1). In order to perform time courses experiments seeds were germinated and cultivated in the dark for 5 d before continuing cultivation in the dark or were exposed to the 16 h photoperiod described above and whole seedlings were harvested at different time points over a 48 h period to be processed for alkaloid analysis or for enzyme assay.

Cultivation of Seedlings for growing plants

Seeds from the 50 flowering cultivars were treated as described above but these Petri dishes were maintained under light in a growth chamber with a 16 h photoperiod and at 25°C, unless otherwise mentioned. Fourteen days after seed germination seedlings were removed from the growth cabinet and were transferred to gardening soil. *C. roseus*

plants were grown under greenhouse conditions for one month prior to replanting to an outdoor flowerbed during the months of May to September.

Preparation of crude protein extract

Freshly harvested young leaves (800 mg fresh weight) as well as seedlings or seedling parts (10 seedlings, about 150 mg fresh weight) exposed to different light/dark treatments were homogenized in 3 mL 0.1 M Tris-HCL buffer, pH 8.0 and 14 mM β-mercaptoethanol using a mortar and pestle as described by St-Pierre & De Luca (1995). The slurry was filtered through miracloth (Calbiochem, La Jolla, CA), the filtrate was desalted on a Sephadex G-25 PD-10 column (GE Healthcare, Piscataway, New Jersey) and the crude protein extract was used directly for enzyme assays. Unless otherwise stated, the extractions and enzyme assays were performed in triplicate for statistical analysis.

Protein determination

Protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) which is based on the method of Bradford (1976). BSA was used as a standard.

Alkaloid extraction and analyses

Seedlings that had been grown for 5 d in the dark followed by 3 d in a 16 h photoperiod as well as young leaves (50 mg fresh weight) were harvested. Ten seedlings (fresh weight about 150 mg) were separated into cotyledons with hypocotyls and into roots before processing for alkaloid extraction. Each of these tissues were extracted by transferring them to 1.5 mL Eppendorf tubes containing MeOH-H_2O (1:1; 400 µL) and by homogenizing with a Kontes pellet pestle (Fisher Scientific, Canada). After homogenization, samples were centrifuged in an Eppendorf centrifuge for 5 min at

15,000 rpm and the supernatant was filtered through a 0.45 μm Pall filter. The filtrate was analyzed by HPLC as described previously (Tikhomiroff & Jolicoeur, 2002; Murata & DeLuca, 2005). In additional developmental time course experiments, five day old etiolated seedlings were grown for a further 48 h in the presence/absence of light and whole seedlings were harvested at different time points. Ten whole seedlings (about 150 mg Fresh weight) from each time point were extracted by transferring them to 1.5 mL Eppendorf tubes containing MeOH-H_2O (1:1; 400 μL) and were homogenized with a Kontes pellet pestle (Fisher Scientific, Canada). To the homogenate was added 100 μL of 10% sulfuric acid followed by extraction with an equal volume of EtOAc as described by Murata & De Luca (2005). After centrifugation as described above, and discarding the organic phase, the aqueous phase was treated with 10 N NaOH (50 μL), and alkaloids were extracted with an equal volume of EtOAc. The organic phase was collected and evaporated to dryness using an SPD Speed Vac (Thermo Savant, Holbrook, New York); the residue was resuspended in MeOH (300 μL) and filtered through a 0.45 μm Pall filter. The filtered alkaloid extracts were analyzed by HPLC as described above.

Enzyme assays

Young leaves (0.8 g) or seedlings (10 seedlings) were ground in 3 mL of extraction buffer (0.1M Tris, pH 8, 14 mM Mercaptoethanol) using a mortar and pestle. The extract was filtered through miracloth and desalted directly by PD-10 Sephadex G-25 column chromatography (GE Healthcare) and this extract was used directly for enzyme assays. TDC, T16H, 16OMT, NMT and DAT enzyme activities were assayed as described previously (De Luca *et al.*, 1986, 1987; 1989; St. Pierre & De Luca, 1995; St. Pierre *et al.*, 1998). All assays were performed in 100 μL reaction volumes containing

desalted crude protein extract together with various substrates and cofactors related to each enzyme assay: TDC assay [20.8 μM (0.1 μCi) L-Tryptophan (side chain-3-^{14}C), Moravek, Brea, CA, USA]; T16H assay [30 μM tabersonine, 8.33 μM S-adenosyl-L-(methyl-^{14}C)Methionine and 1 mM NADPH]; 16OMT assay [approximately 30 μM 16-OH tabersonine and 8.33 μM (0.1 μCi) S-adenosyl-L-(methyl-^{14}C)Met, GE Healthcare, Buckinghamshire, UK]; NMT assay [30 μM 2,3-dihydro-3-hydroxy-tabersonine and 8.33 μM S-adenosyl-L-(methyl-^{4}C)Met]; DAT assay [30 μM deacetylvindoline and16.6 μM (1-^{14}C)Acetyl-coenzyme A, GE Healthcare]. Unlike the other four enzyme assays, the T16H assay is a coupled assay that detects the production of 16-methoxytabersonine (**3**) from tabersonine (**1**) by coupling it with endogenous 16-OMT activity, as described previously (St. Pierre & De Luca, 1995). Standard assays were conducted at 37°C for 60 min, except for the DAT assay that was incubated for only 30 min. The products from each assay were treated with base (10 N NaOH) to facilitate the extraction of labeled alkaloids into an equal volume of EtOAc and the organic phase that was harvested after separation from the aqueous phase by centrifugation and was taken to dryness by vacuum centrifugation using an SPD Speed Vac (Thermo Savant, Holbrook, New York) system. Each dried sample was dissolved in MeOH (5 μL) and reaction products were separated by analytical thin layer chromatography (TLC) [Polygram Sil G/UV254 (Macherey-Nagel)]. TLC plates were developed in various solvent systems depending on the assay: T16H and 16OMT [Et_2O–hexane (1:1, v/v)]; NMT and DAT [EtOAc-MeOH (9:1, v/v)]; TDC [$CHCl_3$-MeOH–25% NH_4OH (5:4:1, v/v)]. The radioactivity was visualized and quantified by exposure of the TLC to a storage phosphor screen (GE Healthcare,

Piscataway, NJ, USA) for 16 h and emissions were detected using a Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge ver. 3.0 (Fujifilm, Tokyo, Japan).

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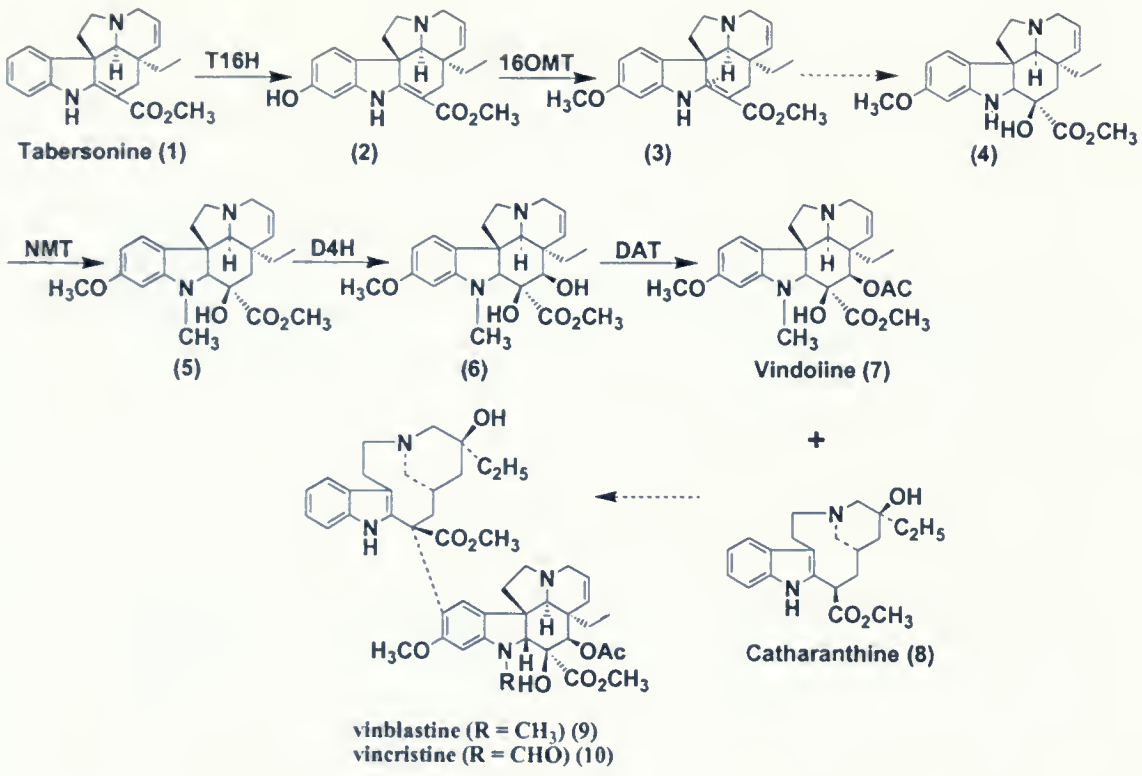

Figure 1. The late stages of vindoline (7) biosynthesis involves hydroxylation of tabersonine by a cytochrome P450-dependent monooxygenase, tabersonine 16-hydroxylase (T16H), O-methylation by a cytosolic S-adenosyl-L-methionine (AdoMet)-16-hydroxytabersonine *O*-methyltransferase (OMT), an uncharacterized hydration of the 2,3-double bond, a thylakoid associated AdoMet: 2,3-dihydro-16-hydroxytabersonine-*N*-methyltransferase (NMT), a cytosolic 2-oxoglutarate dependent dioxygenase, desacetoxyvindoline 4-hydroxlase (D4H), and a cytosolic deactylvindoline-4-*O*-acetyltransferase (DAT)

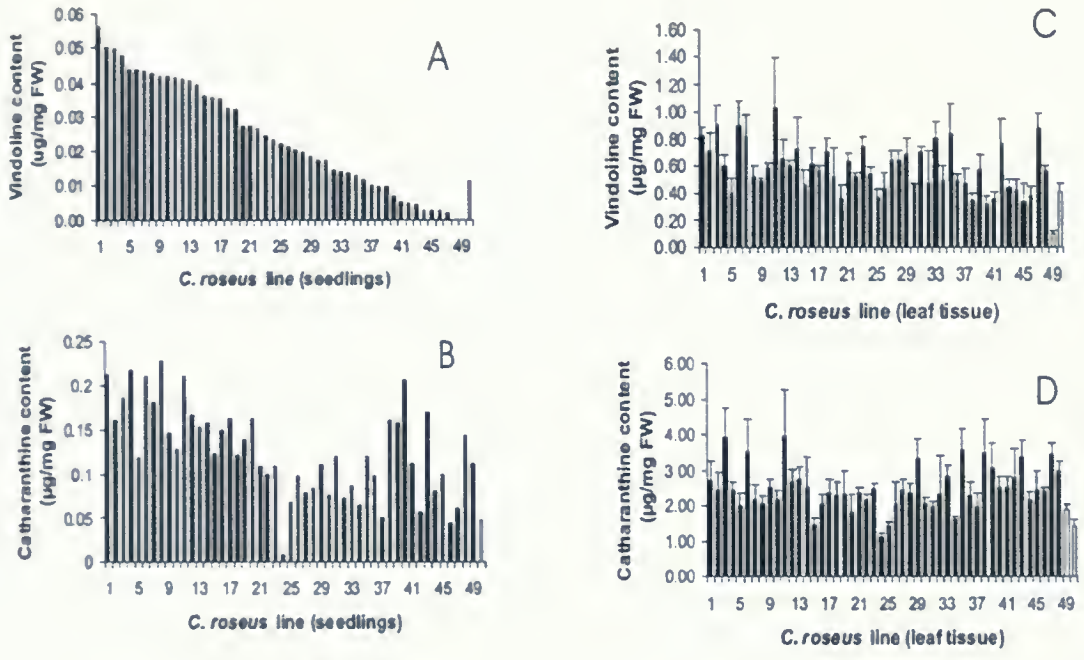

Figure 2. Vindoline (A) (**7**) and catharanthine (B) (**8**) accumulation in cotyledons with hypocotyls of 50 flower cultivars of C. roseus. Vindoline (C) (**7**) and catharanthine (D) (**8**) accumulation in young leaves of 50 cultivars of *Cathatanthus roseus*. The data obtained for the *Catharanthus roseus* cv Little Delicata reference cultivar and the low vindoline accumulating line # 49 are highlighted in white bars compared to the rest of the cultivars that are in black. The cultivars are numbered as follows: 1. Vinca Cooler Coconut; 2. Vinca Blue Pearl; 3. Vinca Pacifica Peach; 4. Vinca Pacific Red; 5, Vinca Cooler Red; 6. Vinca Pacifica Blush; 7. Vinca Cooler Grapes; 8. Vinca Cooler Rose; 9. Vinca Pacifica Apricot; 10. Vinca Pacifica Pink; 11. Vinca Pacifica Cherry Red; 12. Vinca Pacifica Lilac; 13. Vinca Pacifica Burguoy; 14. Vinca Cooler Apricot; 15. Vinca Stardust Orchid; 16. Vinca Cooler Orchid; 17. Vinca Pacifica White; 18. Vinca Cooler

