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The Cloning and Characterization of Two Acetyl Coenzyme A: *O*-Acetyltransferase Genes from *Catharanthus roseus* (L.) G. Don

par

Pierre Laflamme

Département de Sciences Biologiques

Faculté des Arts et des Sciences

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Cette thèse intitulée:

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a été évaluée par un jury composé des personnes suivantes:

David Morse.....président du jury

Vincenzo De Luca.....directeur de recherche

Ragaï K. Ibrahim.....membre du jury

William C. Plaxton.....examinateur externe

Mario Cappadocia.....représentant du doyen

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Abstract

The *O*-acetylation of deacetylvindoline and minovincinine, in *Catharanthus roseus* (L.) G. Don, the Madagascar periwinkle, is catalyzed by leaf- and root-specific acetyl coenzyme A-dependent *O*-acetyltransferases, deactylvindoline 4-*O*-acetyltransferase (DAT) and minovincinine 19-*O*-acetyltransferase (MAT), respectively.

The genes coding for DAT and MAT were successfully cloned from a genomic DNA library, using PCR, and previously obtained protein microsequence data as well as, 3'- and 5'-RACE protocols. Neither gene contained any introns and their open reading frames (ORFs) coded for a 439 and a 443 amino acid protein, respectively, having deduced M_r's of *ca.* 50 kDa each. The identity of the DAT gene was unequivocally established by the identification of nine out of ten, previously obtained, DAT partial peptide sequences in the ORF's deduced amino acid sequence along with the functional expression of DAT activity in *E. coli*. The similar expression of the MAT gene, as a HIS-tagged protein in *E. coli*, exhibited apparent MAT activity. Both genes shared 63 % nucleic acid identity, while their deduced amino acid sequences were 78% identical. The expression of *DAT* was restricted to aerial tissue, predominantly young leaves, while also being present in shoot, and absent in root. In contrast, *MAT* was predominantly expressed within the cortical cells of the root tip. Both genes exhibited tissue- and developmental-specific regulation, while *DAT* showed additional environmental-specific regulation. The expression of *DAT* was only observed in light-exposed tissue, and although very low levels of

DAT enzyme activity were observed in dark-grown tissue, light induced DAT activity by approximately 10-fold, by a process involving phytochrome.

Substrate specificity and kinetic studies demonstrated the recombinant proteins, rMAT and rDAT, to be quite different. While rDAT was specific for catalyzing only the 4-*O*-acetylation of deactylvindoline, rMAT was capable of catalyzing the 19-*O*-acetylation of minovincinine and to a lower extent, the 4-*O*-acetylation of deactylvindoline. The catalytic efficiencies of rDAT and rMAT revealed rMAT to be a poorer acetyltransferase than rDAT whose turnover rates for acetyl-CoA and deacetylvindoline were approximately 240- and 10 000-fold greater than those of rMAT.

The multiple alignment of the deduced amino acid sequences of MAT and DAT with a number of similarly related *O*-acetyltransferases, obtained by searching a protein database (BLAST), revealed the presence of highly conserved HXXXDG and DFGWGKP motifs, previously identified in the chloramphenicol acetyltransferase and dihydrolipoyl *S*-acetyltransferase gene families. The histidine residue in the HXXXDG domain is believed to be essential for catalytic activity because DEPC, a histidine-modifying agent, competed with the acetyl coenzyme A co-substrate for binding to the active site. The finding of plant acyltransferases related to MAT and DAT suggests they evolved from a common ancestral gene and helped define a new 'BAHD' family of plant acyltransferases.

Key words : *Catharanthus roseus*, leaf, root, gene, regulation, light, acetyl coenzyme A, deacetylvindoline 4-*O*-acetyltransferase, minovincinine 19-*O*-acetyltransferase

Résumé

Les plantes ont développé une méthode élégante de survie qui consiste à utiliser certains produits du métabolisme primaire — qui est essentiel pour la vie, la croissance et la reproduction [Dewick (1997)] — pour synthétiser un vaste ensemble de produits que l'on appelle métabolites secondaires. Certains de ces métabolites secondaires, grâce à leur toxicité, protègent la plante contre les prédateurs, d'autres servent comme signaux ou comme colorants volatils qui attirent des pollinisateurs particuliers. Par ailleurs, la plupart des médicaments d'origine naturelle sont les produits du métabolisme secondaire [Dewick (1997)].

Parmi ces composés on retrouve les terpénoïdes, les phéylpropanoïdes et les alcaloïdes. Ces derniers sont un groupe de composés à faible poid moléculaire. Ils contiennent de l'azote et sont dérivés d'acides aminés tels que la phénylalanine, la tyrosine, la tryptophane, la lysine, et l'ornithine. Les alcaloïdes peuvent être toxiques et avoir une action hallucinogène; par contre ils ont une action pharmaceutique lorsqu'utilisé en faibles quantités. Parmi les différents alcaloïdes, notons les bis-indoles, dérivés des précurseurs du tryptophane et de la voie de biosynthèse des terpénoïdes [voir: De Luca *et al.* (1992); De Luca (1993); Meijer *et al.* (1993c)]. Ils sont d'un intérêt particulier car ils sont la source d'agents antinéoplasiques, comme la vinblastine (VBL) et la vincristine (VCR).

Catharanthus roseus (L.) G. Don, la pervenche du Madagascar, est une plante tropicale de la famille des Apocynaceae qui accumule plusieurs alcaloïdes bis-indoles importants pour l'industrie pharmaceutique, parmi lesquels, la VBL et la VCR. La caractérisation de ces agents a démontré qu'ils sont composés d'une molécule de

vindoline et d'une de catharanthine [Neuss *et al.* (1958); Gorman *et al.* (1959)]. Malgré ses effets secondaires, la VCR demeure un agent chimiothérapeutique très efficace contre la leucémie chez les enfants. Quant à la VBL, elle est efficace vis-à-vis d'autres formes de leucémie et du lymphôme d'Hodgkin [Johnson *et al.* (1960)]. Puisque ces composés sont présents en très faibles quantités dans la plante, environ 0.0005% du total des alcaloïdes extraits, leurs coûts de production sont très élevés. Des efforts ont donc été déployés pour produire la VBL et la VCR par l'entremise de la culture *in vitro* de cellules en suspension [Van der Heijden *et al.* (1989); Lounasmaa et Galambos (1989); Moreno *et al.* (1995)]. Quoique ces cultures produisent de grandes quantités de catharanthine [voir références dans Deus-Neumann *et al.* (1987)], l'autre précurseur nécéssaire, la vindoline, n'est pas synthétisée [De Luca *et al.* (1985)]. L'inaptitude de ces cultures a synthétiser la vindoline a été attribuée à l'absence d'activité enzymatique de la désacétoxyvindoline-4-hydroxylase (D4H) et de l'acétyl coenzyme A: déacétylvindoline-4-*O*-acétyltransférase (DAT) [De Luca *et al.* (1987); Eilert *et al.* (1987)]. Ces deux enzymes catalysent les deux dernières étapes dans la voie de biosynthèse de la vindoline chez *Catharanthus roseus*. Par contre, la biosynthèse de la vindoline a été observée dans les tiges régénerées à partir d'un cal, ce qui suggère que les gènes de la biosynthèse de la vindoline sont simplement réprimés chez les cellules en suspension [Constabel *et al.* (1982)].

Le projet de cette thèse s'insère dans le cadre des études sur la régulation de la voie de biosynthèse de la vindoline chez *Catharanthus roseus*, et porte plus particulièrement sur la régulation moléculaire de la DAT.

Résultats

La DAT a été purifiée et caractérisée [Power (1989); Power *et al.* (1990); Fahn et Stöckigt (1990)]. De Luca et Cutler (1986) ont démontré qu'elle était cytosolique. D'après des analyses par SDS-PAGE, la DAT semble se présenter sous forme d'un hétérodimère composé de deux sous-unités de 21 et 33 kDa, respectivement [Power (1989); Power *et al.* (1990)], ce qui concorde avec les résultats obtenus par Fahn et Stöckigt (1990).

Après plusieurs tentatives sans succès [Alarco (1994)], le gène codant pour la DAT a été cloné en utilisant une combinaison de techniques, soit la RT-PCR ainsi que le 3'- et le 5'-RACE. Cette stratégie de clonage se base sur la méthode utilisée pour le clonage du gène de la poly (A) polymérase de *Saccharomyces cerevisiae* [Lingner *et al.* (1991)]. Ceci a permis d'amplifier un fragment de 225 paires de bases, W7-1, de la région 3' de l'ADN complémentaire (ADNc) du gène de la DAT. La séquence de 75 acides aminés correspondants, a révélé que ce fragment contenait les séquences de deux peptides tryptiques déjà connus comme faisant partie de la DAT [Alarco (1994)]. Le criblage d'une banque d'ADN génomique (ADNg) de *Catharanthus roseus* avec le fragment W7-1 a produit quatre clones positifs (*gDAT*4, *gDAT*6, *gDAT*15, et *gDAT*16). Deux d'entre eux, *gDAT*6 et *gDAT*15 se sont révélés particulièrement intéressants.

Les preuves que le clone *gDAT*6 contenait le gène de la DAT ont été fournies: (i) par la présence d'un cadre de lecture ouvert pour une séquence de 439 acides aminés et codant pour neuf des dix séquences peptidiques déjà trouvées dans les sous-unités de 33 et 21 kDa de la DAT [Alarco (1994)], et

(ii) par la démonstration d'activité enzymatique DAT, quand on exprimait cette séquence dans un vecteur d'expression chez *Escherichia coli.*

De plus, la séquence d'acides aminés, deduite du gène de la DAT, démontre que les deux sous-unités de 33 et 21 kDa, obtenues par la purification de la DAT [Power (1989); Power *et al.* (1990); Fahn et Stöckigt (1990)] sont codées par un seul gène. Ceci démontre que l'obtention de deux sous-unités est probablement dûe à un artefact lors de la purification de la DAT, car l'emploi d'un anticorps contre la DAT révèle une seule bande d'une masse moléculaire d'environ 50 kDa sur SDS-PAGE [St-Pierre *et al.* (1998)].

Quant au clone *gDAT*15, sa séquence a révélé un cadre de lecture ouvert pouvant produire un polypeptide de 443 acides aminés. Ce même cadre de lecture ouvert contenait la séquence du clone A-3, un clone partiel (186 acides nucléiques) isolé du criblage de la banque d'ADNc de *Catharanthus roseus* avec le fragment W7-1. L'expression du cadre de lecture ouvert du clone *gDAT*15 chez *E. coli* a produit une protéine ayant une activité enzymatique pour l'acétylation du groupement hydroxyle à la position 19 de la minovincinine donnant l'échitovenine. Donc, ce clone a été identifié comme étant le gène codant pour la minovincinine-19-*O*-acétyltransférase (MAT).

Les séquences des gènes de la MAT et de la DAT ont démontrés 63% d'identité, tandis que leurs séquences d'acides aminés partagent 78% d'identité. Le gène de la DAT est principalement exprimé chez les jeunes feuilles de *Catharanthus* ainsi que chez les tiges et les pétales, mais il n'est pas exprimé dans les racines ou chez les feuilles matures, ce qui est en accord avec la distribution de la protéine de la

DAT et son activité enzymatique [St-Pierre *et al.* (1998)]. Par contre, le gène de la MAT est exprimé principalement chez les racines et plus particulièrement dans les cellules corticales de la pointe des racines. Ces résultats démontrent que l'expression de ces deux gènes est réglée de façon spécifique tant au niveau du tissu que du développement. Par contre, il est à noter que l'expression de la DAT est aussi dépendante de la lumière, car la DAT est présente seulement dans les tissus exposés à la lumière. De plus, même si de très faibles quantités d'activité enzymatique de la DAT ont été détectées dans des tissus provenant de plantules croissant à l'obscurité, la lumière a pu augmenter d'environ 10 fois l'activité DAT. Cet effet inducteur de la lumière sur l'activité de la DAT semble impliquer le phytochrome puisqu'une exposition à la lumière rouge lointaine (*far red light*) est capable d'abolir l'effet d'exposition à la lumière rouge.

La caractérisation des protéines recombinantes, rDAT et rMAT, par rapport a leurs spécificités de substrats ainsi qu'au niveau de leurs paramètres cinétiques a révélé de grandes différences. La DAT recombinante s'est montrée spécifique pour l'acétylation du groupement hydroxyle à la position 4 de la déacétylvindoline, tandis que la MAT recombinante, en plus d'acétyler le groupement hydroxyle à la position 19 de la minovincinine, est aussi capable d'acétyler, à un plus faible niveau, la déacétylvindoline. La comparaison de l'efficacité catalytique, V_{max}/K_m, de la rDAT et de la rMAT a montré que la rMAT est une acétyltransférase faible par rapport à la rDAT, qui a des taux de rendement pour l'acétyl coenzyme A et la déacétylvindoline d'environ 240- et 10 000-fois plus élèvés, respectivement, que celle de la rMAT.

L'expression spécifique de la DAT dans les tissus aériens de la pervenche, ainsi que la présence d'activité MAT chez les cultures de racines de type 'hairy root' — obtenues de Vázquez-Flota *et al.* (1994) — qui ne produisent pas de vindoline, et le fait que la rMAT a la capacité d'*O*-acétyler la déacétylvindoline, peuvent expliquer la présence d'une activité *O*-acétyltranférase, chez les cultures analysées par Bhadra *et al.* (1993) et qui montraient la présence de vindoline. Cette activité qu'ils ont attribuée à la DAT est en réalité probablement dûe à la présence de la MAT.