Raspberry Red; 19. Vinca Pacifica Polka Dot; 20. Vinca Sunglow ScarletW/Eye; 21. Vinca Sunstorm Bright Red; 22. Vinca Sunshower Pink; 23. Vinca Cooler Icy Pink; 24. Vinca Cooler Peppermint; 25. Vinca Sunshower Orchid W/Eye; 26. Vinca Sunstorm Blush; 27. Vinca Sunstorm Pink; 28. Vinca Sunshower White W/Eye; 29. Vinca Pacifica Coral; 30. Vinca Sunshower Lilac; 31. Vinca Cooler Pink; 32. Vinca Sunstorm Orchid; 33. Vinca Mediterranean Polka DT; 34. Vinca Cooler Strawberry; 35. Vinca Sunstorm Violet W/Eye; 36. Vinca Pacifica Deep Orchid; 37. Vinca Sunstorm White W/Eye; 38. Vinca Pacifica Icy Pink; 39. Vinca Sunstorm Apricot; 40. Vinca Pacifica Orchid Halo; 41. Vinca Apricot Delight; 42. Vinca Mediterranean Lilac; 43. Vinca Pacifica Punch; 44. Vinca Sunstorm Rose W/Eye; 45. Vinca Suoress White W/Eye; 46 Vinca Sunstorm Lilac; 47. Vinca Mediterranean Deep Rose; 48. Vinca Mediterranean WH Broad; 49. Vinca Mediterranean DP Orchid; 50. *Catharanthus roseus* cv. Little Delicata

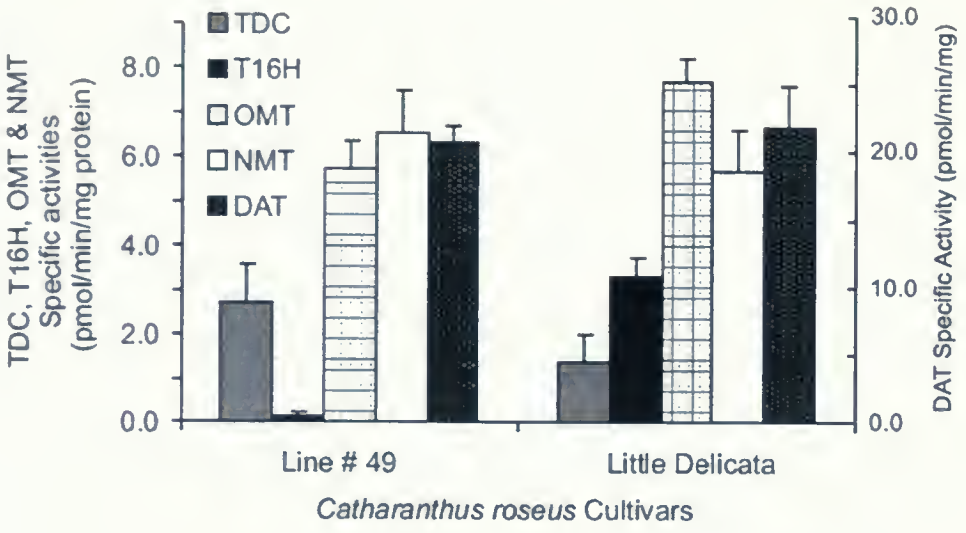

Figure 3. Specific activity of TDC, T16H, NMT, OMT and DAT enzyme activities in young leaves of line 49 and of *Catharanthus roseus* cv Little Delicata.

Table 1: Vindoline (**7**) and tabersonine (**1**) accumulation in line 49 and *Catharanthus roseus* cv Little Delicata 5-day old etiolated seedlings exposed to dark/light for 12, 24, and 48h, respectively.

Catharanthus roseus Cultivar	Light (hrs)	Vindoline (**7**) (μg/mg FW)		Tabersonine (**1**) (μg/mg FW)	
		Light	Dark	Light	Dark
Line 49	0		0		0.048
	12	0	0	0.020 ± 0.006	0.019
	24	0	0	0.017 ± 0.002	0.036
	48	0	0	0.008 ± 0.002	0.039
Little Delicata	0		0		0.026
	12	0	0	0.008 ± 0.004	0.011
	24	0.004 ± 0.001	0	0.006 ± 0.001	0.015
	48	0.018 ± 0.005	0.001	0.002 ± 0.002	0.027

The light exposed seedling data are the means of 3 replicates, whereas only single measurements were taken for dark grown seedlings

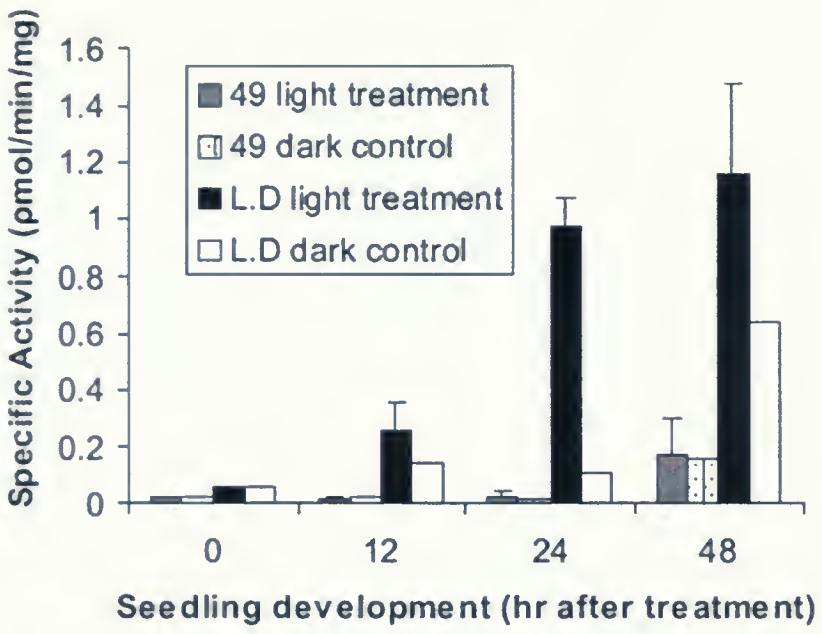

Figure 4. Specific activity of T16H enzyme activity in young seedlings of line 49 and *Catharanthus roseus* cv Little Delicata (L.D.). Five-day old etiolated seedlings were exposed to light or dark conditions and were harvested after an additional 0, 12, 24, and 48 h of growth, respectively.

4. Expression of deacetylvindoline-4-*O*-acetyltransferase in *Catharanthus roseus* hairy roots

This manuscript was submitted to Phytochemistry for review

Expression of deacetylvindoline-4-*O*-acetyltransferase in *Catharanthus roseus* hairy roots

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ABSTRACT

The Madagascar periwinkle [*Catharanthus roseus* (L.) G Don] is a pantropical plant of horticultural value that produces the powerful anticancer drugs vinblastine and vincristine. The present study describes the genetic engineering and expression of the terminal step of vindoline biosynthesis, deacetylvindoline-4-*O*-acetyltransferase (DAT) in *Catharanthus roseus* hairy root cultures. Biochemical analyses showed that several hairy root lines expressed high levels of DAT enzyme activity compared to control hairy root cultures expressing GUS activity. Metabolite analysis using high performance liquid chromotagraphy revealed an altered alkaloid profile with respect to hörhammericine accumulation in DAT expressing lines in comparison to control lines. Further analyses of one hairy root culture expressing high DAT activity suggested a relationship between DAT expression and accumulation of hörhammericine (**9**). It is concluded that expression of DAT in hairy roots altered their MIA profile and suggest that further expression of vindoline pathway genes could lead to significant changes in alkaloid profiles. Evidence is provided that hörhammericine (**9**) accumulates via a DAT interaction with minovincinine-*O*-acteyltransferase (MAT) that inhibits the MAT mediated conversion of hörhammericine (**9**) into 19-*O*-acetyl-hörhammericine (**12**).

INTRODUCTION

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is a tropical plant that belongs to the family Apocynaceae. The presence of the therapeutically valuable alkaloids vinblastine and vincristine in *Catharanthus roseus* has justified many biotechnological studies to increase their production. The low yields of these dimeric monoterpenoid indole alkaloids (MIAs) have also prompted extensive efforts to develop inexpensive and efficient chemical synthesis, but their chemical complexity has made this task difficult and MIAs continue to be obtained from biological sources for commercial uses (Hughes *et al.*, 2004; Laflamme *et al.*, 2001). The possibility for inexpensive production of these *bis*-indole alkaloids by cell and tissue cultures has led to the use of *C. roseus* as an experimental system for plant metabolic engineering (Hughes *et al*, 2004).

Hairy root cultures represent an alternative to cell suspension cultures for production of secondary metabolites (Rijhwani & Shanks, 1998). The advantages of hairy roots in comparison to cell culture systems include their apparent genotypic and biochemical stability, morphological differentiation and growth in hormone-free media (Rijhwani & Shanks, 1998; Rodriguez *et al.*, 2003). Several environmental conditions have been shown to influence the production of MIAs in *C. roseus* cultures (Moreno *et al.*, 1995), including the composition of the culture medium, light treatment and temperature conditions.

C. roseus cell suspension and hairy root cultures do not produce the two *bis*-indole alkaloids due to their inability to make the vindoline (**6**) part of dimeric MIAs, while they do accumulate the catharanthine component of dimeric MIAs as well as

tabersonine (**1**), a precursor of vindoline (**6**) (Hong *et al.*, 2006; Rodriguez *et al.*, 2003). While tabersonine (**1**) is converted into vindoline (**6**) by six enzymatic steps in the above ground plant parts (Fig. 1), hairy roots and cell cultures convert tabersonine (**1**) to the oxygenated derivatives, lochnericine (**8**) and hörhammericine (**9**) (Shanks *et al.*, 1998). The accumulation of these root-derived MIAs has also been shown to increase in response to treatment with jasmonic acid, with the coordinate decrease of tabersonine levels (Morgan & Shanks, 1999; Rijhwani & Shanks, 1998). The jasmonic acid activated conversion of tabersonine (**1**) to lochnericine (**8**) and hörhammericine (**9**) in hairy root cultures is affected by inhibitors of oxygenases and has been reported (Morgan & Shanks, 1999; Rodriguez *et al.*, 2003). While the exact pathway and enzymes responsible for the biosynthesis of tabersonine and its metabolism in roots are presently poorly characterized, the oxidation reactions provide a competitive pathway that may prevent the biosynthesis of vindoline (**6**) by diverting away the flux of tabersonine (**1**) (Rodriguez *et al.*, 2003). In addition, the late steps of vindoline biosynthesis have been studied in detail and require specialized cell types, idioblast and laticifer cells that are located in stems and leaves, for part of their biosynthesis (St. Pierre *et al.*, 1999). Root cultures lack these cell types and are therefore incapable of producing vinblastine and vincristine (Vasquez-Flota *et al*, 2002). For these reasons the metabolic engineering of vindoline production in hairy roots will probably require expression of the last 6 enzymatic steps and the removal of the oxidative reactions for diverting tabersonine (**1**) into lochnericine (**8**) and hörhammericine (**9**).

Since suitable plant transformation and regeneration protocols have yet to been firmly established, tissue culture systems (cell suspension and hairy root cultures) alone

have been useful for genetic engineering of *C. roseus* tissues (Hughes *et al.*, 2004). The present study describes the expression of the last steps in vindoline (**6**) biosynthesis, deacetylvindoline-4-*O*-acetyltransferase (DAT), in hairy root cultures. Enzymatic profiling identified several hairy root lines that expressed high DAT activities compared to non-expressing control lines. Furthermore, high performance liquid chromatography of MIAs extracted from transformed hairy roots revealed that DAT expressing lines had an altered MIA profile. Further analysis of a highly active line, DAT 7, revealed that hörhammericine (**9**) accumulation increased with DAT activity and with root maturation. Additional studies provided evidence that hörhammericine (**9**) accumulates via a DAT interaction with MAT that inhibits the MAT mediated conversion of hörhammericine (**9**) into 19-*O*-acetyl-hörhammericine (**12**). The results obtained provide new unsuspected insights for the problems to be encountered for metabolic engineering of vindoline biosynthesis in hairy root cultures.

RESULTS AND DISCUSSION

Generation of transgenic N. tabacum plants and C. roseus hairy root lines

Leaves of *in vitro* grown *N. tabacum* were infected with *Agrobacterium tumefaciens* LBA 4404 harboring the binary plasmids pBI121/DAT and pBI121/GUS (Fig. 2). The plasmid pBI121/DAT contains the coding region of the wild-type *C. roseus* DAT gene under the control of the CaMV 35S promoter (Fig. 2B). It also contains a marker for kanamycin selection. The plasmid pBI121/GUS permits expression of the GUS reporter gene under the control of the CaMV 35S promoter and also contains a kanamycin selection marker (Fig. 2C). Following co-cultivation of wounded leaves with *Agrobacterium* harboring either plasmid, these were maintained on solid selection media containing 100 mg/L kanamycin and 400 mg/L of cefatoxime (as described in experimental). After several weeks of cultivation, 15 independent pBI/GUS and pBI/DAT transformed calli were transferred to shoot meristem generating medium. Of the 20 green shoots adapted to kanamycin selection media, three DAT lines and three GUS lines were transferred and maintained in Majenta boxes.

Leaves of *C. roseus* were infected with *Agrobacterium rhizogenes* R1000 carrying the plasmids pBI121/GUS and pBI121/DAT. Of the 30 pBI/GUS hairy roots excised, only 15 grew significantly on solid selection media containing 100 mg/L kanamycin and 400 mg/L of cefatoxime. Of these, only 8 adapted to long term maintenance on liquid media. Of the 50 pBI/DAT hairy roots excised, 33 showed strong growth on solid selection media containing antibiotics. As reported by Bhadra *et al.* (1993) the most difficult step is the adaptation of *C. roseus* hairy roots to liquid media. While thirty pBI/DAT hairy root lines grew well on solid selection media containing

antibiotics, only 10 of these lines could be maintained in liquid media. There was significant variability in the morphology and growth patterns in individual root clones (Fig. 3), in spite of continuous cultivation of these pBI/DAT hairy root lines in liquid media for the past 12 months (Bhadra *et al.*, 1993). Similar variable growth morphology was also observed by Batra *et al.* (2004) who speculated that the variation was due to separate transformation events, although there is no experimental evidence for this suggestion. The initial generation of DAT expressing hairy roots in MS medium prior to transfer in half strength B5 medium may have also contributed to the differences observed in the type of branching and lateral root growth of the transgenic lines. This is suggested by subsequent experiments that generation of *Catharanthus* hairy roots directly on half strength B5 medium produced very uniform hairy root cultures that could be more easily transferred to liquid medium after only 6 to 8 weeks of initiating the transformation (data not shown).

An additional *Catharanthus* hairy root line R/J1, generated in 1993 as described in Vazquez-Flota *et al.*, 1994, was also used for comparative analysis.

Expression of deactylvindoline-4-O-acetyltransferase in leaves of N. tabacum and C. roseus hairy roots

The *DAT* gene was expressed and tested for activity in tobacco since this plant does not have this enzyme activity. While several *DAT* and *GUS* expressing transgenic tobacco were produced, the leaves of only 3 plants were assayed for the presence of DAT enzyme activities and these were compared with the activities occurring in negative control *GUS* expressing tobacco and in positive control *Catharanthus roseus* cv. Little

delicata leaves (Fig. 4). The results showed that *DAT* transformed tobacco produced variable but low levels of DAT activity, depending on the tobacco plant assayed compared to the GUS expressing tobacco that did not have any activity. While the specific activity of DAT in transformed tobacco was at least 10 fold lower than the levels of enzyme found in *C. roseus* leaf extracts (Fig. 4), these studies suggested that the vector pBI121/DAT should produce a functionally active DAT in *C. roseus* hairy roots.

DAT enzyme activities were assayed in extracts obtained from 10 independently *DAT* transformed *C. roseus* hairy root lines in comparison to the activities found in *Catharanthus roseus* leaves, in a GUS expressing hairy root culture and from the well-established hairy root line R/J1 (Fig. 5). All lines were harvested in triplicate for enzyme assay. Significant variation in DAT activity was observed in independently transformed DAT expressing hairy roots (specific activities between 1 to 200 pmol/min/mg of protein) compared with *Catharanthus* leaves that displayed a specific activity of 100 pmol/min/mg protein. In contrast, neither GUS expressing hairy roots nor line R/J1 expressed any detectable DAT activity. For example, lines DAT 3 and 7 contain DAT activities that were 100 and 200 times higher than the control hairy root lines whereas other *DAT* expressing lines were 20 times more active than those of control lines. In contrast, the high DAT expressing line 7 expresses 2-fold higher DAT activity than enzyme extracted from *C. roseus* leaves (Fig. 5). These results suggest that over-expression of *DAT* in hairy roots of *C. roseus* can produce high enzymatic activity of DAT and this raised the question whether this modification could alter the patterns of MIAs produced in DAT expressing hairy root.

Accumulation of tabersonine (1), lochnericine (8) and hörhammericine (9) in hairy roots

Hairy roots (R/J1, GUS and lines DAT 1 to DAT 10) were cultivated over a 4 week period and triplicate cultures of each line were harvested in preparation for alkaloid extraction and analysis by HPLC. Root tissues from all DAT transformed lines accumulated similar levels of tabersonine (**1**) and lochnericine (**8**) compared to the levels found in control hairy root lines, varying between 0 to 0.2 ng/mg fresh weight of tissue for tabersonine (**1**) and 0 to 1.0 ng/mg fresh weight of tissue for lochnericine (**8**) (Fig. 6). In contrast the hörhammericine (**9**) levels varied significantly among the hairy root lines compared to both the GUS expressing and R/J1 line. For example, line DAT 7 exhibited 4 times higher levels of hörhammericine (**9**) compared to either control line while other DAT lines accumulated lower levels of this alkaloid (Fig. 6). The important increase in accumulation of hörhammericine (**9**), a non *O*-acetylated MIA, in line DAT 7 is difficult to explain since this pattern of accumulation does not appear to be correlated with the variations in DAT specific enzyme activities observed between different DAT transformed lines (Fig. 7) and it is known that hörhammericine (**9**) is a substrate for MAT but not for DAT (Laflamme *et al.* (2001).

Expression of DAT activity and accumulation of hörhammericine are correlated with root maturation in hairy root line DAT 7

In order to further analyze the high DAT activities and increased hörhammericine (**9**) accumulation of hairy root line DAT 7, individual hairy roots were harvested from a 4-week-old culture. Each hairy root was divided into 0.5 to 1 cm sections that were

combined to generate 4 separate developmental stages from young root tips to the more mature parts of the root (Fig. 7). Each stage of root development was extracted for MAT and DAT enzyme assays (Fig. 7A), MAT and DAT gene expression (Fig. 7B) and for MIA analysis (Fig. 7C). The enzyme activity of MAT was highest in the root tip (3.5 pkat/mg protein) and it decreased rapidly in developmentally older root sections for both hairy root lines (Fig. 7A). These results correlated with previous studies by Laflamme *et al.* (2001) where *MAT* gene expression was localized to the root cortex and epidermal tissues of the root tip. The lack of DAT enzyme activity in line R/J1 (Fig. 7A) was expected since previous studies localized DAT expression only to laticifers and idioblasts of aerial tissues (St. Pierre *et al.*, 1999). In contrast DAT enzyme activity in line DAT 7 increased significantly with root maturity (Fig. 7A), perhaps as a result of expression of *DAT* driven by the constitutive CaMV 35S promoter. These effects of the CaMV 35S promotor were confirmed by histochemical staining of control GUS expressing hairy roots. The distribution of GUS obtained in the stained tissue of stably transformed roots (Fig. 8) suggests a similar distribution of DAT enzyme activity driven by the CaMV 35 promoter (Fig. 7A). This is also consistent with previous histochemical localization studies of GUS activity confirming that the CaMV 35S promoter is active in the root cortex and confers comparable levels of GUS expression and activity in transgenic root tissue (Battraw & Hall, 1990). RT-PCR analysis to measure relative *DAT* mRNA levels showed consistent expression profiles (Fig. 7B) with the enzyme results (Fig. 7A). The expression of *DAT* transcripts throughout the length of the root in line DAT 7 appeared similar. In contrast, *MAT* expression was preferentially distribution in younger root tissue (root tip-1cm) whereas it declined with root maturity (the section 3cm from the root tip)

(Fig. 7B). The results for MAT expression in lines DAT 7 and R/J1 were consistent with those obtained by northern blot analysis of hairy root tissue line R/J1 obtained by Laflamme *et al.* (2001) and correlated with MAT activities observed in both hairy root lines (Fig. 7A).

MIA analyses clearly showed an increasing accumulation of hörhammericine (**9**) with root maturity in both hairy root cultures, but hörhammericine levels were much higher in line DAT 7 (Fig. 7C). For example R/J1 accumulated 5.5 ng of hörhammericine (**9**)/mg fresh weight of tissue in developmentally older hairy root sections (3.0 cm) whereas line DAT 7 accumulated 3 times more of this MIA. In contrast, the tabersonine (**1**) content in line DAT 7 was similar to that found in R/J1, varying between 0.01 to 0.1 ng/mg fresh weight of tissue in the root tip to older section 3.0 cm from the root tip (Fig. 7C). Similarly, there was little variation observed in lochnericine (**8**) levels in different one cm-sections in either of the hairy root lines. The increased accumulation of hörhammericine (**9**) in line DAT 7 is clearly correlated with the increase in DAT enzyme with root maturation (Fig. 7), but it is difficult to explain how expression of this gene in hairy roots could lead to these results.

Expression of DAT activity in Catharanthus hairy roots interferes with MAT activity and leads to the accumulation of hörhammericine (9).

Southern blot analysis have suggested that DAT and MAT occur in *Catharanthus roseus* as single-copy genes, with the root-specific MAT sharing 63% nucleic acid and 78% amino acid identities with the above-ground specific *DAT* gene (Laflamme *et al.*, 2001). *In vitro* kinetic studies with proteins produced in *E. coli* showed that rDAT only

accepted deacetylvindoline (**5**) as a substrate, while rMAT did accept minovincinine, hörhammericine (**9**) and deacetylvindoline (**5**) as substrates (Laflamme *et al.*, 2001). In addition, the catalytic efficiencies and turnover rates of rMAT were very poor compared to those of rDAT whose turnover rate for Acetyl CoA and DAV was approximately 240- and 10,000-fold greater than that of rMAT (Laflamme *et al.*, 2001). In order to address the unusual effects of DAT on the accumulation of hörhammericine (**9**), rDAT and rMAT were produced in *E. coli* and their respective enzyme activities were compared. Remarkably, incubation of MAT with varying amounts of pure rDAT inhibited MAT activity in a dose dependent manner (Fig. 9A). Similarly, incubation of DAT with varying amounts of crude rMAT inhibited DAT activity in a dose dependent manner (Fig. 9B). In contrast when either experiment was repeated with varying levels of bovine serum albumin, this protein did not inhibit the activities of either rDAT or rMAT (data not shown). Additional studies with DAT showed that enzyme activity was not inhibited by varying concentrations of hörhammericine (**9**) (0, 3.75, 7.5, 15 and 30 μM) this suggests that the mechanism of accumulation of this alkaloid does not involve its binding to DAT and preventing its conversion to 19-*O*-acetyl-hörhammericine (**12**) by MAT (data not shown). Together the results suggest that inhibition of MAT appears to be caused by an interaction between the MAT and DAT proteins.

In summary, the present report describes the first step in the expression of late stages of vindoline biosynthesis in hairy root cultures. While it was not surprising that expression of the terminal step in vindoline biosynthesis was not sufficient to make vindoline in hairy root cultures, it was surprising that a non-*O*-acetylated MIA, hörhammericine, accumulated to 4-fold higher levels in line DAT 7 than in control lines.