L'alignement des séquences déduites d'acides aminés de la MAT et la DAT avec un nombre d'*O*-acétyltranférases semblables, obtenues d'une banque de données de séquences de protéines, BLAST, a révèlé la présence de deux motifs très bien conservés, soit HXXXDG et DFGWGKP. Ces motifs ont été identifiés dans la famille de gènes comprenant la chloramphénicol acétyltranférase (CAT) ainsi que la dihydrolipoyl *S*-acétyltranférase (DHLAAT). L'histidine du motif HXXXDG est considérée éssentielle pour l'activité catalytique, car elle lie le co-substrat acétyl coenzyme A. Ceci a été démontré avec la DAT et la MAT en présence du diéthylpyrocarbonate (DEPC). Ce réactif, qui réagit spécifiquement avec les résidus histidine, a inactivé les deux enzymes et cette inactivation a pu être renversée en présence d'acétyl coenzyme A, mais pas en présence des substrats alcaloïdes. Les résultats de cet alignement suggèrent que la MAT et la DAT ont évolué à partir d'un gène commun ancestral, relié à la CAT. Finalement, ces résultats aident a définir une nouvelle famille d'acétyltranférases de type 'BAHD' chez les végétaux [voir St-Pierre et De Luca (2000)].

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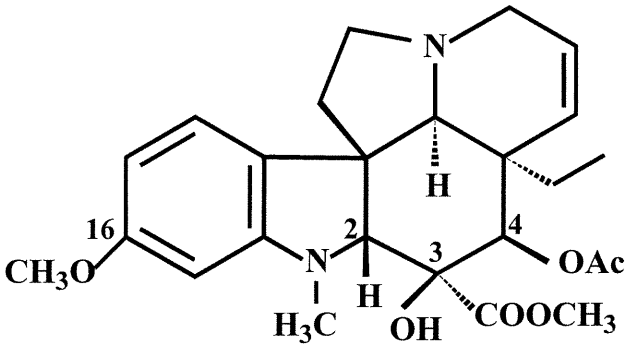
List of Abbreviations

Abbreviation	Name
BLAST	basic local alignment search tool
bp	base pair
CAS	ceric ammonium sulphate
cDNA	complementary DNA
D4H	desacetoxyvindoline 4-hydroxylase
DAT	deacetylvindoline 4-*O*-acetyltransferase
DAV	deacetylvindoline
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FR light	far-red light
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid
kDa	kilodalton
MAT	minovincinine 19-*O*-acetyltransferase
MeJa	methyl jasmonate
MIA	monoterpene indole alkaloid
min	minute
M_r	molecular weight
mRNA	messenger RNA
O-AT	*O*-acetyltransferase
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RACE	rapid amplification of cDNA ends
R light	red light
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STR1	strictosidine synthase
TDC	tryptophan decarboxylase
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
VBL	vinblastine
VCR	vincristine
v/v	volume per volume
w/v	weight per volume

List of Generic Names[a]



Vindoline

Compound Name	Generic Name
deacetylvindoline	methyl 6,7-didehydro-3,4-dihydroxy-16-methoxy-1-methyl-aspidospermidine-3-carboxylic acid
desacetoxyvindoline	methyl 6,7-didehydro-3-hydroxy-16-methoxy-1-methyl-aspidospermidine-3-carboxylic acid
echitovenine	methyl 20-acetoxy-2,3-didehydroaspidospermidine-3-carboxylic acid
hörhammericine	methyl 2,3-didehydro-6,7-epoxy-20-hydroxyaspidospermidine-3-carboxylic acid
lochnericine	methyl 2,3-didehydro-6,7-epoxy-aspidospermidine-3-carboxylic acid
minovincinine	methyl 2,3-didehydro-20-hydroxyaspidospermidine-3-carboxylic acid
tabersonine	methyl 2,3,6,7-tetrahydroaspidospermidine-3-carboxylic acid
vindoline	methyl 4-acetoxy-6,7-didehydro-3-hydroxy-16-methoxy-1-methyl-aspidospermidine-3-carboxylic acid

[a]The numbering system used is as for aspidospermidine alkaloids in Chemical Abstracts (Collective Substance Index V 106-115 12CS3 p.5731CS, 1987-1991).

".....guérissez-vous avec votre bonne pervenche,
bien verte, bien amère, mais bien spécifique à vos
maux, et dont vous avez senti de grands effets,
rafraîchissez-en cette poitrine enflamée,....."
[from: Schlittler (1964) *Lloydia* **27**:277-279]

To Mom & Dad

To Zeina

To the Saïkalis

— for their love, encouragement and support —

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A. Introduction

The fundamental unity of all living organisms can be demonstrated biochemically by the uniform presence of primary metabolic processes, that are required for life, growth and reproduction. Primary metabolism encompasses those pathways which synthesize essentially important molecules such as carbohydrates, proteins, fats and nucleic acids as well as pathways for cellular maintenance [Dewick (1997)]. On the other hand, secondary metabolism produces a large number of small molecules that arise from biochemical pathways characteristic of only a few species of organisms of 'single chemical race' which appear at a certain stage of differentiation of specialized cells [Luckner (1990)]. Secondary metabolites are not essential for organisms to live but provide a means of survival. The role of secondary metabolites ranges from providing defence against predators due to their toxicity, to acting as volatile signals or colorants which attract particular pollinators. In addition, this group of compounds provides most of the naturally occurring pharmacologically active molecules [Dewick (1997)].

Among the various classes of secondary metabolites, alkaloids are a group of nitrogen-containing, low molecular weight compounds mostly derived from the amino acids phenylalanine, tyrosine, tryptophan, lysine and ornithine. They possess toxic as well as hallucinogenic properties, yet in small dosages are known for their pharmacological benefits. Of the various sub-classes of alkaloids, the monoterpene indole alkaloids (MIAs), derived from tryptophan and terpenoid precursors, are of particular interest since they are the source of the anti-neoplastic agents vinblastine (VBL) and vincristine (VCR) (Fig.1). These compounds were first isolated from

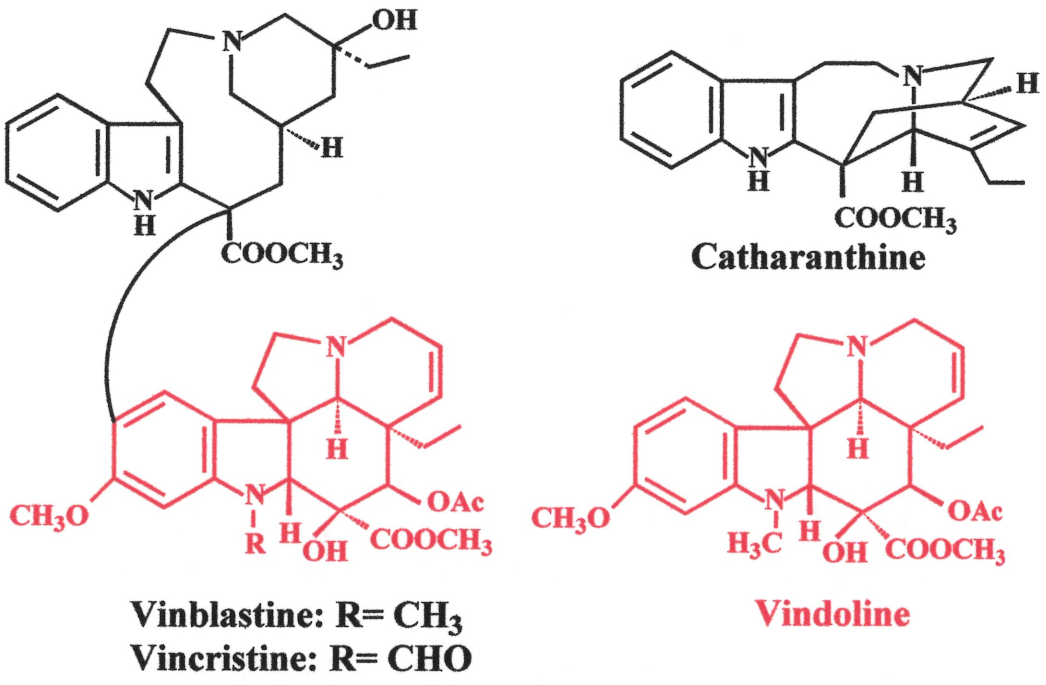

Figure 1. The dimeric antineoplastic agents, vinblastine (VBL) and vincristine (VCR) and their monomeric constituents vindoline and catharanthine.

Catharanthus roseus (L.) G. Don, (Madagascar periwinkle), by the Canadian R.L. Noble in collaboration with scientists from Eli Lilly in the late 1950's [Noble (1990)] and after structure elucidation were shown to be composed of a molecule of vindoline and a molecule of catharanthine [Neuss *et al.* (1958); Gorman *et al.* (1959)]. Despite some secondary effects, VCR is still the most effective chemotherapeutic agent in use today for fighting leukaemia in children, whereas VBL is more effective towards other forms of leukaemia and Hodgkin's lymphoma.

Due to their low abundance (*ca.* 0.0005% of dry weight) and high price, extensive efforts were undertaken to establish a *Catharanthus roseus* cell culture capable of producing VCR and VBL in larger quantities [Van der Heijden *et al.* (1989)]. Although these efforts produced cell lines which accumulated large quantities of catharanthine [Deus-Neumann *et al.* (1987) and references therein], these cell lines were unable to accumulate vindoline. Subsequent studies characterizing the last six steps in the biosynthesis of vindoline revealed a significant amount of information regarding pathway regulation, product accumulation and the different cell types involved in indole alkaloid biosynthesis.

This thesis describes the strategy used to clone the gene coding for acetyl coenzyme A : deacetylvindoline-4-*O*-acetyltransferase (DAT) which catalyzes the last step in vindoline biosynthesis, as well as its characterization. In addition, the cloning and characterization of a root-specific, minovincinine 19-*O*-acetyltransferase (MAT) gene is also described, with comparisons to the leaf-specific DAT. Both acetyl CoA-dependent *O*-acetyltransferases are compared with respect to their expression patterns, substrate specificities, and kinetic parameters. Lastly, results obtained from

investigating the effect of light, phytohormones and secondary messenger inhibitors on DAT activity are presented.

B. Literature Review

B.1. Early Stages of *Catharanthus* Alkaloid Biosynthesis

The MIAs of *Catharanthus roseus* are composed of the corynanthe, iboga and aspidosperma types, based on the various rearrangements in the terpenoid moeity of the strictosidine molecule (Fig. 2). Their biosynthesis is initiated from the amino acid tryptophan (Trp), a product of the shikimate pathway, and geraniol, a product of the mevalonic acid pathway. Decarboxylation of Trp by tryptophan decarboxylase (TDC) yields tryptamine while geraniol undergoes several enzyme-catalyzed biosynthetic conversions to yield secologanin (Fig. 3). Tryptamine and secologanin are condensed together by the action of strictosidine synthase (STR1) to produce the central intermediate strictosidine that leads to the formation of the various classes of MIAs (Fig. 2). The enzymology and regulation of indole alkaloid biosynthesis has been extensively reviewed [De Luca *et al.* (1992); De Luca (1993); Meijer *et al.* (1993c)].

B.1.1. Tryptophan Decarboxylase (TDC)

The decarboxylation of tryptophan to tryptamine is catalyzed by a pyridoxal phosphate and pyrroloquinone quinone-dependent trytophan decarboxylase (TDC) (EC 4.1.1.28), an enzyme at the crossroads between primary and secondary metabolism. In *C. roseus* this cytosolic soluble protein [De Luca & Cutler (1987)] occurs as a homodimer of M_r 115 kDa consisting of 54 kDa monomer subunits, with a pI of 5.9 [Alvarez-Fernandez *et al.* (1989b); Noé *et al.* (1984)]. TDC exists as a stable dimeric form *in vivo*, in equilibrium with a monomeric form which can undergo irreversible inactivation via a proteolytic process requiring ATP [Alvarez-