Catharanthus hairy roots are well known to accumulate hörhammericine rather than 19-acetoxyhörhammericine and studies with inhibitors suggested that both lochnericine and hörhammericine turnover and breakdown during growth (Morgan and Shanks, 1999). The direct inhibition of rMAT activity by rDAT protein provides a plausible explanation for the results observed in this study. The inhibition of rMAT activity by rDAT suggests that these proteins interact in a novel manner that remains to be investigated.

This study also illustrates the potential of future efforts to express the last six steps in vindoline biosynthesis in order to generate transgenic vindoline accumulating hairy roots. The results show that several unexpected factors may negatively affect efforts to achieve this objective unless reactions competing for tabersonine can be eliminated.

EXPERIMENTAL

Construction of plant expression cassettes

Expression cassettes for *DAT* were constructed by amplifying the *DAT* cDNA from plasmid pQE30 expression vector using primers for the addition of BamHI and SacI restriction enzyme sites. A BamHI/SacI fragment containing the *DAT* cDNA was cloned into the binary vector pBI121 downstream of the CaMV 35S promoter following the initial removal of the GUS reporter gene from the vector. This construct was named pBI121/DAT.

Preparation and growth of sterile plant material

Seeds from *Catharanthus roseus* cv. Little Delicata and *Nicotiana tabacum* SR1 were surface-sterilized by treatment with a solution of 5% (w/v) NaOCl (commercial bleach, 4% active chlorine) containing a few drops of detergent and shaken for 20min. Seeds were rinsed 3x with sterile distilled water. Sterilized seeds were germinated on solidified nutrient medium composed of MS basal salts (Murashige & Skoog), 3% sucrose, 3 g/L gelrite. The pH of all media was adjusted to 5.8 before autoclaving. Medium (25 mL) was dispensed into each plastic Petri dish (100 mm^2). Approximately 25 seeds were used per plate and care was taken to avoid any contact between seeds. The plates were sealed with laboratory film and were grown using a 16 h photoperiod at 25°C. Individual fourteen day old seedlings were transferred to Majenta boxes containing 50 mL of solidified nutrient medium composed of WPM basal salts (Woody Plant Medium), 3% sucrose, 2.6 g/L gelrite, 2.5 g/L activated charcoal, 2g/L IBA (indole 3-butyric acid) and 1 mM of silver thiosulfate following autoclaving (pH 5.8). Majenta boxes were sealed with laboratory film and continued to grow in the same conditions (16 h photoperiod at 25°C). Young leaves from 6 week old plants were harvested for hairy root induction.

Generation of transformed *Catharanthus* hairy roots and tobacco plants

The vectors, pBI121/GUS and pBI121/DAT were transformed into *Agrobacterium rhizogenes* strain R1000 and *Agrobacterium tumefaciens* LBA 4404 by electroporation. A single transformed colony from each combination growing on solid LB kanamycin (50 mg/L) agar medium were harvested and were used to start a 50 mL culture grown at 28°C and 200 rpm for 16 h in liquid LB/kanamycin medium. Leaves from *C. roseus* and *N. tabacum* were wounded, infected with one of each respective *Agrobacterium*/construct strain and incubated in the dark for 48 h on solid MS/kanamycin (100 mg/L) medium.

Following the co-cultivation period, *Catharanthus* leaves/*Agrobacterium rhizogenes* cocktails were transferred to fresh plates containing cefatoxime (400 ppm) to kill the remaining bacteria and after 6 weeks of cultivation, the hairy roots that were formed were excised from independently transformed leaves and selected as independent hairy root clones on liquid MS media containing kanamycin and cefatoxime. Once no further signs of contamination of *Agrobacterium* appeared, hairy root cultures were then maintained in an antibiotic free sterilized solution of 50 mL half strength Gamborg's B5 salts, 2% sucrose (pH 5.8) into a 250 mL Erlenmeyer flask. The cultures were grown on rotary shakers at 25°C at 100 rpm in the dark and transferred routinely to fresh medium every 5-6 weeks.

Following the co-cultivation period, *N. tabacum* leaves/*Agrobacterium tumefaciens* cocktails were transferred to solid MS medium, composed of 3% sucrose, 3 g/L gelrite (pH 5.8) and filter-sterilized zeatin (1 ppm), kanamycin (100 ppm) and cefatoxime (400 ppm) following autoclaving. After 3-4 weeks calli began to form and were transferred to a shoot meristem generating medium containing NN basal salts (Nitsch & Nitsch), 2% sucrose, 3 g/L gelrite (pH 5.8), kanamycin (100 ppm) and cefatoxime (400 ppm). Antibiotic resistance shoots were maintained in Majenta boxes

containing 50 mL of solidified NN media (2% sucrose, 3 g/L gelrite, pH 5.8). Majenta boxes were sealed with laboratory film and continued to grow in a 16 h photoperiod at 25°C.

Preparation of crude protein extracts from *Catharanthus* hairy roots and tobacco leaves

Freshly harvested *Catharanthus* hairy roots (about 500 mg fresh weight) and young leaves (1.0 g fresh weight) from transformed tobacco plants were homogenized in 3 mL of extraction buffer (0.1 M Tris-HCl buffer, pH 8.0 and 14 mM β-mercaptoethanol) using a mortal and pestle. The slurry was filtered through miracloth (Calbiochem, La Jolla, CA), the filtrate was centrifuged in an Eppendorf centrifuge for 4 minutes at 15,000 rpm and the supernatant was desalted on a Sephadex G-25 PD-10 column (GE Healthcare, Piscataway, New Jersey). This crude desalted protein extract was directly used for DAT enzyme assays. Unless otherwise stated, the extractions and enzyme assays were performed in triplicate for statistical analysis.

Alkaloid extraction and analyses of *Catharanthus* hairy roots transformed pBI121/GUS and pBI/DAT

Whole hairy roots (about 500 mg fresh weight) were harvested and used directly for alkaloid extraction. Each tissue was homogenized in 2 mL of 50% MeOH using a mortar and pestle and the slurry was filtered through miracloth (Calbiochem, La Jolla, CA). To the filtrate was added 0.5 mL of 10% sulfuric acid followed by extraction with an equal volume of ethyl acetate. After centrifugation for 5 min at 15,000 rpm, the organic phase was discarded and the aqueous phase was treated with 0.25 mL of 10 N NaOH, and alkaloids were extracted with an equal volume of ethyl acetate. The organic phase was collected and evaporated to dryness using an SPD Speed Vac (Thermo Savant,

Holbrook, New York): the residue was resuspended in 0.3 mL MeOH and filtered through 0.2 μm Pall filter. The filtered alkaloid extracts were analyzed by UPLC.

In addition, twenty 3.0 cm long hairy roots from DAT expressing line 7 and R/J1 hairy root lines were harvested into 0.5-cm sections from the developmentally young root tip to more mature parts of each root and were analyzed for variations in alkaloid content as described above. However, due to the small amounts of tissue used, samples were homogenized in 400 μL of 50% MeOH using Kontes pellet pestle (Fisher Scientific, Canada). To the homogenate was added 100 μL of 10% sulfuric acid followed by extraction with equal volume of ethyl acetate. Samples were centrifuged for 5 min at 15,000 rpm, and the organic phase was discarded and the aqueous phase was treated with 50 μL of 10 N NaOH, and alkaloids were extracted with an equal volume of ethyl acetate. The organic phase was collected and filtered as described above for analysis by UPLC.

HPLC Analysis

Alkaloid extracts were analyzed by HPLC basically as described by Uniyal *et al.* (2001). Briefly, the solvent system was composed of solvent A [methanol:acetonitrile:5mM ammonium acetate:triethlyamine (5.8:14.2:80:0.2 by volume)] and solvent B [methanol:acetonitrile:5mM ammonium acetate:triethlyamine (23:57:20:0.2 by volume)] and solvent C [methanol:acetonitrile:5mM ammonium acetate:triethlyamine (26:64:10:0.2 by volume)], each of which were pre-filtered through a 0.2 μm Ultipor N 66 filter (Pall Science, Mississauga, Canada). The ratio of the solvent A to B was; 0-3 min, linear gradient from 99.9:0.1 to 0.1:99.9 at the increasing flow rate from 0.047 to 0.075 mL/min; 3-5.5 min, isocratic with 0.1:99.9 at flow rate 0.075 mL/min; 5.5-6.5 min, linear gradient from 0.1:99.9 to 99.9:0.1 at the decreasing flow rate from 0.075 to 0.047 mL/min; 6.5-8.0 min, isocratic with 99.9:0.1 at flow rate 0.047

mL/min. Samples were run on Acquity UPLC BEH C_{18} 1.7 µm column (1.0 x 50 mm) (Waters, Milford, MA).

Preparation of recombinant DAT and MAT enzymes expressed in *E. coli*

A single colony of *E. coli* BL21 (DE3) (Invitrogen, Burlington, Canada) harboring either pQE30-MAT or pQE30-DAT, growing on LB ampicillin (LBA) (50 mg/L) agar medium, was used to inoculate 3 mL of the LBA liquid media, and the cells were grown for 16 h at 37°C, shaking at 200 rpm. 0.5 mL of saturated 3 mL cultures were used to inoculate 50 mL of LBA liquid medium, and the cells were grown at 37°C, shaking at 200 rpm until the OD_{600} absorbance was 0.5. Recombinant protein expression was then induced with the addition of 2 mM isopropyl thiogalactose (IPTG), followed by the incubation at 90 rpm for 16 h at 25°C. After the induction, the cells were harvested by the centrifugation at 3000 rpm for 10 min at 4°C. The cell pellet was resuspended in 3 mL of 0.1 M Tris-HCl buffer (pH 8.0) containing 14 mM β-mercaptoethanol, and then sonicated. The slurry was centrifuged in microfuge tubes for 5 min at 15,000 rpm and the supernatant was desalted using a Sephadex G-25 PD-10 column (GE Healthcare, Piscataway, New Jersey).

The recombinant DAT and MAT proteins were purified from the crude desalted extracts using Ni-NTA Agarose resin (Qiagen, Mississauga, Canada), according to the manufacturer's procedures. Unlike affinity purified rDAT, affinity purified rMAT was not stable and no enzyme activity was detected. Enzyme assays were therefore performed using affinity purified rDAT and the crude desalted rMAT.

DAT and MAT enzyme assays

Transformed DAT expressing *Catharanthus* hairy roots (500 mg), R/J1 hairy roots or young leaves of tobacco (1.0 g) were homogenized in 3 mL of extraction buffer (0.1 M Tris-HCl, pH 8, 14 mM Mercaptoethanol) using a Mortar and Pestle. The extract was filtered through miracloth and 2.5 mL of crude extract was desalted directly by PD-10 column chromatography (GE Healthcare) and this extract (3.5 mL) was used directly for enzyme assays. DAT and MAT enzyme activities were assayed as described previously (Laflamme *et al.*, 2001; St-Pierre *et al.*, 1998). All DAT assays were performed in 100 µL reaction volumes whereas MAT assays were performed in 400 µL reaction volumes containing the desalted crude protein extract together with various substrates and cofactors related to each enzyme assay: DAT assay [30 µM deacetylvindoline and 16.6 µM (1-^{14}C) Acetyl-coenzyme A, GE Healthcare]; MAT assay [30 µM hörhammericine and 16.6 µM (1-^{14}C) Acetyl-coenzyme A, GE Healthcare]. MAT assays were conducted at 37°C for 60 min and DAT assays were conducted at 37°C for 30 min, unless otherwise mentioned. The reactions were terminated by adding 50 µL 10 N NaOH, radio-labeled alkaloids were extracted into an equal volume of ethyl acetate, the organic phase was harvested after separation form the aqueous phase by centrifugation and was taken to dryness by vacuum centrifugation using an SPD Speed Vac (Thermo Savant, Holbrook, New York) system. Each dried sample was dissolved in 5 µL methanol and the reaction products were separated by analytical thin layer chromatography (TLC) [Polygram Sil G/UV254 (Macherey-Nagel)]. TLC plates were developed in an ethylacetate-methanol (9:1, v/v) solvent system. The radioactivity was visualized and quantified by exposure of the TLC to a storage phosphor screen (GE Healthcare, Piscataway, NJ, USA) for 16 h and emissions were detected using a Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge ver. 3.0 (Fujifilm, Tokyo, Japan).

Inhibition of MAT activity by DAT and Inhibition of DAT activity by MAT

MAT inhibition assays were performed in a total reaction volume of 120 µL containing 62.3 µg of crude desalted protein extract (100 µL), 30 µM hörhammericine, 16.6 µM [1-^{14}C] Acetyl-coenzyme A (GE Healthcare) and varying amounts of purified recombinant DAT protein (0, 0.1, 0.5, 1.0 and 2.0 µg). The inhibitory effect of MAT over DAT activity was studied in a reaction mixture as follows; 10 µg of the purified DAT protein (100 µL), 30 µM deacetylvindoline, 16.6 µM [1-^{14}C] Acetyl-coenzyme A and varying amounts of crude desalted MAT protein extract (0, 0.1, 0.5, 1.0 and 2.0 µg) in a total volume 120 µL. The inhibitory effect of hörhammericine to DAT enzyme activity was determined in a total reaction volume of 120 µL containing 10 µg of purified DAT protein (100 µL), 30 µM deacetylvindoline and 16.6 µM [1-^{14}C] Acetyl-coenzyme A with the addition of varying amounts of hörhammericine (0, 3.75, 7.5, 15 and 30 µM). All the MAT and DAT enzyme assays were conducted at 37°C for 30 min. The reactions were terminated by the addition of 50 µL of 10 M NaOH, and the radio-labeled assay products were extracted to an equal volume of ethyl acetate. The organic phase was harvested after the centrifugation, and was dried to dryness by SPD Speed Vac (Thermo Savant, Holbrook, New York) system. Each dried sample was dissolved in 5 µL of methanol and analyzed by thin layer chromatography (TLC) using Polygram Sil G/UV254 (Macherey-Nagel, Düren, Germany). TLC plates were developed in an ethylacetate-methanol (9:1, v/v) solvent system. The radioactivity was visualized and quantified by the exposure of the TLC to a storage phosphor screen (GE Healthcare) for 16 h and emissions were

detected using Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) with Multi Gauge ver. 3.0 software (Fujifilm, Tokyo, Japan).

RNA extraction and RT-PCR amplification of DAT and MAT mRNA from R/J1 and DAT transformed *Catharanthus* hairy root cultures

Catharanthus hairy roots (50 mg) were harvested and extracted for mRNA by homogenizing samples with a kontes pellet pestles (Fisher Scientific, Canada) in 1.5 mL Eppendorf tubes containing 1 mL of Trizol reagent. After homogenization, 0.2 mL of chloroform was added and samples were centrifuged in an Eppendorf centrifuge for 15 min at 12,000 rpm and the supernatant was recovered. Following isopropanol precipitation the pellet was dissolved in 45 µL DEPC water and 5 µL of 10 x TE (10 mM Tris-HCl, 1 mM EDTA) and the RNA was treated with 10U of DNase I FPLCpure (GE Healthcare, Piscataway, New Jersey) for 30 min at 37°C followed by phenol/chloroform extraction and concentration by ethanol precipitation. RNA was quantified by spectrophotometry and used for RT-PCR as described by Murata *et al.*, 2005.

The mRNA levels of DAT and MAT were analyzed using gene specific oligonucleotides as follows; CrDAT-RT01 5'-GTGCGTATCCGTTGGTTTCT-3', CrDAT-RT02 5'-CGAACTCAATTCCATCGTCA-3', CrMAT-RT01 5'AGGATTGGGCTGCTTCTACA-3', CrMAT-RT02 5'-CCGTAGCACATCGACAGAGA-3'. Reverse transcriptase was performed using an RNA PCR Kit (AMV) ver. 2.1 (Takara, Otsu, Japan) and the PCR reactions were carried out for 25, 27 and 30 cycles respectively, of 15 sec at 94°C, 20 sec at 57°C and 30 sec at 72°C. ExTaq DNA polymerase (Takara, Otsu, Japan) was used in place of rTaq which was included in the kit. Amplified cDNA fragments were run on 1.5% agarose gel and visualized by ethidium bromide staining.

Protein determination

Protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) which is based on the method of Bradford (1976). BSA was used as a standard.

Histochemical localization of GUS activity in transformed hairy root cultures

GUS activity was localized in *C. roseus* hairy root tissue with the histochemical substrate X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt; Indofine Chemical Company, Inc., Belle Mead, New Jersey) as described in Weigel & Glazebrook (2002). Incubation was performed overnight at 37°C.

ACKNOWLEDGEMENTS:

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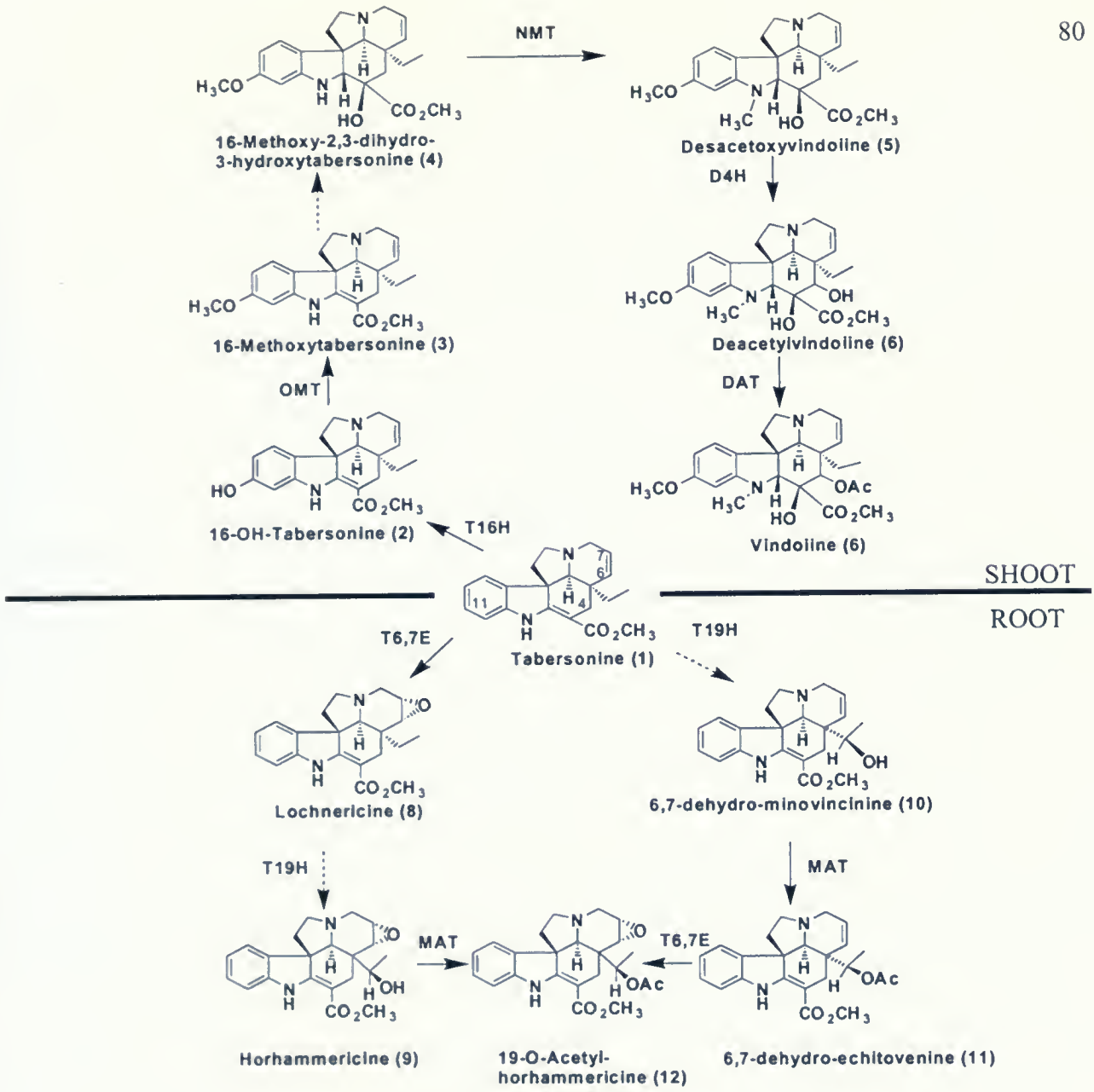
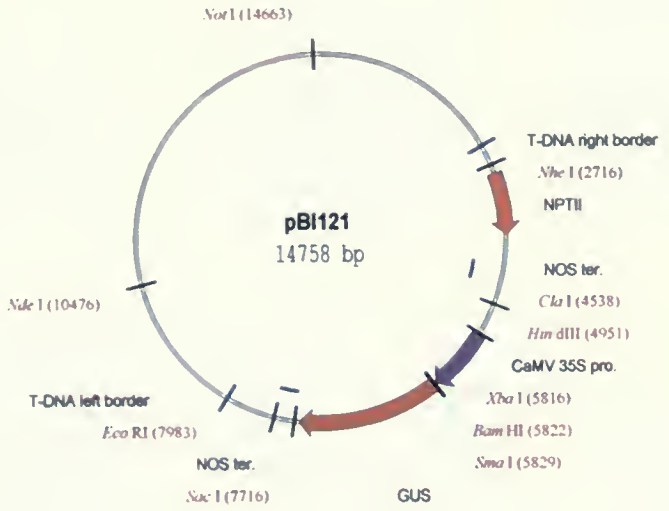


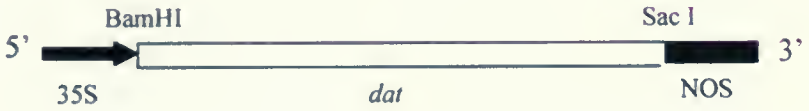
Figure 1- Biosynthesis of tabersonine derived metabolites in *C. roseus* organs [adapted from Rodriguez *et al.* (2003)]. In the aerial parts, tabersonine is converted to vindoline via six enzymatic steps. In the roots, tabersonine is converted to various oxidized tabersonine intermediates leading to the formation of 19-*O*-Acetyl-hörhammericine. The abbreviations stand for tabersonine 16-hydroxylase (T16H); 16-hydroxytabersonine-16-*O*-methyltransferase (16-OMT); *N*-methyltransferase (NMT); deacetoxyvindoline-4-

hydroxylase (D4H); deacetylvindoline 4-*O*-acetyltransferase (DAT); tabersonine-6,7-epoxydase (T6,7E); tabersonine 19 hydroxylase (T19H), minovincinine-19-*O*-acetyltransferase (MAT). Dotted arrows describe uncharacterized enzyme reactions.

A)



B)



C)

Figure 2- Cassettes for *DAT* and *GUS* expression *in N. tabacum* plants and *C. roseus* hairy roots. (a) pBI121 binary plasmid (b) pBI121/DAT: 35S, cauliflower mosaic virus (CaMV) 35S promoter: *DAT*, *C. roseus* cDNA sequence of deactylvindoline-4-*O*-acetyltransferase: NOS, nopaline synthase (nos) terminator (c) pBI121/GUS: 35S, cauliflower mosaic virus (CaMV) 35S promoter: *GUS*, β-glucuronidase (GUS) reporter gene: NOS, nopaline synthase (nos) terminator.