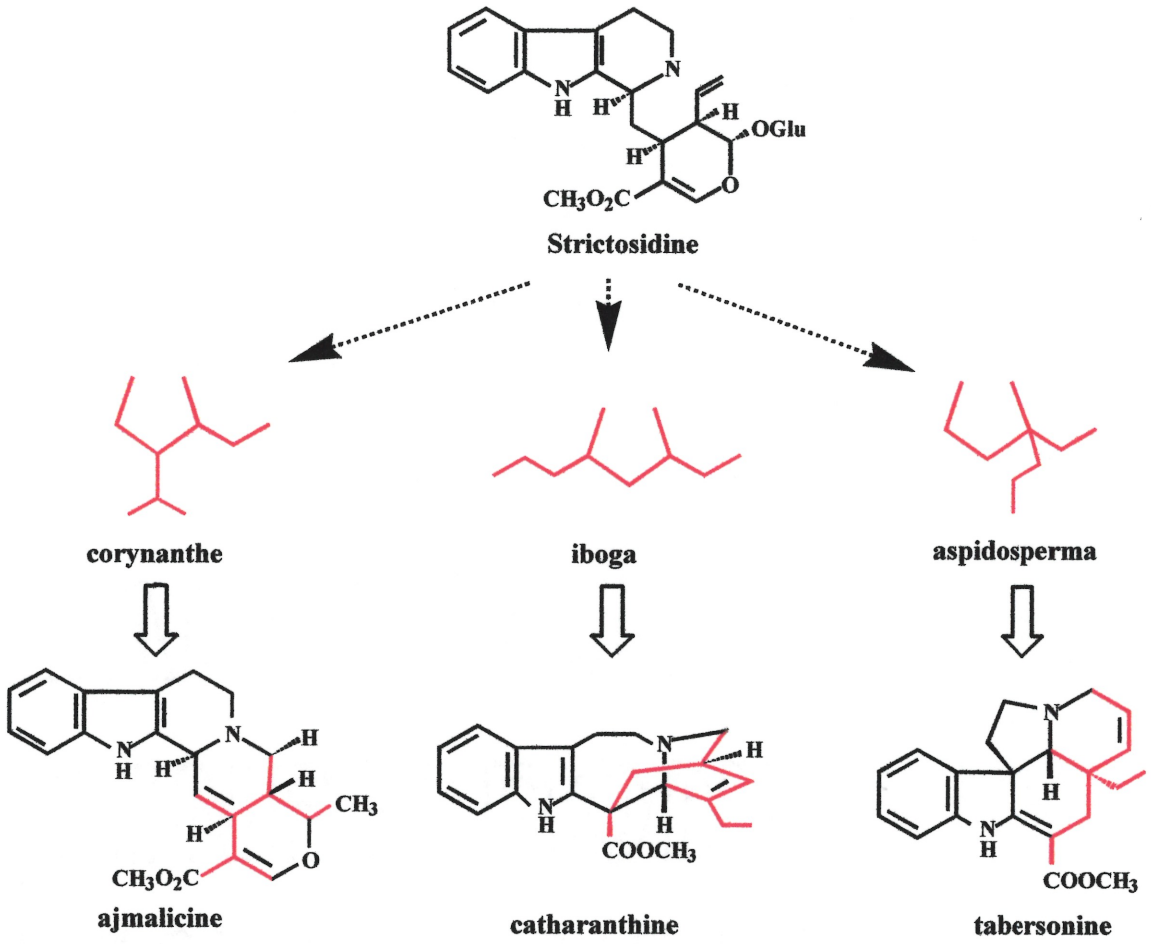

Figure 2. Formation of corynanthe, iboga and aspidosperma backbones (illustrated in red) derived from strictosidine and representing the different classes of monoterpene indole alkaloids found in *Catharanthus roseus*.

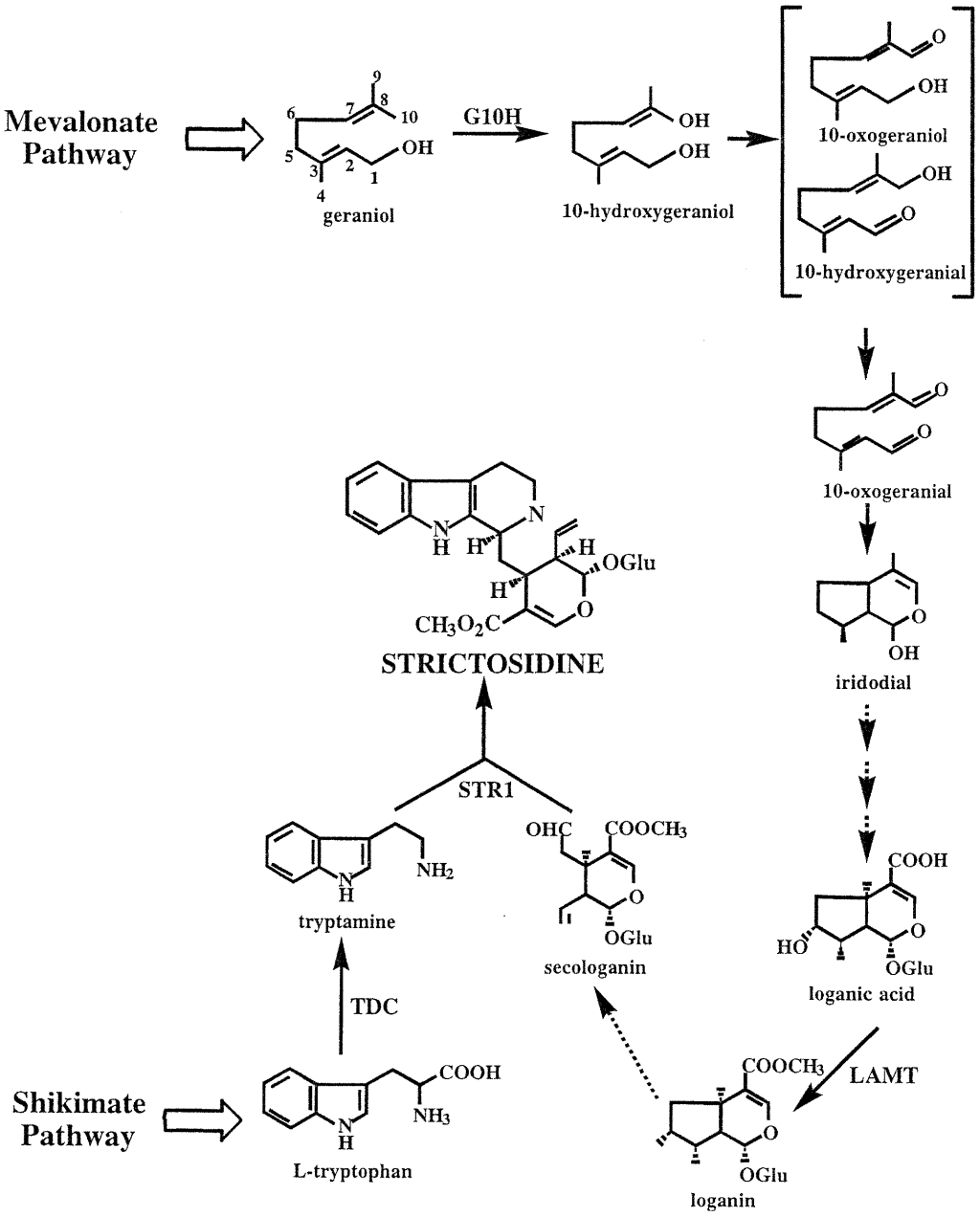

Figure 3. The early steps in the biosynthesis of vindoline [adapted from : De Luca (1993)]. The formation of strictosidine from L-tryptophan and geraniol. Dotted lines represent uncharacterizaed enzyme reactions.. **TDC**= tryptophan decarboxylase; **G10H**= geraniol –10- hydroxylase; **LAMT**= *S*-adenosyl-L-methionine :loganic acid methyltransferase; **STR1**= strictosidine synthase

Fernandez *et al.* (1989a)]. Antibody screening of a cDNA library made from 7-day-old, light-induced *Catharanthus* seedlings permitted the isolation of full-length TDC cDNA clones [De Luca *et al.* (1989)]. Expression of the clones in *E.coli* [De Luca *et al.* (1989)] and in *Nicotiana tabacum* [Songstad *et al.* (1990)] was verified by assay for TDC activity and confirmed the identity of the clone. Southern blot analysis of the genomic DNA revealed TDC to be a single copy gene [Goddijn *et al.* (1994)] and its deduced amino acid sequence was shown to be 39% identical to L-DOPA decarboxylase from *Drosophila melanogaster* [De Luca *et al* (1989); see review by Facchini *et al.* (2000)].

B.1.2. Geraniol-10-Hydroxylase (G10H)

Secologanin provides the C_9-C_{10} moiety in the biosynthesis of MIAs in *Catharanthus roseus* [Meehan and Coscia (1973)]. This precursor is formed via an initial hydroxylation of geraniol by geraniol-10-hydroxylase (G10H) that leads to the formation of 10-hydroxygeraniol (Fig. 3) [Madyastha and Coscia (1979)]. More recently, it has been found, through the use of ^{13}C-glucose feeding experiments to *Catharanthus* cell cultures, that the triose phosphate/pyruvate pathway may be an alternate route to secologanin biosynthesis [Contin *et al.* (1998)].

G10H was initially isolated from *C. roseus* seedlings and found to be an NADPH-dependent cytochrome P-450 monoxygenase associated with the endoplasmic reticulum (ER) [Meehan and Coscia (1973); Madyastha *et al.* (1977)]. Partial purification of G10H was initially carried out by Madyastha *et al.* (1976), while Meijer *et al.* (1993a) purified it to near homogeneity. The purified enzyme had

a M_r of 56 kDa, a pI = 8.3 and accepted geraniol as well as its *cis* isomer nerol as substrates [Meijer *et al.* (1993a)]. Therefore, it may well be that G10H can hydroxylate either geraniol or nerol, or that the nerol-specific enzyme has similar purification properties to G10H [Meijer *et al.* (1993a)]. Solubilization of the G10H protein from the membrane fraction resulted in separating G10H from the NADPH:cytochrome P450 reductase using ion exchange chromatography. G10H activity was reconstituted by incubating the reductase with a crude preparation of *Catharanthus* lipids containing the cytochrome P450 component [Madyastha *et al.* (1976)].

Since it catalyzes the first committed step in secologanin biosynthesis, it is believed that G10H may be a potential site of regulation, since catharanthine was shown to be a reversible, linear, non-competitive inhibitor of its activity [McFarlane *et al.* (1975)]. Such a feedback mechanism may operate *in vivo* provided that catharanthine is produced and/or accumulates in the same site(s) as is G10H. Developmental control of G10H activity in *C. roseus* seedlings appears to be similar to that observed for TDC [Meijer *et al.* (1993c)] (see section on regulation of TDC). It is interesting to note that in *Catharanthus* cell suspension cultures, both TDC and G10H activities can be induced when cells are transferred to alkaloid production media [Knobloch *et al.* (1981)]. However, only induction of G10H was strongly correlated with alkaloid accumulation, suggesting that regulation of secologanin biosynthesis may be rate limiting [Schiel and Berlin (1986); Schiel *et al.* (1987)].

Although several cytochrome P450 cDNA sequences have been cloned from a *C. roseus* cDNA library [Meijer *et al.* (1993b)], it is only recently that Mizutani *et*

al. (1998) have successfully cloned a G10H gene from *Arabidopsis* [Ohta and Mizutani (1998)].

B.1.3. Strictosidine Synthase (STR1)

The stereospecific condensation of secologanin and tryptamine is catalyzed by the enzyme strictosidine synthase (STR1) (EC 4.3.3.2) to produce the key intermediate in MIA biosynthesis, H-3-α-(S)-strictosidine (Fig. 3). The enzyme is present in cell suspension cultures and seedlings of *C. roseus* [Scott and Lee (1975); see review by Kutchan (1993)].

Strictosidine synthase, which has been purified to apparent homogeneity from *C. roseus* cell suspension cultures, was resolved into four [Pfitzner and Zenk (1989)] and six charge isoforms [de Waal *et al.* (1995)], respectively. Since multiple isoforms of strictosidine synthase were also detected in *C. roseus* leaves, this suggests that they were not an artefact derived from the growth of cell cultures. In addition to being more abundant than in *C. roseus* cultures, STR1 was found to exist as a single isoform in cell cultures of *Rauvolfia serpentina* [Hampp and Zenk (1988)] and was more stable than the enzyme purified from *Catharanthus* [Hampp and Zenk (1988); Pfitzner and Zenk (1989)].

Full-length cDNA STR1 clones have been isolated from both *Rauvolfia* and *Catharanthus* cDNA libraries [Kutchan *et al.* (1988); McKnight *et al.* (1990); Pasquali *et al.* (1992)]. The translated product of the *Rauvolfia* cDNA contains a single putative *N*-terminal glycosylation site [Kutchan *et al.* (1988)], while 2 sites were identified in the *Catharanthus* cDNA [McKnight *et al.* (1990)]. However,

expression of the *STR*1 clone in *Escherichia coli* yielded a single enzymatically active recombinant enzyme [Roessner *et al.* (1992)]. Early biochemical studies reported that STR1 was a cytosolic enzyme [Deus-Neumann and Zenk (1984); De Luca and Cutler (1987)]. However, the presence of glycosylation sites and a vacuolar targeting signal peptide in both cDNAs, as well as immunogold localization studies, suggest that STR1 is a vacuolar enzyme [McKnight *et al.* (1991)]. Recently, *in situ* RNA hybridization and immunocytochemistry studies revealed that *str1* transcripts are expressed exclusively within epidermal tissue of stems, leaves and flower buds, as well as in protoderm and cortical cells near the apical meristem of root tips [St-Pierre *et al.* (1999)].

Studies on the regulation of STR1 enzyme activity indicated that this enzyme does not represent a rate-limiting step in alkaloid biosynthesis [Meijer *et al.* (1993c)]. STR1 activity levels remained relatively constant in experiments carried out with *C. roseus* cell cultures grown in alkaloid production medium, whereas TDC and G10H activities rose rapidly with a concomitant increase in alkaloid accumulation [Knobloch *et al.* (1981); Schiel *et al.* (1987)]. However, the regulation of strictosidine synthase appears to resemble that of TDC at the mRNA level (see section on regulation of TDC and STR1).