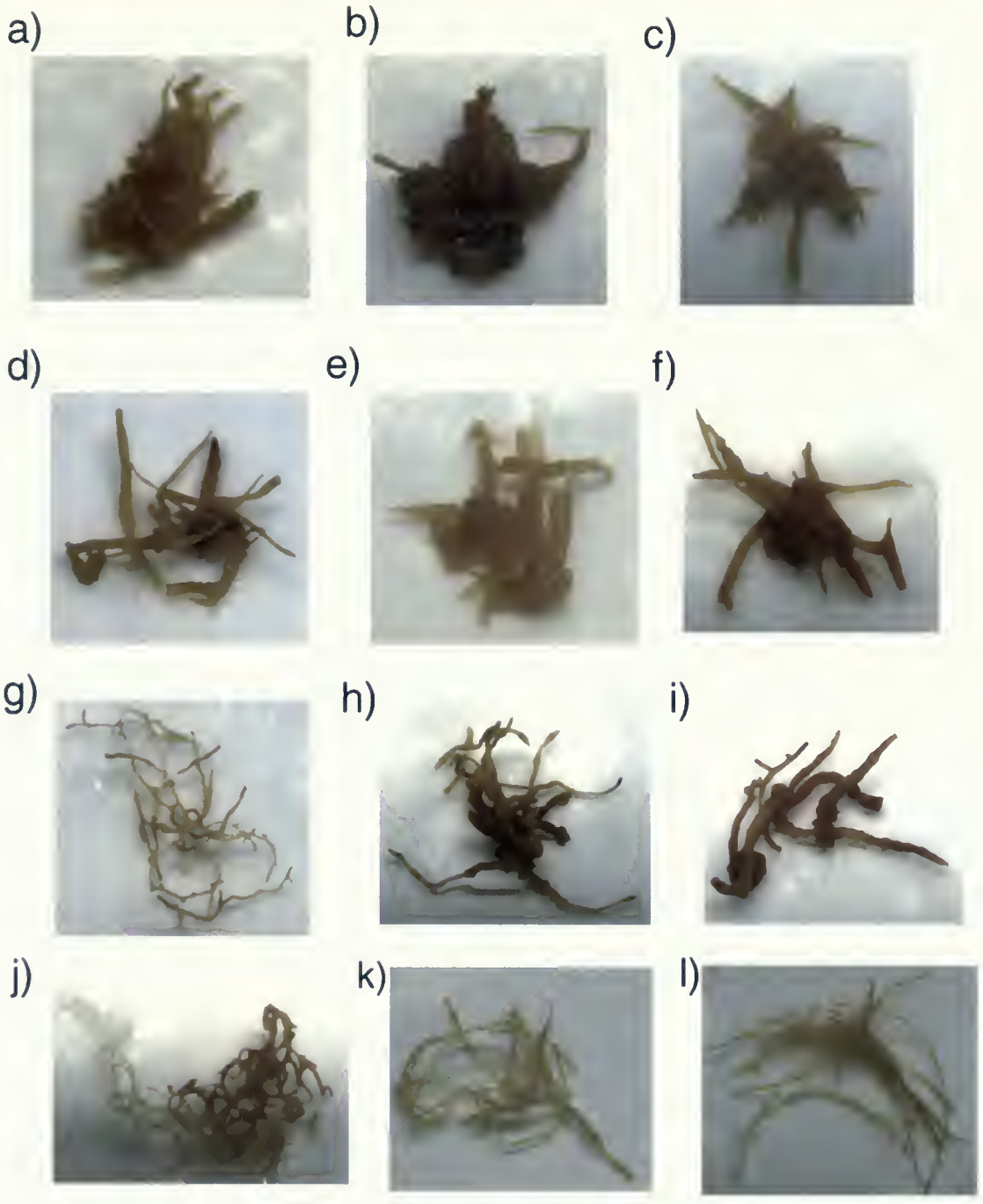


Figure 3- Morphological features of different transgenic *Catharanthus roseus* hairy root lines. A-J) Line DAT1-DAT10, k) GUS hairy root line, l) R/J1 hairy root line.

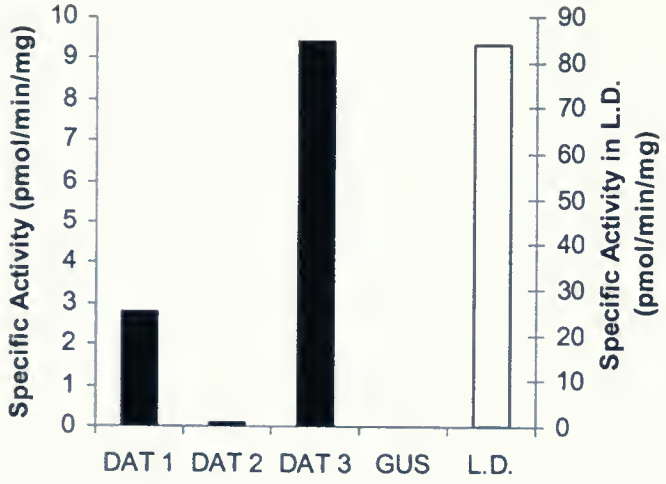


Figure 4 – Relative DAT enzyme activities in leaves of 3 *Nicotiana tabacum* lines DAT 1 to DAT 3 compared to a GUS expressing control line and to *Catharanthus* (Little Delicata) leaves (L.D.).

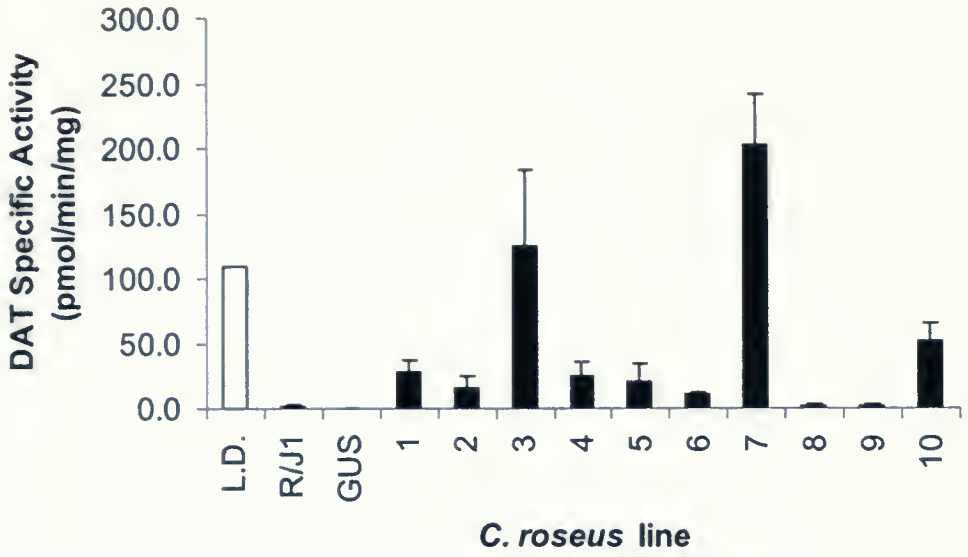


Figure 5 – Relative DAT enzyme activities found in *Catharanthus* (cv. Little delicata) leaves (L.D.), hairy roots (R/J1), GUS expressing hairy roots (GUS) and in Lines DAT 1-DAT 10 transformed with the DAT gene. In the case of lines DAT 1-10, 3 separate extractions of roots were made and assayed for DAT activity for each line in order to generate the standard errors displayed in the figure.

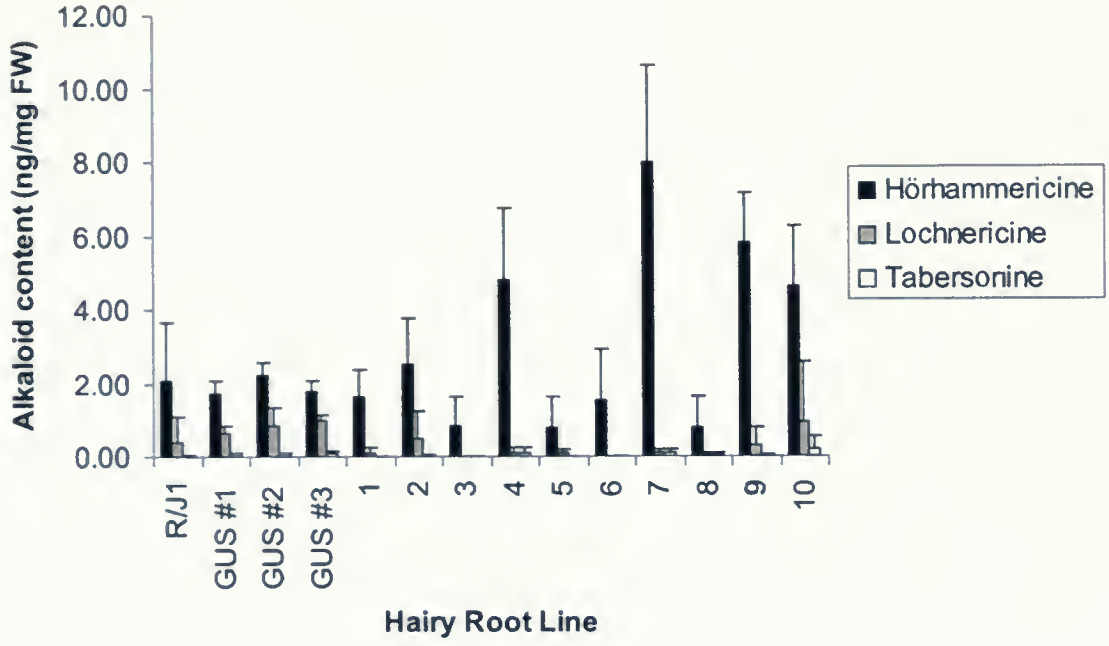
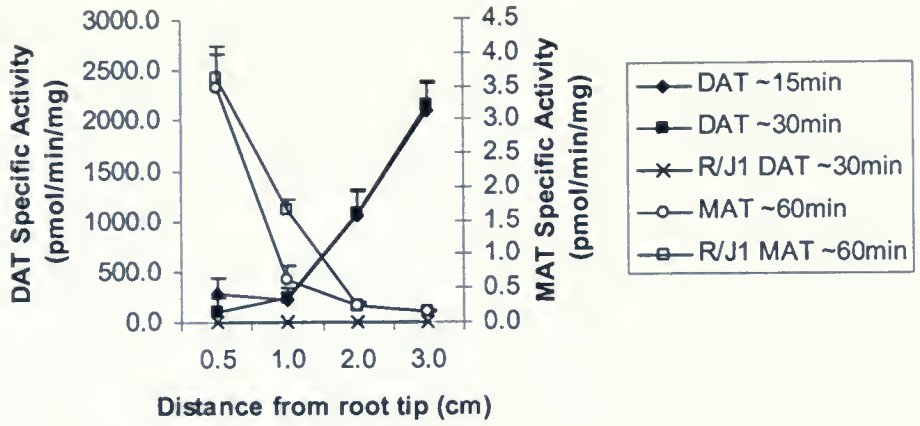
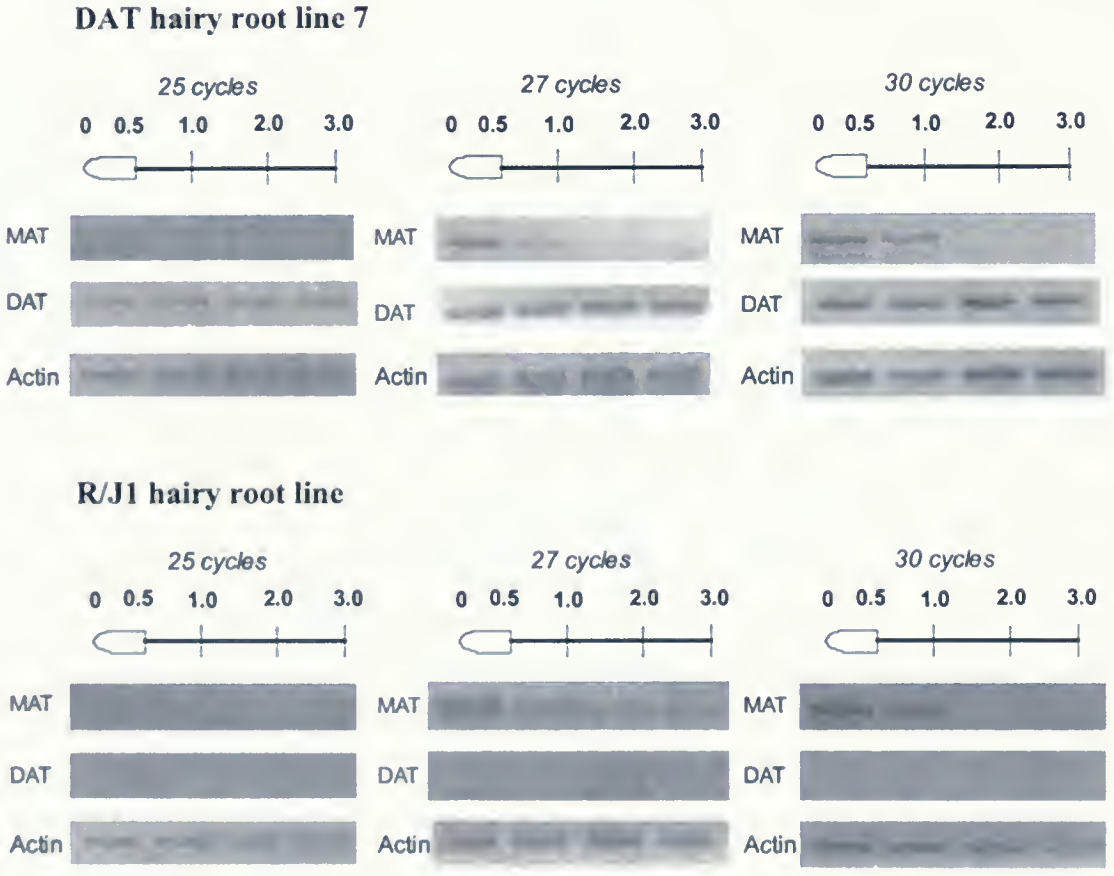


Figure 6 – Hörhammericine, Lochnericine and Tabersonine accumulation in hairy root cultures of *C. roseus*: DAT expressing lines labeled 1-10; GUS, *C. roseus* hairy roots expressing *GUS*; R/J1, *C. roseus* hairy roots. Three separate sets of roots were extracted for MIAs to provide a measure of the variability of alkaloid profiles found in each hairy root line.

A)

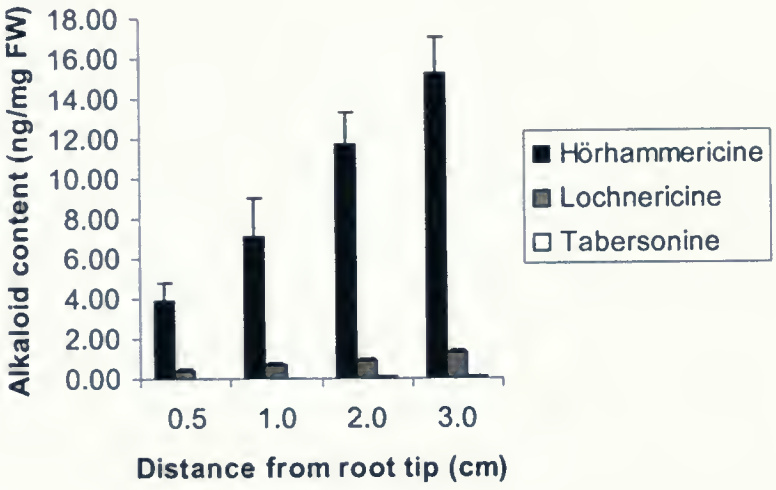

DAT Specific Activity (pmol/min/mg)
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MAT Specific Activity (pmol/min/mg)
0.5
1.0
2.0
3.0
Distance from root tip (cm)
DAT ~15min
DAT ~30min
R/J1 DAT ~30min
MAT ~60min
R/J1 MAT ~60min

B)


DAT hairy root line 7
25 cycles
27 cycles
30 cycles
0 0.5 1.0 2.0 3.0
MAT
DAT
Actin
R/J1 hairy root line
25 cycles
27 cycles
30 cycles
0 0.5 1.0 2.0 3.0
MAT
DAT
Actin

C)

DAT hairy root line 7



R/J1 hairy root line

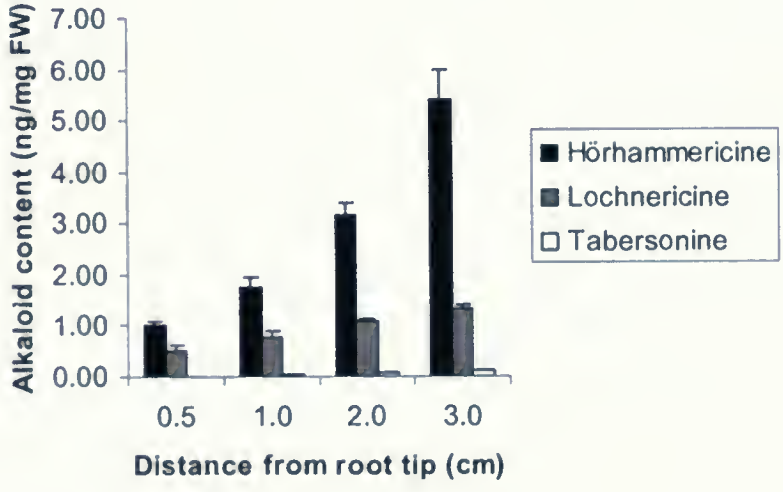


Figure 7- Enzyme, mRNA and MIA profiles in different parts of *Catharanthus* hairy roots in lines DAT 7 and R/J1, respectively. A) DAT and MAT enzyme activities were assayed in extracts from sequential 0.5 cm sections of hairy root tissues. DAT enzyme assays were incubated for 15 or 30 minutes, while MAT assays were incubated for 60 min. Sections from each stage of development were harvested in triplicate and each lot was extracted and assayed separately for DAT and MAT activities in order to generate the standard errors displayed in the figure B) The relative expression of MAT and DAT transcripts isolated from different hairy root tissue sections were monitored by RT-PCR (25, 27 and 30 cycles). The schematic diagram shows where the sections were obtained in relation to the root tip. C) MIAs (Tabersonine (**1**), Lochnericine (**8**) and Hörhammericine (**9**) were extracted from different hairy root tissue sections and their levels were quantitated by UPLC. Sections from each stage of development were harvested in triplicate and each lot was extracted for MIAs and analyzed by UPLC to generate the standard errors displayed in the figure.

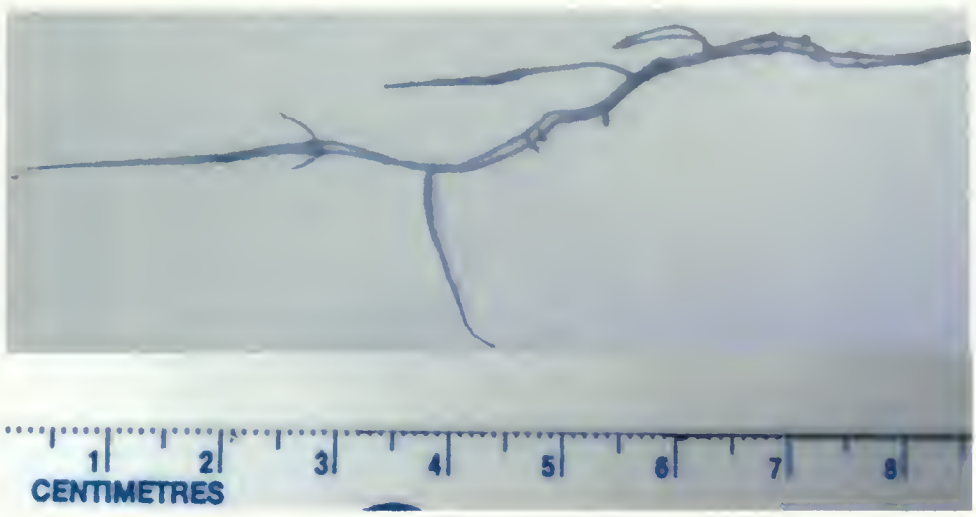
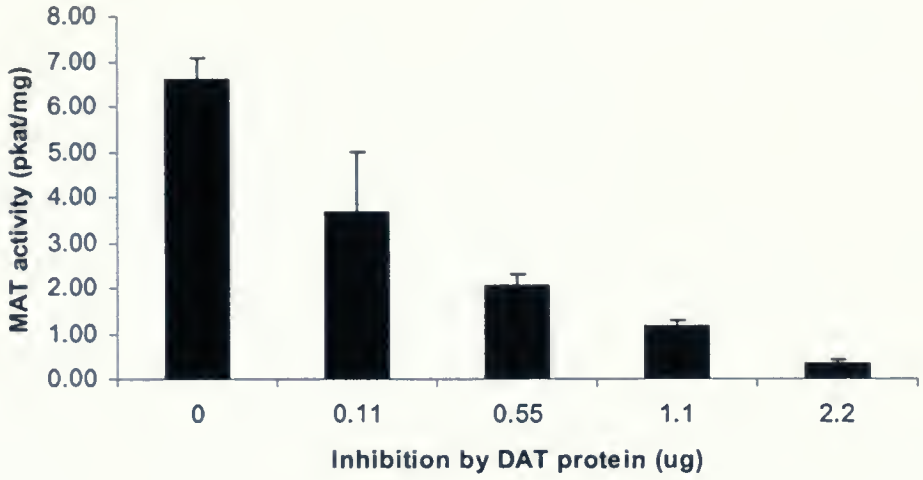


Figure 8- Histochemical localization of GUS in hairy root tissue transformed with pBI121/GUS plasmid. The plasmid expresses the GUS reporter gene under the control of the CaMV 35S promoter.

A)



B)

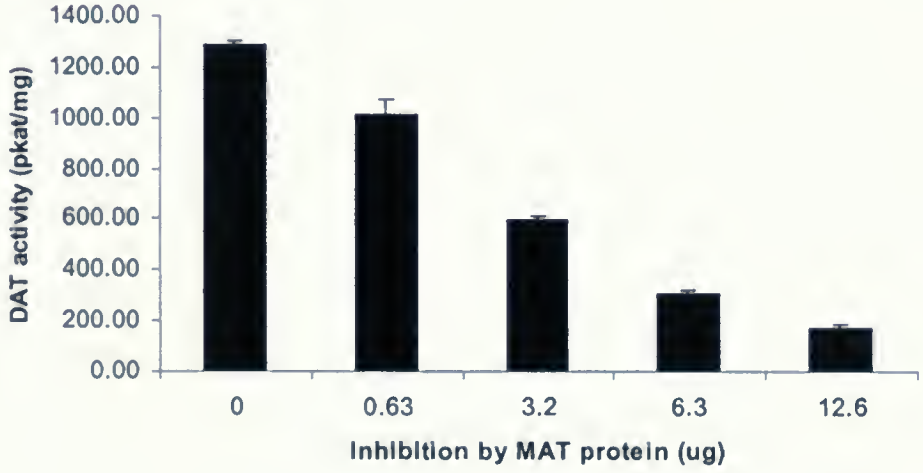


Figure 9- A) Inhibition of rMAT enzyme activity by adding different amounts of affinity purified rDAT protein to the incubation mixture. Enzyme assays were done in triplicate B) Inhibition of rDAT enzyme activity by adding different amounts of unpurified rMAT protein to the incubation mixture. Enzyme assays were performed in triplicate.

General Conclusion

The therapeutically valuable cytotoxic alkaloids vinblastine and vincristine found in *Catharanthus roseus* (L.) G. Don., a plant originally from Madagascar, has made it an area of major interest in modern plant cell biotechnology. At present, however, it is not yet possible to produce these dimeric indole alkaloids in plant tissue culture systems of *C. roseus*. Various aspects of the regulation of secondary metabolite biosynthesis in *Catharanthus* are discussed in Chapter 2.

In Chapter 3, a low vindoline accumulating cultivar of *Catharanthus roseus* is identified by MIA and enzymatic profiling in order to highlight these techniques for the rapid identification of a range of candidate lines modified for alkaloid content. The technology can easily be scaled-up for extensive screening of mutant populations of *Catharanthus roseus*. Furthermore, this study indicates the value of seedlings and young leaf material of mature plants when screening for altered MIA production and/or related pathway enzymes which could then be analyzed in greater detail for basic studies or for possible commercial commercialization.

Chapter 4 focuses on metabolic engineering to express a leaf-specific enzyme (deacetylvindoline-4-*O*-acetyltransferase; DAT) to produce *Catharanthus roseus* hairy roots with high DAT activity that led to modified MIA profiles. While the effects of expressing enzymes specific for vindoline production in hairy root cultures remain to be fully exploited, this study is a promising first step in metabolic engineering studies for vindoline production from tabersonine in hairy root cultures.

These studies improve our understanding of the complex regulation of vindoline biosynthesis in the Madagascar periwinkle and provide necessary tools to gain further

understanding of indole alkaloid biosynthesis. Moreover, the combination of bioengineering and biochemical approaches afforded valuable information on the scaling up problems in an industrial process. Eventually, the understanding obtained from such studies will provide suitable targets and approaches for genetic engineering in order to improve production of particular commercially relevant MIAs.

References

Aerts, R.J. & De Luca, V. (1992) Phytochrome is involved in the light-regulation of vindoline biosynthesis in *Catharanthus*. *Plant Physiol.* **100**: 1029-1032.

Aerts, R.J., Gisl, D., De Carlois, E., De Luca, V. & Baumann, T. (1994) Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. *The Plant Journal.* **5**: 635-643.

Avila, J. (1997) The use of microtubule poisons on tumor cells. *The Cancer Journal.* **10**: 34-40.

Balsevich, J., De Luca, V., Kurz, W.G.W. (1986). Altered alkaloid pattern in dark grown seedlings of *Catharanthus roseus*. The isolation and characterization of 4-desacetoxyvindoline. A novel indole alkaloid and proposed precursor of vindoline. *Heterocycles* **24** : 2415-2421.