The *STR1* gene was also isolated and partially characterized in *R. serpentina* and *R. mannii* [Bracher and Kutchan (1992)]. Northern analysis, enzyme assays and indole alkaloid analysis suggested that all plant parts were capable of strictosidine biosynthesis, but the highest expression occurred in roots. Similar studies revealed that expression of *STR1* was highest in *Catharanthus* roots. More recently, the complete genomic clone was isolated and sequenced [Pasquali *et al.* (1992)]. The

STR1 gene occurred as a single copy in the *Catharanthus* genome, confirming that the multiple isoforms are produced by post-translational modifications [Pasquali *et al.* (1992)] such as differential glycosylation. Deglycosylation of strictosidine results in the formation of a highly reactive aglycone leading to the production of a variety of MIAs [reviewed by De Luca (1993); Stöckigt (1980)].

B.1.4. Strictosidine β-D-Glucosidase (SGD)

Strictosidine is deglucosylated by the ER-associated protein SGD (EC 3.2.1.105) to yield a highly reactive dialdehyde. Various products can be formed from this dialdehyde depending on solvent and pH conditions within the cell. In *C. roseus*, the major product is cathenamine [Geerlings *et al.* (2000)].

Early studies showed SGD to exist as two isoforms with a high specificity for strictosidine [Hemscheidt and Zenk (1980)]. More recently, SGD was purified from *C. roseus* cultured cells and characterized [Geerlings *et al.* (2000)]. It has a M_r of 63 kDa by denaturing SDS-PAGE, but is resolved as three high molecular weight bands of *ca.* 250, 500 and 630 kDa on native PAGE, suggesting the formation of either a 4, 8 or 12 monomer aggregate [Geerlings *et al.* (2000)]. *SGD* was cloned from a cDNA library and found to be a single copy gene with homology to other plant β-glucosidases such as prunasin hydrolase and amygdalin hydrolase from *Prunus serotina* and cyanogenic β-glucosidase from *Trifolium repens* [Geerlings *et al.* (2000)]. Expression of the *SGD* cDNA in yeast resulted in a recombinant protein with SGD activity. The *sgd* mRNA and SGD activity levels were most abundant in leaf and root tissue [Geerlings *et al.* (2000)]. Treatment of cell suspension cultures with

methyl jasmonate (MeJa) transiently induced *SGD* expression similar to results obtained for *TDC* [Aerts *et al.* (1994); Vazquez-Flota and De Luca (1998b)] and *STR1* [Aerts *et al.* (1994)]. Maximum *SGD* expression levels were observed at 2-8 h and decreased by 24 h after exposure to MeJa [Geerlings *et al.* (2000)]. Despite some ambiguity regarding the localization of SGD outside the vacuole, SGD was localized to the ER by sucrose density gradient analysis together with marker protein assays, in addition to immunocytochemical localization studies using a monoclonal antibody specific for the (peptide) ER retention signal HDEL [Geerlings *et al.* (2000)]. These findings further suggest that metabolite trafficking occurs between the different cellular compartments involved in MIA biosynthesis in *C. roseus*.

B.2. Late Stages of Vindoline Biosynthesis

The late stages of vindoline biosynthesis involve a six step conversion of tabersonine to vindoline (Fig. 4), involving hydroxylation of tabersonine to yield 16-hydroxytabersonine, *O*-methylation to form 16-methoxytabersonine, hydration of the 2,3 double bond, *N*-methylation and hydroxylation to yield deacetylvindoline, and finally *O*-acetylation to form vindoline. For the purpose of this thesis, only the studies on the last two steps in vindoline biosynthesis will be reviewed.

B.2.1. Desacetoxyvindoline 4-Hydroxylase (D4H)

The penultimate step in vindoline biosynthesis is catalyzed by a cytosolic, 2-oxoglutarate-dependent dioxygenase which hydroxylates the C-4 position of desacetoxyvindoline (16-methoxy-2,3-dihydroxy-3-hydroxy-*N*(1)-methyltabersonine)

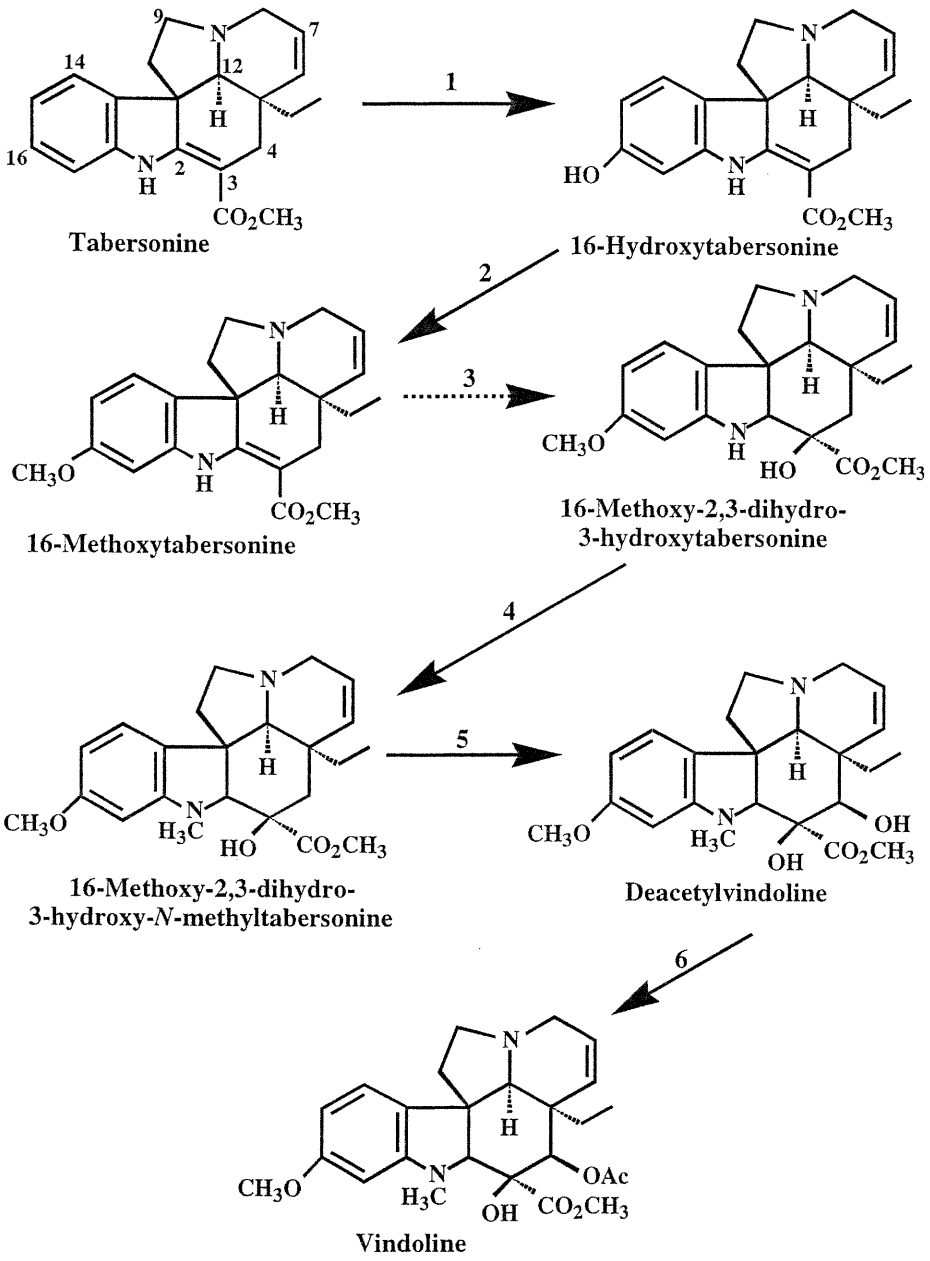

Figure 4. The last six steps in vindoline biosynthesis stemming from tabersonine. (**1**) tabersonine-16-hydroxylase; (**2**) 16-hydroxytabersonine-16-*O*-methyltransferase; (**3**) an uncharacterized hydroxylase; (**4**) 2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase; (**5**) desacetoxyvindoline 4-hydroxylase, D4H; (**6**) acetyl CoA :deactylvindoline 4-*O*-acetyltransferase, DAT.

to yield the 3,4-dihydroxy derivative, deactylvindoline (Fig. 4) [De Carolis *et al.* (1990)]. Typical of this class of dioxygenase, D4H showed an absolute requirement for 2-oxoglutarate and Fe^{2+} ions, and its activity was enhanced by ascorbate [De Carolis and De Luca (1993)]. The enzyme was purified to near homogeneity and a detailed kinetic analysis was carried out [De Carolis and De Luca (1993)]. Three charge isoforms of D4H with pI values 4.6, 4.7, and 4.8 were resolved by denaturing, two-dimensional isoelectric focusing (IEF—SDS-PAGE) of the purified enzyme [De Carolis and De Luca (1993)]. Data from kinetic analyses suggest an ordered Ter Ter mechanism, a general feature of 2-oxoglutarate-dependent dioxygenases, with K_m values of 45.0, 45.0, and 0.03 μM for 2-oxoglutarate, O_2, and desacetoxyvindoline, respectively [De Carolis and De Luca (1993)]. In addition, the K_m values for ascorbate and Fe^{2+} were found to be 0.2 mM and 8.5 μM, respectively. The V_{max} for the conversion of desacetoxyvindoline to DAV was calculated to be 3.85 picokatal/mg protein [De Carolis and De Luca (1993)]. D4H was shown to be developmentally regulated and was induced in developing seedlings after light exposure, involving a phytochrome-mediated mechanism [De Carolis *et al.*(1990); Vazquez-Flota and De Luca (1998a)]. Cloning of the D4H gene was carried out using a degenerate oligonucleotide derived from one of three partial peptide sequences obtained from microsequencing of the purified D4H. Sequence analysis of this clone revealed it to have a high degree of homology to *Hyoscyamus niger* hyoscyamine 6β-hydroxylase [Vazquez-Flota *et al.* (1997)] and to the super family of 2-oxoglutarate-dependent dioxygenases [De Carolis and De Luca (1994)]. *Escherichia coli* transformed with a histidine-tagged expression vector (pQE-30) harbouring the open-

reading frame segment of clone *cD4H-3* expressed D4H activity, thereby confirming the identity of the clone [Vazquez-Flota *et al.* (1997)]. D4H assays along with Southern blot analyses showed that enzyme activity closely followed *d4h* transcripts, which occurred predominantly within young leaves [Vazquez-Flota *et al.* (1997)]. Furthermore, although etiolated seedlings had considerable levels of *d4h* transcripts and D4H protein, hydroxylase activity was undetectable. Exposure of dark-grown seedlings to light resulted in a rapid increase of enzyme activity while *d4h* transcript levels remained unchanged as compared with dark-grown controls [Vazquez-Flota *et al.* (1997)]. The results obtained by Vazquez-Flota *et al.* (1997) suggest that D4H is dependent on light for its activity and is possibly regulated by post-transcriptional and post-translational modifications. Further studies have revealed that the expression of D4H is regulated by cell-, tissue-, development- and environment-specific controls.

B.2.2. Acetyl Coenzyme A-Dependent Deacetylvindoline-4-*O*-Acetyltransferase (DAT)

The final step in vindoline biosynthesis is catalyzed by DAT (Fig. 4). This cytosolic enzyme, which catalyzes the conversion of deacetylvindoline to yield vindoline [De Luca *et al.* (1985); Fahn *et al.* (1985a)], was initially purified 365-fold, from *Catharanthus roseus* leaves and partially characterized [De Luca *et al.* (1985)]. DAT was also purified *ca.* 3500-fold from *Catharanthus* leaf tissue and characterized by Fahn and Stöckigt (1990) whose results suggested that DAT exists as a 20 and 26 kDa heterodimer. Similar findings were obtained by [Power (1989); Power *et al.* (1990)] who purified DAT 3300-fold using an agarose-hexane-coenzyme A Type 1

affinity matrix. SDS-PAGE of the purified protein revealed two major proteins with M_r values of 33 kDa and 21 kDa, while native PAGE resolved three proteins [Power (1989); Power *et al.* (1990)]. The SDS-PAGE results suggested that DAT existed as a heterodimer [Power (1989); Power *et al.* (1990)]. When subjected to IEF-PAGE, purified DAT was resolved into three isoforms. One major isoform had a pI = 4.7-5.3, while the other two had pI values of 5.7 and 6.1 [Power (1989); Power *et al.* (1990)]. DAT had a pH optimum between 7.5 and 9, a K_m of 6.5 μM and 1.3 μM for acetylcoenzyme A (AcCoA) and deacetylvindoline (DAV), respectively, and V_{max} values of 12.6 pkat/μg protein for AcCoA and 10.1 pkat/μg protein for DAV [Power (1989); Power *et al.* (1990)]. The enzyme activity could be maintained and stabilized in the presence of dithiothreitol (DTT), whereas inactive enzyme could be reactivated by an overnight incubation with 1mM DTT [De Luca *et al.* (1985)]. DAT activity could be inhibited by tabersonine, CoA and K^+, Mg^{2+} and Mn^{2+} [Power (1989); Power *et al.* (1990)].

B.3. Regulation of Expression of Vindoline Biosynthetic Enzymes

Vindoline biosynthesis is regulated during plant development, is expressed in particular tissues and responds to environmental cues. This section will deal with the early steps in the pathway catalyzed by TDC and STR1, in addition to the penultimate step catalyzed by D4H and the last step catalyzed by DAT. Various studies have been carried out with respect to elucidating the developmental and environmental factors involved in regulating vindoline biosynthesis, as well as the tissue-specific

localization of the above-mentioned enzymes and their respective genes by *in situ* localization.

B.3.1. Regulation of TDC

Tryptophan decarboxylase activity is highly regulated at the transcriptional, translational and post-translational levels [as reviewed in : De Luca (1993); Meijer *et al.* (1993c); Facchini *et al.* (2000)]. TDC appears transiently within developing *C. roseus* seedlings and its presence coincides with the accumulation of MIAs in this plant [De Luca *et al.* (1986)]. The enzyme can also be transiently induced in cell suspension cultures by transfer of cells to an alkaloid-production medium consisting of a high concentration of sucrose and low levels of growth regulators, phosphate, and nitrogen-containing compounds [Knobloch *et al.* (1981)] or by treating the cells with fungal elicitors [Eilert *et al.* (1987)]. Since the induction of TDC under such circumstances does not always coincide with the accumulation of indole alkaloids, it has been suggested that the synthesis of indole alkaloids is not controlled by the supply of tryptamine precursor [De Luca (1993)].

B.3.1.1. Elicitors and TDC

The effect of elicitation on TDC activity in relation to that of STR1 in young *Catharanthus* seedlings was investigated. It was found that MeJa had a less pronounced effect on inducing TDC activity than it did on STR1 activity [Aerts *et al.* (1994)]. However, 7d-old seedlings exposed to 12 hours of MeJa produced a 400%

increase in TDC in both dark- and light-grown seedlings, as compared to untreated controls [Vazquez-Flota and De Luca (1998b)].

More recently, functional dissection of the promoter of the *TDC* gene using T-DNA activation tagging and *C. roseus* cell suspension cultures, revealed the presence of a jasmonate/elicitor responsive element (JERE) [van der Fits (2000)]. Furthermore, sequencing of the rescued transgenic plasmid revealed an ORF for an octadecanoid-derivative responsive *Catharanthus* AP2-domain protein (ORCA3) transcription factor [van der Fits (2000); van der Fits and Memelink (2000)]. This factor is related to a previously cloned ORCA2, which is enhanced by JA or elicitor [Menke *et al.* (1999a)]. However, further characterization of ORCA3 revealed its specificity for binding to the promoter of *STR1* [van der Fits (2000); van der Fits and Memelink (2000)].

B.3.2. Regulation of STR1

It has been observed by a number of researchers that *tdc* and *str1* are coordinately regulated at the transcriptional level and that they may share some common regulatory mechanisms [Roewer *et al.* (1992); Pasquali *et al.* (1992)]. However, it has been speculated that the lower stability of *tdc* mRNA and *in vitro* enzyme activity, compared to that of *str1* [Treimer and Zenk (1979); Noé *et al.* (1984); Noé and Berlin (1985)], suggest that TDC rather than STR may represent an important control point in regulating alkaloid biosynthesis [Meijer *et al.* (1993c)].

Within *Catharanthus* plants, the steady state levels of both *tdc* and *str1* were highest in roots while activity levels were highest in the aerial parts of seedlings

[Pasquali *et al.* (1992)]. Studies on the time-induced/developmental appearance of TDC, STR1, *N*-methyltransferase (NMT) and DAT in developing *C. roseus* seedlings revealed that despite the order of the enzyme-catalyzed reactions in this biosynthetic pathway, STR1 activity appeared before that of TDC. STR1 activity appeared relatively early in seedling development, attaining approximately 50% of its maximal level of activity by day 3, while that of TDC began to increase after day 3 and attained its maximum by day 5, along with that of STR1 [De Luca *et al.* (1988)]. In addition, STR1 activity was present in all plant parts and was not influenced by light treatment [De Luca *et al.* (1988)].

B.3.2.1. Elicitors and STR1

As observed with *tdc*, *str1* was trancriptionally down-regulated by auxin, in cell suspension cultures, while cytokinins did not exert any effect [Pasquali *et al.* (1992)]. Of the various elicitors tested on the induction of *str1* mRNA in *C. roseus* cell cultures, a *Pythium aphanidermatum* culture filtrate and a yeast extract, respectively, had the maximum effect in causing a transient increase in *str1* mRNA levels, by possibly acting upon pre-existing transcription factors [Roewer *et al.* (1992); Pasquali *et al.* (1992)].

Other studies showed that the effect of MeJa treatment of *Catharanthus* seedlings markedly enhanced STR1 enzyme activity [Aerts *et al.* (1994)]. Promoter analysis studies of the *STR1* gene carried out with *Catharanthus* cell cultures, revealed that a GCC-box-like domain was necessary and sufficient to activate *STR1* expression by treatment with jasmonic acid (JA) or with yeast fungal elicitor [Menke

et al. (1999a)]. Further characterization of the promoter region using a yeast one-hybrid screen revealed that two octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) proteins bind the JA- and elicitor-responsive element in a sequence-specific manner. Studies with ORCA2 showed it could *trans*-activate the *str1* promoter, in the presence of JA and fungal elicitor and rapidly induce *STR1* expression [Menke *et al.* (1999a)]. More recently, ORCA3 was cloned through the use of T-DNA activation tagging and was shown to be related to ORCA2 with only slight differences in their sequences [van der Fits (2000); van der Fits and Memelink (2000)]. Further yeast one-hybrid screening of a *Catharanthus* cDNA library, using the *STR1* promoter as bait identified a MYB-like protein with high homology to parsley Box P Binding Factor-1 (PcBPF-1), CrBPF-1 [van der Fits (2000)]. CrBPF-1 differed from the ORCA transcription factors since its expression was rapidly activated by elicitor treatment, but not by JA [van der Fits (2000)]. These findings clearly illustrated the presence of two distinct transcription factors capable of affecting *STR1* expression.

The yeast elicitation of *STR1* was further investigated in *C. roseus* cell cultures and the results demonstrated that in addition to activating *TDC* and *STR1*, jasmonic acid biosynthesis was also induced [Menke *et al.* (1999b)]. In addition, the protein kinase inhibitor K-252a abolished elicitor-induced biosynthesis of JA, as well as JA-induced expression of *STR1* and *TDC*. These results suggest that the JA biosynthetic pathway may coordinate expression of *TDC* and *STR1* and that protein kinases are involved [Menke *et al.*(1999b)]. In order to improve the yield of monoterpene indole alkaloids in cell cultures, the effects of over-expressing STR1

and TDC on alkaloid production in *Catharanthus roseus* cell cultures was investigated [Canel *et al.* (1998)]. The results showed that over-expressing TDC was toxic to the cells and was unnecessary for increasing the production of MIAs. In contrast, STR1 over-expression appeared to be necessary yet insufficient to maintain a high level of alkaloid biosynthesis and was well-tolerated by the cells [Canel *et al.* (1998)].

Other *STR1* promoter studies in transgenic tobacco plants revealed the presence of a putative CACGTG *cis*-acting element known as a G-box, which directed seed-specific expression of *STR1* [Ouwerkerk and Memelink (1999)]. In addition, Pasquali *et al.* (1999) found enhancer sequences within the *STR1* promoter region capable of binding the tobacco nuclear protein factor GT-1.

B.3.3. Regulation of D4H

The availability of an enzyme assay for detecting D4H activity [De Carolis *et al.* (1990); De Carolis and De Luca (1993)], D4H cDNA clones [Vazquez-Flota *et al.* (1997)], and a highly specific anti-D4H antibody made it possible to investigate the expression and regulation of D4H in more detail. Earlier studies carried out during the cloning of the D4H gene revealed that *d4h* transcripts accumulated predominantly in very young *C. roseus* leaves, whereas very low levels were observed in stems and fruits while no transcripts were observed in roots [Vazquez-Flota *et al.* (1997)]. Interestingly, although detectable amounts of *d4h* transcripts were observed in etiolated seedlings, D4H activity was negligible. However, upon exposing dark-

grown seedlings to light, a rapid increase in hydroxylase activity was observed without any significant increases in transcript levels [Vazquez-Flota *et al.* (1997)].

B.3.3.1. Elicitors and D4H

Previous studies have shown that treatment of *Catharanthus* and *Cinchona* seedlings with MeJa transiently activated TDC activity [Aerts *et al.* (1994)]. Similar experiments were also performed to measure the effects of salicylic acid (SA) and MeJa on the induction of D4H activity in relation to that of TDC in 7d-old *Catharanthus roseus* seedlings [Vázquez-Flota and De Luca (1998b)]. The findings showed that neither compound was effective in inducing either D4H transcripts or enzyme activity in dark-grown seedlings [Vázquez-Flota and De Luca (1998b)]. However, exposing 7d-old etiolated seedlings to both light and MeJa produced a *ca.* 85% increase in D4H activity, as compared to a simple exposure of seedlings to light. The levels of *d4h* transcripts were enhanced 6-fold by light treatment, regardless of whether MeJa was present [Vázquez-Flota and De Luca (1998b)]. However, treating light-exposed seedlings with MeJa caused maximal D4H protein levels to be detected 24 hours earlier than usual and this coincided with the time of highest D4H activity [Vázquez-Flota and De Luca (1998b)].

B.3.3.2. Light and D4H

Light treatment of 5d-old etiolated seedlings showed that a brief 12 h light exposure produced a *ca.* 3-fold increase in D4H activity, while exposure for a further 12 h resulted in a *ca.* 7.5-fold increase in activity, compared to etiolated seedlings of

the same age. The level of D4H protein increased in the presence of light to a maximum after *ca.* 12 h and remained relatively constant thereafter [Vazquez-Flota and De Luca (1998a)]. Similar light treatments carried out with 7d-old dark-grown seedlings only increased D4H activity *ca.* 3-fold over their dark-grown counterparts [Vazquez-Flota and De Luca (1998a)]. These results illustrated that 5d-old *Catharanthus* seedlings were more responsive to light than 7d-old seedlings and that D4H activity was not only under light regulation but it was also influenced by the stage of development. Furthermore, when 7d-old seedlings were returned to darkness following a 24h exposure to light, *d4h* transcripts as well as D4H protein and enzyme activity levels decreased. Re-exposure to light resulted in an increase in all three components, thus clearly illustrating that light is necessary for maintaining D4H activity during seedling development [Vazquez-Flota and De Luca (1998a)]. In addition, 5- and 7d-old seedlings were exposed to pulses of red light, to show that a minimum of 30 min of red light exposure was sufficient to saturate the D4H response at the transcript, protein and activity levels. The involvement of phytochrome in activating this process was also strongly suggested by the ability of far-red light to reverse the activation observed by the initial red light exposure [Vazquez-Flota and De Luca (1998a)]. Since previous studies had revealed that D4H existed as three charged isoforms [De Carolis and De Luca (1993)], it was suggested that post-translational modifications of D4H might be involved in regulating D4H activity. Studies involving IEF—SDS-PAGE and Western immunoblotting illustrated that a pI= 4.7 (putative inactive) D4H isoform was predominant in dark-grown seedlings, whereas it decreased in intensity to be replaced with a more acidic pI= 4.6 (active)

D4H isoform upon exposure to light [Vazquez-Flota and De Luca (1998a)]. These results suggest that D4H expression is under a complex form of post-translational control. It is interesting to note that DAT also appears to exist as isoforms with various activities [Fahn *et al.* (1985b); Power *et al.* (1990)] and may perhaps also rely on a similar regulatory mechanism.

B.3.4. Regulation of DAT

Expression of DAT activity also appears to be regulated by development-, tissue- and environment-specific controls. DAT activity is highest within young leaf/cotyledon tissue, with low levels occuring in stem/hypocotyl tissue and is absent in roots [Fahn *et al.* (1985a); De Luca *et al.* (1986); De Luca *et al.* (1988)]. Trace DAT activity has been observed within 5d-old dark-grown seedlings, which increase *ca.* 10-fold after light treatment [De Luca *et al.* (1986); De Luca *et al.* (1988)]. The light induced increase in DAT activity occurred approx. 24 h after the appearance of TDC and STR1 in developing seedlings, whereas the appearance of TDC and STR1 was not light-dependent [De Luca *et al.* (1988)]. In addition, the appearance of DAT activity coincided with vindoline accumulation [De Luca *et al.* (1986)].

B.3.4.1. Light and DAT

In order to learn more about the inducing effect of light on DAT activity in *Catharanthus* cotyledons, the effect of red light on DAT activity was studied [Aerts and De Luca (1992)]. The results suggested that phytochrome was indeed involved in regulating DAT activity. When 7d-old, dark-grown seedlings were treated with a 30

min pulse of red light ($\lambda \geq 575$ nm), enzyme activity increased *ca.* 2.3-fold over dark-grown seedlings [Aerts and De Luca (1992)]. A 30 min pulse of red light followed by a 30 min pulse of far-red light ($\lambda \geq 710$ nm) reversed the induction process and further suggested the involvement of phytochrome [Aerts and De Luca (1992)].

B.4. Tissue- and Cell-Specific Localization of the Vindoline Pathway

In situ hybridization studies were combined with immunolocalization to identify the sites of biosynthesis of the vindoline pathway. The results showed that at least three types of cells are involved and movement of indole alkaloid intermediates must occur between cells to allow the biosynthesis of vindoline [St-Pierre *et al.* (1999)].