Batra, J., Dutta, A., Singh, D., Kumar, S. & Sen, J. (2004) Growth and terpenoid indole 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.

Battraw, M. & Hall, T. (1990) Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. *Plant Molecular Biology.* **15**: 527-538.

Bhadra, R., Vani, S. & Shanks, J. (1993) Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus*. *Biotech. and Bioengin.* **41**: 581-592.

Bradford, M.M., (1976). A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical *Biochemistry* **72**: 248-254.

Burlat., V., Oudin, A., Courtois, M., Rideau, M, & St-Pierre, B. (2004) Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of Catharanthus roseus implicates multicellular transclocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. *The Plant Journal.* **38**: 131-141.

Canel, C., Ines Lopes-Cardoso, M., Whitmer, S., van der Fits, L., Pasquali, G., van der Heijden, R., Hoge, J.H.C. & 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.

Chahed, K., Oudin, A., Guivarc'h, N., Hamdi, S., Chenieux, J., Rideau, M. & Clastre, M. (2000) 1-Deoxy-D-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. *Plant Physiol. Biochem.* **38**: 559-566.

Collu, G., unver, N., Peltenburg-Looman, A., van der Heijden, R. Verpoorte, R & Memelink, J. (2001) Geraniol 10-Hydroxylase, a cytochrom P450 enzyme involved in terpenoid indole alkaloid biosynthesis. *FEBS Letters.* **508**: 215-220.

Contin, A., van der Heijden, R., Lefeber, A.W.M., Verpoorte, R. (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. *FEBS Letters*. **434:** 413-416.

Cutts, J.H., Beer, C. T. & Noble, R.L. (1957) Effects on hematopoeisis in rats of extracts of *Vinca rosea*. *Rev. Can. Biol.* **16**: 476-484.

De Carlois, E., Chan, F., Balsevich, J. & De Luca, V. (1990) Isolation and characterization of a 2-oxoglutarate dependent dioxygenase involved in the second-to-last step in vindoline biosynthesis. *Plant Physiol.* **94**: 1323-1329.

De Carlois, E. & De Luca, V. (1993) Purification, characterization, and kinetic analysis of a 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis from *Catharanthus roseus*. *J. Biol. Chem.* **268**: 5504-5511.

De Luca, V., Balsevich, J., Tyler, B., Eilert, U., Panchuk, B., Kurz, W.G.W., 1986. Biosynthesis of indole alkaloids: developmental regulation of the biosynthetic pathway from tabersonine to vindoline in *Catharanthus roseus* seedlings. *Plant Physiol.* 125,147–156.

De Luca, V., Balsevich, J., Tyler, R.T., Kurz, W.G.W. (1987). Characterization of a novel N-methyltransferase from *Catharanthus roseus* plants. *Plant Cell Reports*. **6**: 458–461.

De Luca, V., Marineau, C., Brisson, N. (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: comparison with animal dopa decarboxylases. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 2582-2586.

De Luca, V. (1993) Enzymology of indole alkaloid biosynthesis. *Methods in Plant Biochemistry*. **9**: 345-369.

De Luca, V. & Laflamme, P. (2001) The expanding universe of alkaloid biosynthesis. *Current Opinion in Plant Biology*. **4**: 225-233.

De Luca, V. & St-Pierre, B. (2000) The cell and developmental biology of alkaloid biosynthesis. *Trends in Plant Science*. **5**: 168-173.

Dethier, M. & De Luca, V. (1993) Partial purification of an N-methyltransferase involved in vindoline biosynthesis in *Catharanthus roseus*. *Phytochemistry*. **32**: 673-678.

De Waal, A., Meijer, A.H. & Verpoorte, R. (1995) Strictosidine synthase from *Catharanthus roseus*: purification and characterization of multiple forms. *Biochem. J.* **306**: 571-580.

Dumontet, C. & Sikic, B. (1999) Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J. Clinical Oncology*. **17**: 1061-1070.

Dutta A., Batra, J., Pandey-Rai, S., Singh, D., Jumar, S. & Sen, J. (2004) Expression of terpenoid indole alkaloid biosynthetic pathway genes corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) G. Don. *Planta*. **220**: 376-383.

Facchini, P. (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation and metabolic engineering applications. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**: 29-66.

Gamborg, O.L., Miller, R.A., Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**: 151-158.

Geerlings, A., Martinez-Lozano Ibanez, M., Memelink, J., van der Heijden, R. & Verpoorte, R. (2000) Molecular cloning and analysis of strictosidine β-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *J. Biol. Chem*, **275**: 3051-3056.

Hashimoto, T. & Yamada, Y. (1994) Alkaloid biogenesis: molecular aspects. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**: 257-85.

Herrmann, K. & Weaver, L. (1999) The shikimate pathway. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**: 473-503.

Hong, S., Peebles, C., Shanks, J., San, K. & Gibson, S. (2006) Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. *J. Biotech.* **122**: 28-38.

Hughes, E., Hong, S., Gibson, S., Shanks, J. & San, K. (2004) Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. *Metabolic Engineering*. **6**: 268-276.

Irmler, S., Schroder, G., St-Pierre, B., Crouch, N., Hotze, M., Schmidt, J., Strack, D., Matern, U. & Schroder, J. (2000) Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. *The Plant Journal*. **24**: 797-804.

Kutchan, T. (1993) Strictosidine: from alkaloid to enzyme to gene. *Phtyochemistry*. **32**: 493-506.

Laflamme, P., St-Pierre, B. & De Luca, V. (2001) Molecular and biochemical analysis of a madagascar periwinkle root-specific minovincininine-19-hydroxy-O-acetyltransferase. *Plant Physiol.* **125**: 189-198.

Leech, M., May, K., Hallard, D., Verpoorte, R., De Luca, V. & Christou, P. (1998) Expression of two consecutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. *Plant Mol. Biol.* **38**: 765-774.

Luijendijk, T., Stevens, L. & Verpoorte, R. (1998) Purification and characterization of strictosidine β-D-glucosidase from *Catharanthus roseus* cell suspension cultures. *Plant Physiol. Biochem.* **36**: 419-425.

Madyastha, KM., Guarnaccia, R., Baxter, C. & Coscia CJ. (1973) S-Adenosyl-L-methionine: loganic acid methyltransferase. A carboxyl alkylating enzyme from *Vinca rosea*. *J. Biol. Chem.* **248**: 2497-2501.

Meijer, A., Verpoorte, R., & Hoge, H. (1993) Regulation of enzymes and genes involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *J. Plant Res.* **3**: 145-164.

Menke, F., Champion, A., Kijne, J., & Memelink, J. (1999) A novel jasmonate- and elixitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor- inducible AP2-domain transcription factor, ORCA2. *The EMBO Journal.* **18**: 4455-4463.

Moreno, P., van der Heijden, R. & Verpoorte, R. (1995) Cell and tissue cultures of *Catharanthus roseus*: a literature survey. *Plant Cell Tissue and Organ Culture.* **42**: 1-25.

Moreno-Valenzuela, O., Galaz-Avalos, R., Minero-Garcia, Y., & Loyola-Vargas, V. (1998) Effect of differentiation on the regulation of indole alkaloid production in *Catharanthus roseus* hairy roots. *Plant Cell Reports.* **18**: 99-104.

Morgan, J. & Shanks, J. (1999) Inhibitor studies of tabersonine metabolism in *Catharanthus roseus* hairy roots. *Phytochemistry.* **51**: 61-68.

Murashige, T., Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum.* **15**: 473-497.

Murata, J. & De Luca, V. (2005) Localization of tabersonine 16-hydroxylase and 16-OH tabersonine-16-*O*-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. *The Plant Journal.* **44**: 581-594

Noble, R.L. (1990) The discovery of the vinca alkaloids-chemotherapeutic agents against cancer. *Biochem, Cell Biol.* **68**: 1344-1351.

O'Keefe, B., Mahady, G., Gills, J. & Beecher, C. (1997) Stable vindoline production in transformed cell cultures of *Catharanthus roseus*. *J. Nat. Prod.* **60**: 261-264.

Pandey-Rai, S., Luthra, R., Kumar, S. (2003). Salt tolerant mutants in glycophytic salinity response (GSR) genes in *Catharanthus roseus*. *Theor. Appl. Genet.* **106**: 221-230.

Rijhwani, S. & Shanks, J. (1998) Effect of elicitor dosage and exposure time on bosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. *Biotechnol. Prog.* **14**: 442-449.

Rodriguez, S., Compagnon, V., Crouch, N., 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.

Schroder, G., Unterbusch, E., Kaltenbach, M., Schmidt, J., Strack, D., De Luca, V. & Schroder, J. (1999) Light-induced cytochrome P450-dependent enzyme in indole alkaloid biosynthesis: tabersonine 16-hydroxylase. *FEBS Letters*. **458**: 97-102.

Shanks, J.V., Bhadra, R., Morgan, J., Rijhwani, S., Vani, S. (1998) Quantification of metabolites in the indole alkaloid pathways of Catharanthus roseus: implications for metabolic engineering. *Biotech. & Bioeng.* **58**: 333-338.

Songstad, D.D., De Luca, V., Brisson, N., Kurz, W.G.W. & Nessler, C.L. (1990) High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. *Plant Physiology* 94:1410-1413

Sottomayor, M., Lopez-Serrano, M., DiCosmo, F. & Barcelo, A. (1998) Purification and characterization of α-3',4'-anhydrovinblastine synthase (peroxidase-like) from *Catharanthus roseus* (L.) G. Don. *FEBS Letters*. **428**: 299-303.

Stockigt, J., Husson, H.P., Kan-Fan, C. & Zenk, M.H. (1977) Cathenamine, a central intermediate in the cell free biosynthesis of ajmalicine and related indole alkaloids. *J. Chemical Society, Chemical. Communications*. **1977**: 164-166.

St. Pierre, B. & De Luca, V. (1995) A cytochrome P-450 monooxyenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthus roseus*. *Plant Physiol.* **109**: 131-139.

St. Pierre, B., Laflamme, P., Alarco, A.M., De Luca, V. (1998) The terminal *O*-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A dependent acyl transfer. *Plant J.* **14**: 703–713

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

Sundberg, R. & Smith, S. (2002) The iboga alkaloids and their role as precursors of anti-neoplastic bisindole *Catharanthus* alkaloids. *The Alkaloids*. **59**: 281-376.

Svoboda, G.H., Johnson, I.S., Gorman, M. & Neuss N. (1962) Current status of research on the alkaloids of *Vinca rosea* Linn. *J. Pharm. Sci.* **51**: 707-720.

Tikhomiroff, C., Jolicoeur, M., (2002) Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. *J. Chromatogr.* A, 955, 87–93

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

van der Heijden, R., Jacobs, D.I., 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. (1998) Developmental and light regulation of desacetoxyvindoline 4-hydroxylase in *Catharanthus roseus* (L.) G. Don. *Plant Physiol.* **117**: 1351-1361.

Vazquez-Flota, F., Moreno-Valenzula, OA., Mirands-Ham, ML., Coello-Coello, J. & Loyola-Vargas, Vm. (1994) Catharanthine and ajmalicine synthesis in *Catharanthus* hairy root cultures. Medium optimization and elicitation. *Plant Cell Tiss. Org. Cult.* **38**: 273-279.

Vazquez-Flota, F., St-Pierre, B. & De Luca, V. (2000) Light activation of vindoline biosynthesis does not require cytomorphogenesis in *Catharanthus roseus* seedlings. *Phytochemistry*. **55**: 531-536.

Vaszquez-Flota, F., De Luca, V., Carrillo-Pech MR., Canto-Flick, A. & Miranda-Ham ML. (2002). Vindoline biosynthesis is transcriptionally blocked in *Catharanthus roseus* cell suspension cultures. *Mol Biotechnol.* **22**: 1-8

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.

Whitmer, S. Verpoorte, R. & Canel, C. (1998) Influence of auxins on alkaloid accumulation by a transgenic cell line of *Catharanthus roseus*. *Plant Cell Tissue and Organ Culture*. **53**: 135-141.

Whitmer, S., van der Heijden, R. & Verpoorte, R. (2002) Effect of precursor feeding on alklaloid accumulation by a tryptophan decarboxylase over-expressing transgenic cell line T22 of *Catharanthus roseus*. *J. Biotechnology*. **96**: 193-203.

Yamamoto, H., Katano, N., Ooi, A., & Kenichiro, I. (2000) Secologanin synthase which catalyzes the oxidative cleavage of loganin into secologanin is a cytochrome P450. *Phytochemistry*. **53**: 7-12.