B.4.1. TDC and STR1

Early studies have shown high levels of *tdc* and *str1* mRNA present in roots, while low but detectable levels were found in stems and flowers [Pasquali *et al.* (1992)]. More recently, *in situ* RNA hybridization and immunocytochemical studies carried out on various *C. roseus* tissues have localized *tdc* and *str1* to the epidermis of stems, leaves and flower buds, and in the apical meristem of the root tips [St-Pierre *et al.* (1999)]. More specifically, in leaf tissue, *tdc* and *str1* transcripts along with the expression of TDC protein was localized within the upper and lower epidermal surfaces. The expression of the *tdc* and *str1* genes along with TDC protein decreased significantly with the developmental age of leaf tissue [St-Pierre *et al.* (1999)]. The leaf base exhibited the highest level of *tdc* and *str1* transcripts as well as TDC

protein, while the middle portion of the leaf showed decreasing transcript and protein levels and no expression was noticeable near the tip of the leaf blade. These results suggest a basipetal expression pattern for genes involved in the early steps of MIA biosynthesis. In root tissue, *tdc* and *str1* transcripts together with TDC protein expression was restricted to the protodermal and cortical cells as observed along a longitudinal section of a root tip, while absent in the root cap and stele [St-Pierre *et al.* (1999)]. As was observed in leaf tissue, *tdc* and *str1* transcripts also decreased in older developmental stages of roots. These results are in agreement with earlier studies [Pasquali *et al.* (1992)] which revealed the strict coordinate regulation between *tdc* and *str1* and illustrate that particular cells in roots, stems and leaves are capable of expressing TDC and STR1 activity in order to make the precursors to indole alkaloids. Furthermore, this latter study [St-Pierre *et al.* (1999)] demonstrates how the coordinate regulation of these genes is associated with cell-type specific and developmental control.

More recently, TDC protein immunolocalization studies carried out within young seedlings, demonstrated the presence of TDC protein in cells forming the upper epidermis of both dark- and light-grown cotyledons [Vazquez-Flota *et al.* (2000)], findings similar to the earlier results of St-Pierre *et al.* (1999), who observed the presence of TDC protein in both upper and lower epidermal layers of young leaves of mature plants.

B.4.2. D4H

Investigations regarding *in situ* RNA hybridization and immunocytochemical localization of *d4h* transcripts and D4H protein, respectively, have shown *d4h* is expressed within laticifer and idioblast cells of leaves, stems and flower buds [St-Pierre *et al.* (1999)]. Complementing the *in situ* results, immunocytochemical studies also revealed the presence of D4H protein within the idioblasts and laticifers of leaves, stems and flowers [St-Pierre *et al.* (1999)]. In the case of laticifers, these specialized, latex-containing cells whose primary function is protection against herbivores or microorganisms, are typical of the latex-producing plant families such as the Apocynaceae [Fahn (1979)]. Laticifers mostly occur in aerial plant organs, although they have been known to occur within roots, and are most commonly associated with the phloem [Fahn (1979)]. As already described for *tdc* and *str1* transcripts, a basipetal gradient of *d4h* expression was also observed within the leaf blade [St-Pierre *et al.* (1999)]. Epifluorescence microscopic analysis of cotyledons from young etiolated and light-grown seedlings demonstrated the presence of laticifers and idioblasts, without any noticeable differences in their shape or number [Vazquez-Flota *et al.* (2000)]. Immunolocalization studies of D4H in cotyledons from etiolated seedlings revealed the presence of D4H protein [Vazquez-Flota *et al.* (2000)]. Although D4H protein was present within etiolated tissue, it was not correlated with the level of enzyme activity. These results further corroborated earlier findings that an inactive D4H isoform exists in etiolated *Catharanthus* seedlings [Vazquez-Flota and De Luca (1998a)] and suggest that light is essential for triggering D4H enzyme activity [De Carolis *et al.* (1990); Vazquez-Flota *et al.* (1997);

Vazquez-Flota and De Luca (1998a)]. It has also been suggested that light may cause laticifer-specific factors to interact either at the transcription, translation, and/or post-translation level to ultimately induce D4H activity [Vazquez-Flota *et al.* (2000)].

These studies demonstrated the roles played by cells and tissues in the expression of some key enzymes and transcripts involved in vindoline biosynthesis. The results provide insight about why cell suspension cultures, which are a mass of undifferentiated cells lacking specialized laticifers and idioblasts, are not capable to produce VBL and/or VCR.

B.5. Metabolism of Monoterpene Indole Alkaloid Biosynthesis in *C. roseus*

B.5.1. Cell Suspension Cultures

The very low abundance of VBL and VCR in plant tissues have prompted extensive efforts to produce these compounds in cell culture in order to increase their supply and in turn decrease their cost of production [as reviewed by : Van der Heijden *et al.* (1989); Lounasmaa and Galambos (1989); Moreno *et al.* (1995)]. This strategy has proven ineffective since cell cultures are unable to synthesize consistent levels of vindoline. In contrast, several laboratories have shown that cell suspension cultures accumulate catharanthine when they are transferred to alkaloid production medium [see references in: Deus-Neumann *et al.* (1987)]. In cell suspension cultures, neither NMT nor DAT activities could be detected, even after cells were transferred to an alkaloid producing medium or following the addition of a fungal elicitor, which is known to activate both TDC and STR activities [De Luca *et al.* (1987)]. It was concluded that the inability of cell cultures to produce vindoline may be due to a

block of expression of a few enzymes involved in the late stages of biosynthesis [Meijer *et al.* (1993c)].

When cell cultures were allowed to undergo differentiation to produce shoots and whole plants, vindoline accumulation re-appeared in shoots. These results show that cell cultures contain all the necessary machinery to carry out vindoline biosynthesis but their undifferentiated state may suppress its synthesis [Constabel *et al.* (1982)]. Recently, a study using *Catharanthus* cell cultures transformed with either *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* showed that these transformed cultures could accumulate low levels of vindoline. However, these findings are questionable since the only culture producing a significant amount of vindoline was in fact a transformed shoot culture partly composed of differentitated cells exhibiting shoot growth, typical of a shooty teratoma, rather than an undifferentiated cell suspension culture [O'Keefe *et al.* (1997)].

B.5.2. Seedlings

Catharanthus roseus seedlings have proven to be ideal for studying the complex regulatory mechanisms governing vindoline biosynthesis, particularly with respect to the last stages in the pathway derived from tabersonine. Early studies showed that 6 to 9d-old etiolated seedlings accumulated tabersonine, 16-methoxytabersonine, and catharanthine as the major alkaloids, whereas 16-hydroxytabersonine, 4-desacetoxyvindoline, deacetylvindoline and vindoline were indentified as minor constituents [Balsevich *et al.* (1986)]. Light treatment, however, converted these intermediates quantitatively into vindoline. This study demonstrated

the important role of light in establishing the late stages of this pathway and also gave an idea of the order of reactions leading to the terminal vindoline product [Balsevich *et al.* (1986)]. Similar studies with dark- and light-exposed seedlings clearly showed that vindoline biosynthesis occurred predominantly in cotyledons [De Luca *et al.* (1986)]. Furthermore, the results showed that an increase in TDC activity coincided with the accumulation of tabersonine while an increase in DAT activity coincided with the accumulation of vindoline. This led to the suggestion that the early and late stages of the pathway are coordinately regulated. In addition, the enzymes involved in tabersonine biosynthesis were present in all plant parts whereas those involved in the last five steps in vindoline biosynthesis were restricted to the aerial parts of seedlings (Fig.4) [De Luca *et al.* (1986)].

Catharanthus seedlings were also ideal to study the light-dependent regulation of D4H expression [Vazquez-Flota and De Luca (1998a); Vazquez-Flota and De Luca (2000)] in relation to the modulatory effects of MeJa and SA treatment [Vazquez-Flota and De Luca (1998b)].

B.5.3. Roots and Hairy Root Cultures

Since the discovery of the antineoplastic agent VBL from *Catharanthus roseus* [Noble *et al.* (1958)], all parts of the plant were studied for their chemical constituents. Alkaloid extracts from root tissue were initially analyzed by thin-layer chromatography (TLC) and later by high performance liquid chromatography (HPLC) along with infra-red (IR) spectroscopy and mass spectrometry (MS), to characterize and identify them. Numerous reports revealed that *Catharanthus* roots accumulate a

large variety of alkaloids [Svoboda *et al.* (1963) and references therein] (Fig. 2), such as the *Corynanthe* alkaloid ajmalicine, the *Aspidosperma* alkaloid tabersonine and the *Iboga* alkaloid catharanthine [for a review on the pharmacognosy of *Catharanthus* as well as *Vinca* and its various organs'/tissues' compounds, see: Farnsworth (1961)].

Vindoline is not present in *Catharanthus* roots, despite the presence of many other MIAs which have been isolated. *C. roseus* roots were transformed with *Agrobacterium rhizogenes* to produce hairy root cultures. Such cultures tend to be genetically and biochemically more stable and they possess a higher level of differentiation than cells in suspension cultures, in addition to being amenable to genetic transformation [Hamill *et al.* (1987); Shanks *et al.* (1998); Shanks and Morgan (1999)]. The other advantage of such cultures is their ability to produce higher levels of secondary metabolites than callus or cell suspension cultures, which may be important to achieve the ultimate goal of engineering the production of VBL and VCR in culture.

Among several studies with *Catharanthus roseus* hairy roots, one reported that two separate cell lines were capable of producing vindoline [Bhadra *et al.* (1993)]. However there was some controversy concerning the indirect method of detection used to measure the presence of vindoline. In addition, other studies with *C. roseus* hairy root cultures [Parr *et al.* (1988); Toivonen *et al.* (1989); Vazquez-Flota *et al.* (1994)] demonstrated that they were incapable of producing vindoline. Furthermore, fungal elicitor treatment of such cultures stimulated the accumulation of tabersonine, hörhammericine and lochnericine, but not vindoline [Shanks *et al.* (1998)]. Lochnericine and hörhammericine appear to be derived from tabersonine by

oxidation (Fig. 5). Exposure of these hairy root cultures to light caused a decrease in the level of tabersonine compared to dark-grown cultures and while light is necessary for the induction of the later steps in vindoline biosynthesis in seedlings, vindoline was not present in light-adapted hairy root cultures [Shanks *et al.* (1998)]. Dose-response studies carried out with elicitors such as pectinase and chitinase caused a *ca.* 2.5-fold increase in tabersonine and a 50% increase in ajmalicine levels, respectively, compared to non-elicited control cultures. Treatment with jasmonic acid (JA) increased the specific yields of ajmalicine by 80%, while the level of serpentine, lochnericine and hörhammericine increased by 60, 150 and 500%, respectively, above their respective basal levels within non-treated cultures [Rijhwani and Shanks (1998)]. On the other hand, low dosage JA treatment of *C. roseus* hairy roots initially caused the level of tabersonine to decrease below control levels, followed by a gradual increase when JA was increased from 0.25 mg to 1.25 and 2.5 mg [Rijhwani and Shanks (1998)]. However, tabersonine levels still remained below control levels possibly due to the flux of tabersonine and tabersonine-derived intermediates towards the JA-induced accumulation of ajmalicine, serpentine, lochnericine and hörhammericine.

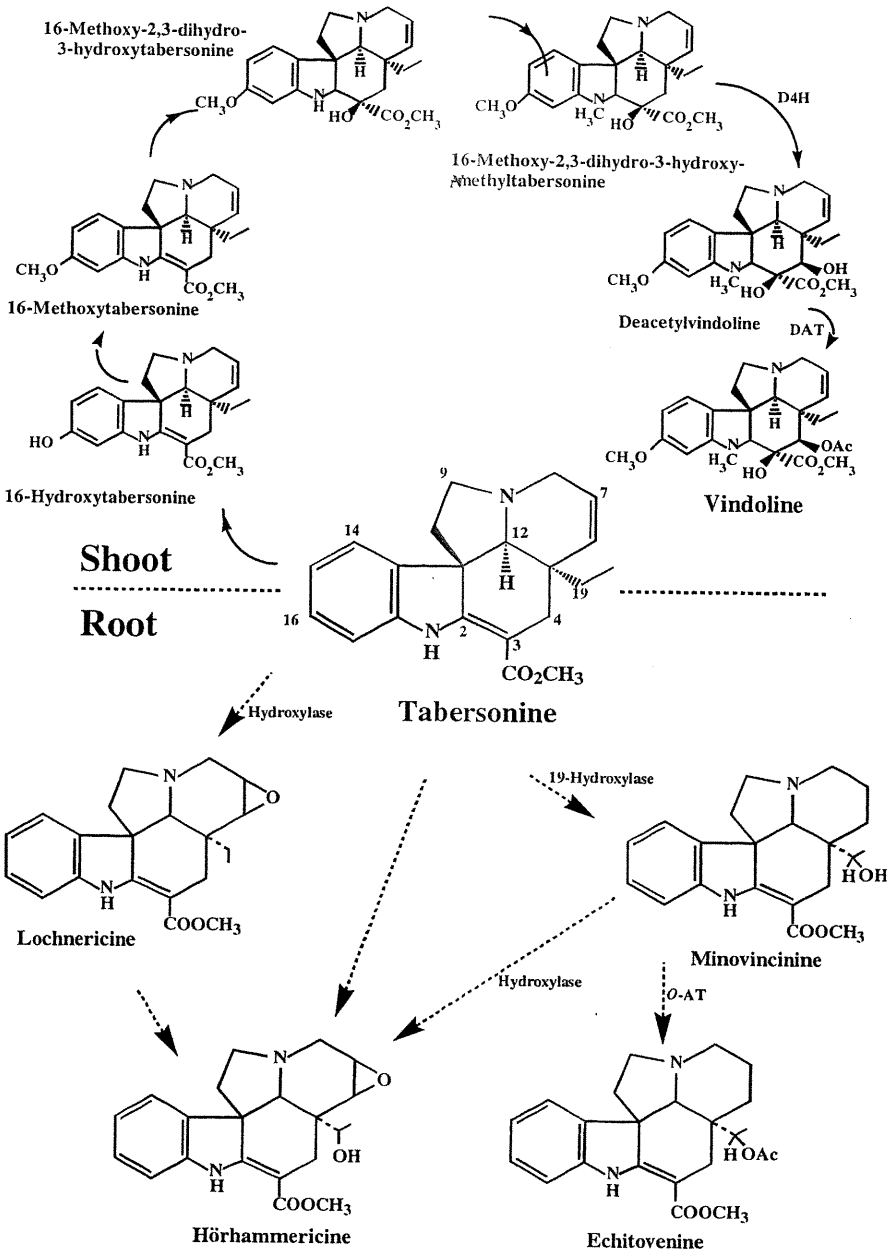

Figure 5. Biosynthesis of tabersonine derived indole alkaloids in *Catharanthus roseus* organs. Tabersonine is converted into vindoline via six enzymatic steps. Tabersonine is converted into lochnericine, hörhammericine, and minovincinine via uncharacterized hydroxylations and 19-hydroxy-indole alkaloids are converted into their respective products by an uncharacterized *O*-acetylation (*O*-AT). These tabersonine analogues are known to accumulate under certain conditions within cell cultures [Kutney *et al.* (1980)] and roots [Shanks *et al.* (1998)] of *C. roseus*. Dashed arrows represent uncharacterized reactions.

C. Material and Methods

C.1. Plant Material

C.1.1. *Catharanthus roseus* Seedlings and Hairy Root Cultures

Catharanthus roseus (L.) G. Don cv. Little Delicata seeds (W.H. Perron, Laval, Québec) were sterilized in 70% (v/v) ethanol for 30 s and then washed thoroughly in sterile water. After imbibition for 12 h in sterile water, the seeds were germinated at 25°C in the dark on H_20-moistened, sterile filter paper (1 layer of Whatman #1 filter paper + 3 layers of commercial paper towels) in 9 mm petri dishes. Seedlings were sectioned into radicles, hypocotyls and cotyledons prior to harvesting at different times of development. Harvested material was frozen in liquid nitrogen and kept at -80° C until required for analysis. *Catharanthus roseus* plants were grown under standard greenhouse conditions and were harvested immediately prior to analysis.

Catharanthus roseus hairy root cultures [Vázquez-Flota *et al.* (1994)] were grown in the dark in half strength Gamborg B5 medium containing 3% (w/v) sucrose on an orbital shaker set at 130 rpm at 25° C. Hairy roots were subcultured every 21 days and analyses were performed with material grown for 14 days.

C.1.2. Alkaloid Extraction

Alkaloids were extracted from *C. roseus* leaves, hairy roots and roots, according to the method described in [Monforte-González *et al.* (1992)]. Plant material was freeze-dried using a LABCONCO freeze dryer, ground to a fine

powder using a mortar and pestle and extracted with MeOH (50 mL per gram d.wt.) in a Polytron homogenizer. The homogenate was incubated at 50° C in a shaking water bath for 2 h and then filtered through two layers of Whatman #1 filter paper, followed by a wash with 50° C MeOH. The extract was then evaporated to dryness using a Büchi Rotavapor rotary evaporator. The residue was resuspended with a minimal volume of 2.5% (v/v) H_2SO_4 and extracted three times with EtOAc. The aqueous phase was adjusted to *ca.* pH 9.0-10.0 with NH_4OH (29.5%) and extracted three times, each with EtOAc (1:1 v/v). The organic phase was evaporated to dryness and resuspended with 1 mL MeOH. The root and/or hairy root crude alkaloid extract was used as the source of minovincinine for enzyme assays.

C.2. Cloning of *Catharanthus O*-Acetyltransferase Genes

C.2.1. PCR Amplification, 3' RACE and 5' RACE

Four pairs of degenerate primers corresponding to the N- and C- terminal amino acid sequences of peptide 54 of the small subunit of purified *C. roseus* DAT (see Table II) [Alarco (1994)] were used for PCR amplification of a specific 73 bp DAT fragment (Fig. 6). The 50 μL PCR reaction contained: 1X TAQ buffer (10 mM TRIS-HCl pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100), 1.5 mM $MgCl_2$, 20 μM of each dNTP, 2 μM of 54-3 or 54-4 primer, 2-3 μM of 54-5 or 54-6 primer, 5 μCi of 54-3 or 54-4 [^{32}P] end-labeled primer, 1 unit of TAQ DNA polymerase (Promega Biotech, Madison, WI) which was added after the first annealing step, and 0.1 μg of polyadenylated mRNA that had been reverse transcribed in presence of 50 ng oligo d(T)$_{12-18}$ (see below). After denaturation at 96° C for 3 min, the reaction was carried

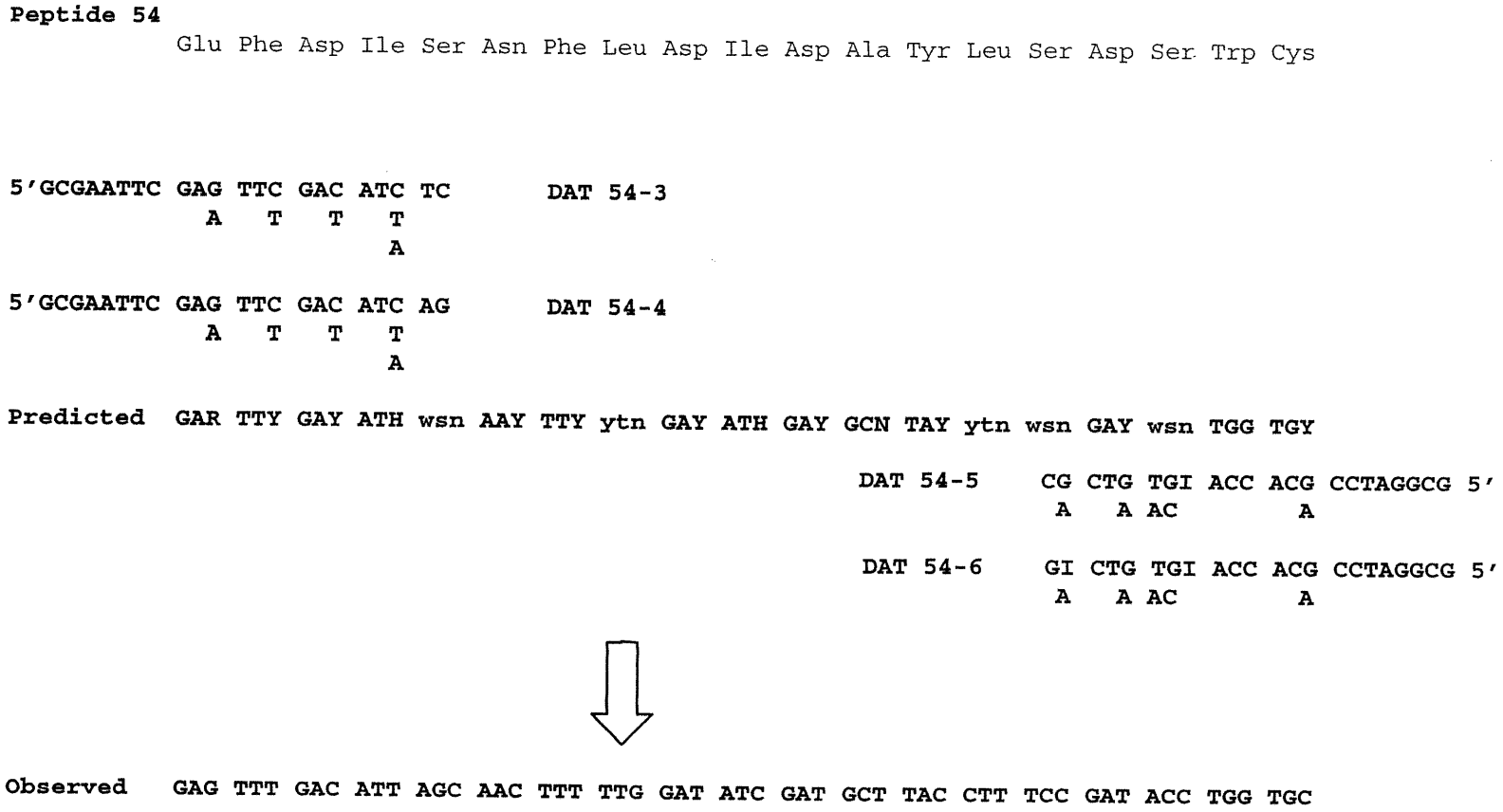

Figure 6. Strategy for amplification of the sequence encoding DAT peptide 54. [Adapted from: St-Pierre *et al.* (1998)].

out for 35 cycles at 94° C for 15 s, 40° C for 30 s, and 72° C for 30 s. The PCR products were separated by urea-PAGE on an 8% (w/v) acrylamide gel, and products were detected by autoradiography. The PCR product of the expected size (73 bp) was purified by native PAGE on a 12% (w/v) acrylamide gel, restricted with both *Eco*RI and *Bam*HI and ligated to the pBluescript II SK+ (pBSIISK+) vector (Stratagene, La Jolla, CA).

For 3' RACE (Fig. 7), poly(A)+ RNA isolated from young leaves of *Catharanthus roseus* was reverse transcribed according to Frohman *et al.* [1988] in the presence of 2 μM XBC-T17 adaptor (Table I) and M-MuLV- reverse transcriptase (Promega, Madison, WI). After incubation at 37°C for 1 hour, the reaction was stopped by dilution in 1 ml TE and stored at 4° C. For isolation of the 3'cDNA end, a PCR amplification was performed with 1 μL of reverse-transcribed RNA, 0.5 μM XBC adaptor (Table I), 0.5 μM DAT 54-8 primer (Table I) according to Frohman *et al.* (1988). A second PCR was performed with 0.2 μM of primer DAT 54-9 (Table I), 0.2 μM XBC adaptor and 0.1 μL of the primary 3'RACE reaction.

For amplification of the 5' end [Frohman *et al.* (1988)] of DAT cDNA, poly(A)+ RNA was reverse transcribed as described for the 3'RACE protocol except that the DAT-17 primer (Table I) was substituted for XBC-T17 adaptor. After completion of the reaction, excess primer was removed using a NanoSpin Plus 100K MWCO filter (Gelman Sciences, Ann Arbor, MI) and the first strand cDNA was tailed. For 5'RACE, the PCR reaction included the tailed cDNA (5 μL), 0.08 μM XBC-T17 adaptor, 0.5 μM XBC adaptor, 0.5 μM DAT-17 primer (Table I) and 2

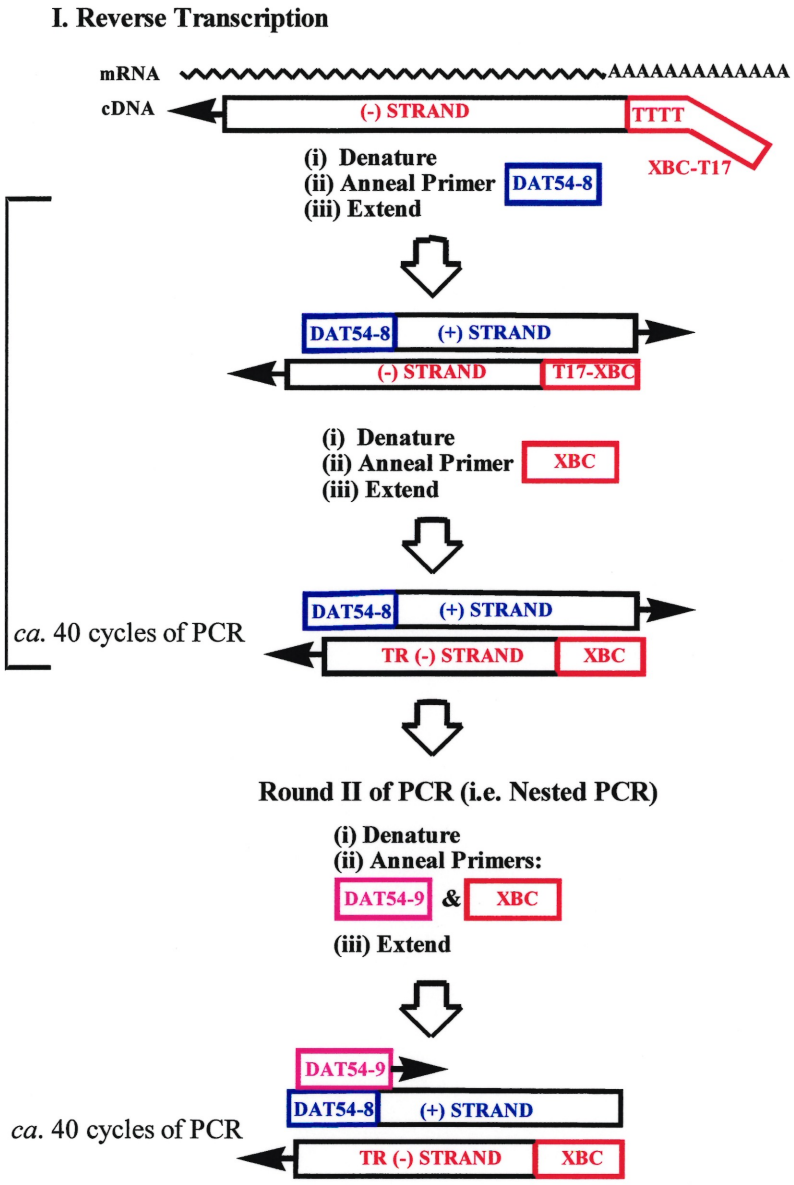

Figure 7. Schematic representation of the 3'RACE technique [adapted from: Frohman (1990)]. **DAT 54-8** — primer specific to DAT gene, complementary to (-) strand; **DAT 54-9** — primer specific to DAT gene, complementary to (+) strand; **XBC-T17** — dT17 adapter containing *Xba* I, *Bam* HI and *Cla* I restriction sites; **XBC** — primer containing the above-mentioned restriction sites; **TR** — designates a 'truncated' strand which is shorter than the original (-) or (+) strand.

Table I. Oligonucleotides used in the cloning and sequencing of the *O*-acetyltransferases. Highlighted sections correspond to the restriction sites. **A**= adenine; **T**= thymine; **C**= cytosine; **G**= guanine; **I**= inosine ; **H**= A, T, or C; **R**= A or G; **S**= C or G; **W**= A or T; **Y**= T or C. **n.a.**= not applicable. Size is given in units of bases.

Name	Sequence (5'→3')	Size	Restriction Site
DAT 14	GCGGATCCATGGAGTCAGGAAAAATATCGG	30	*Bam*HI
DAT 15	GCCTGCAGACAATAACGCAGAAGGTCAAG	29	*Pst*I
DAT 16	GCCTGCAGATATCATTCATGCGGCAA	26	*Pst*I
DAT 17	CCGTTTGGGAAGGACTAG	18	n.a.
DAT 54-3	GCGAATTCGARTTYGAYATHTC	22	n.a.
DAT 54-4	GCGAATTCGARTTYGAYATHAG	22	*Eco*RI
DAT 54-5	GCGGATCCRCACCAISWRTCRC	22	n.a.
DAT 54-6	GCGGATCCRCACCAISWRTCIG	22	*Bam*HI
DAT 54-8	CAACTTTTTGGATATCGATG	20	n.a.
DAT 54-9	GCGAATTCATCGATGCTTACCTTTC	25	*Eco*RI
DAT 54-11	GCGAATTCGGGAAATCTACACCAACT	26	*Eco*RI
HOM 1	TCCCTTTTATGACATTG	17	n.a.
HOM 2	CAACCATTGTTATTGAGTCCA	21	n.a.
HOM 3	GCCTGCAGAAGAAACCAGCGAATATATACTC	31	*Pst*I
HOM 4	CTCACGAATTGTGGAAATTTCAT	23	n.a.
HOM 5	GCGGATCCATGGACTCAATAACAATGGTTG	30	*Bam*HI
HOM 6	GCTGCAGAGAGACAATCATGCTGAAACTC	29	*Pst*I
HOM 7	CTTGACGTGGAAATATGG	18	n.a.
HOM 8	GACAATTTCCTGCCTCCCC	19	n.a.
HOM 9	TAATATTTTAAAGTTGGGTGGG	22	n.a.
HOM 10	TTAAAATGGGGTTGAAACATGC	22	n.a.
XBC-T17	GACTCGAGGATCCAATCGATTTTTTTTTTTTTTTT	36	*Xho*I, *Bam*HI, *Cla*I
XBC	GACTCGAGGATCCAATCG	18	*Xho*I, *Bam*HI, *Cla*I

units of TAQ DNA polymerase which was added after the initial denaturation step. After incubation at 97° C for 5 min, 48° C for 2 min and 72° C for 40 min, the reaction was carried out for 35 further cycles at 94° C for 15 s, 51° C (increased by 0.1° C every cycle) for 45 s and 72° C for 90 s. A secondary PCR reaction was performed with DAT-16 primer, XBC-T17 adaptor and XBC adaptor (Table I) as described above.

C.2.2. Gene Isolation and Sequence Determination

A genomic library prepared from *C. roseus* [Vazquez-Flota *et al.* (1997)] was screened with a 225-bp 3'RACE clone (W7-1) (see Fig. 8 in results). Two of the four isolated genomic clones, g*DAT*4 and *gDAT*16, shared similar restriction patterns, while clones *gDAT*6 and *gDAT*15 revealed a different restriction pattern (see Fig. 10 in results).

An *Eco*RI fragment of the *gDAT6* clone was inserted into the pBSIISK+ vector and sequenced on both strands using the dideoxynucleotide chain-termination method [Sanger *et al.* (1977)] with a recombinant T7 DNA polymerase (Pharmacia Biotech, Baie d'Urfé, QUE).

The g*DAT*15 clone was digested with *Eco*RI and 3 fragments (0.6, 2.4 and 4.0 kb) were sub-cloned into pBSIISK+, and sequenced on both strands as was performed with the *gDAT*6 fragment.

C.2.3. Expression of the Cloned *O*-Acetyltransferases in *Escherichia coli*

The 439 amino acid ORF of the *gDAT6* clone and the 443 amino acid ORF of the *gDAT15* clone were amplified by PCR with *Pwo* DNA polymerase (Boehringer Mannheim). The ORF of clone *gDAT6* was amplified using the primers DAT-14 and DAT-15 (Table I), while that of clone *gDAT15* was amplified using the primers HOM5 and HOM6 (Table I) according to the manufacturer's instructions. The primers were designed to incorporate a 5'- *Bam*HI (DAT-14; HOM5) and a 3'- *Pst*I (DAT-15; HOM6) restriction site into the amplified PCR product (Table I). The respective amplified fragments were restriction digested with *Bam*HI and *Pst*I endonucleases, and inserted into the corresponding restriction sites of the pQE30 expression vector (QIAgen, Chatsworth, CA.) as well as pBSIISK+. *Escherichia coli* BB4 cells (Stratagene) harboring the ORFs of clone *gDAT6* (pQE-DAT6), clone *gDAT15* (pQE-DAT15), or the pQE30 vector, were grown at 37° C in 25 mL of Luria-Bertani medium to OD_{600} = 0.6 - 0.7. Expression of the recombinant proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 2 mM. Cells were collected 3 h post-induction, centrifuged to remove the medium and frozen at –80° C until used for analysis. The cellular pellet was thawed, washed once and then resuspended with 2 mL of extraction buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF), followed by freeze-thawing in liquid N_2. The bacterial suspension was sonicated, on ice, using a Branson Sonifier Model 250 (Branson Ultrasonic Corporation, Danbury, CT) set at 40% duty cycle with an output control of 4, for 3 pulses of 30 s with 30 s pauses in between. The cellular debris was removed by centrifugation and the supernatant desalted on a

PD-10 column (Pharmacia Biotech) pre-equilibrated with 100 mM HEPES, pH 7.6 containing 2 mM ascorbate, followed by rechromatography on a PD-10 column pre-equilibrated with 50 mM Na-$PO_4^=$, pH 8.0 + 300 mM NaCl + 10% (v/v) glycerol (Buffer A) prior to Ni-affinity chromatography. These recombinant enzyme extracts were then used to test for *O*-acetyltransferase activity using either DAV, crude leaf alkaloids or crude root or hairy root alkaloids as substrate.

C.2.4. Ni-affinity chromatography

The desalted protein was submitted to nickel-nitrilotriacetic acid metal-affinity chromatography (QIAgen) (column dimensions: 10.0 mm i.d. x 3.0 cm) using an FPLC system (Pharmacia Biotech), in the presence of Buffer A containing 5 mM β-mercaptoethanol (β-ME) (Buffer B). The column was washed with Buffer B containing 5 mM imidazole until the O.D. 280 readout was less than 0.01. Elution of the HIS-tagged protein was carried out using a 30 mL, 5 to 180 mM imidazole linear gradient in Buffer A containing 5 mM β-ME . Enzyme assays were carried out as described in a following section and protein purity was estimated by SDS-PAGE on 10% gels stained with Coomassie blue R-250 and silver (data not shown) [Wray *et al.* (1987)] (see Appendix I, Figs. 30-31). Fractions showing approximately 80% purity were pooled and stored in elution buffer at -20° C for further analysis.

C.3. Isolation and Gel Blot Analysis of Nucleic Acids

C.3.1. Genomic DNA and Southern Blotting

Leaf genomic DNA was isolated according to Murray and Thompson (1980), digested with various restriction endonucleases, electrophoresed on 1% (w/v) agarose gels and transferred to nylon membranes (Hybond-N^+, Amersham, Arlington Heights,

IL) [Sambrook *et al.* (1989)]. Membrane hybridization was carried out with either a randomly primed ^{32}P-labeled *Bam*HI-*Pst*I fragments from the *gDAT6* or *gDAT15* ORFs, under high stringency conditions (65° C in 250 mM sodium phosphate buffer, pH 8.0, 7% (w/v) SDS, 1% (w/v) bovine serum albumin, 1 mM EDTA) for two days. The blots were washed at 65° C, twice with 2X SSC, 0.1% (w/v) SDS and twice with 0.5X SSC, 0.1% (w/v) SDS [Sambrook *et al.* (1989)] (1X SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Autoradiography of the membranes on X-ray film was performed at -80° C in the presence of intensifying screens.

C.3.2. RNA and Northern Blotting

Total RNA was isolated according to Jones *et al.* (1985). RNA concentrations were determined spectrophotometrically at 260 nm and adjusted following analysis by agarose gel electrophoresis and staining with ethidium bromide. Electrophoresis of 20 μg of total RNA was carried out on 7.7% (v/v) formaldehyde/ 1% (w/v) agarose gels and transferred to nitrocellulose (BA-85, Schleicher and Schuell, Keene, NH) as described in [Sambrook *et al.* (1989)]. The membranes were hybridized under high stringency conditions with ^{32}P-labeled probe for 24 h at 65° C, washed and exposed to film as described for genomic DNA.

C.3.3. *In situ* RNA hybridization

RNA *in situ* hybridization for *mat* transcripts within *C. roseus* lateral hairy root tissue was performed as described in [St-Pierre *et al.* (1999)]. The pBSMAT

construct containing a *ca.* 1.4 kb fragment representing the coding region of *mat*, obtained by PCR amplification of *gDAT*15 (see section on: Cloning of *Catharanthus O*-acetyltransferase genes), was used for generating the antisense RNA probe inserted into either pBluescript SK- or SK+. The RNA probe was synthesized by *in vitro* transcription with digoxigenin-UTP and T7 or T3 RNA polymerase, according to the manufacturer's instructions (Boehringer Mannheim). The RNA probe was submitted to alkaline hydrolysis for 20 min at 60° C [Jackson (1992)]. Rehydrated sections of root tissue were prepared for *in situ* hybridization by treatment with proteinase K (2 μg mL^{-1} in 100 mM Tris-HCl and 50 mM EDTA, pH 8.0) for 30 min at 37° C, followed by two rinses with TBS buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.5), by blocking of proteinase K with glycine (2 mg mL^{-1} in TBS) for 2 min, and by two rinses in TBS buffer. Sections were postfixed with 3.7% (v/v) formaldehyde in PBS buffer for 20 min and washed in TBS for 5 min. Finally, sections were acetylated with acetic anhydride (0.25% v/v in 0.1 M triethanolamine-HCl, pH 8.0) for 10 min, washed with TBS, dehydrated in an ethanol series, and air dried.

Hybridization was carried out by dispersing portions of hybridization mixture (60 μL) on a cover slip (22 x 50 mm) and inverting the slides onto the droplet of probe. The hybridization mixture included 200 ng mL^{-1} of hydrolyzed digoxigenin-labeled RNA transcripts, 40% (w/v) formamide, 10% (w/v) dextran sulfate, 1mg mL^{-1} yeast tRNA, 0.5 mg mL^{-1} polyadenylic acid, 0.3 M NaCl, 0.01 M Tris-HCl, pH 6.8, 0.01 M Na-phosphate, pH 6.8, 5 mM EDTA, and 40 U mL^{-1} RNasin ribonuclease inhibitor (Promega). Hybridization was for 16 to 18 h at 50° C in an atmosphere of 50% (w/v) formamide. Cover slips were then detached by soaking in 2 X SSC at 37°

C (1 X SSC is 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) for 30 min at 37° C and then washed in 2 X SSC for 1 h, in 1 X SSC for 1 h, and in 0.1 X SSC for 1 h at 65° C.

For immunolocalization of hybridized transcripts, slides were washed in TBST (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.3% v/v Triton X-100) for 10 min and blocked with 2 % (w/v) BSA fraction V (Boehringer Mannheim) in TBST for 16 h at 4° C. Portions (60 μL) of sheep anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) at a 1:200 dilution in a solution of 1% (w/v) BSA in TBST were dispensed onto cover slips, and the slides were inverted onto the droplet. After a 2 h incubation at room temperature, the unbound conjugates were washed twice for 15 min with TBST and twice for 10 min with AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM $MgCl_2$). For color development, slides were immersed in 175 μg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 350 μg mL^{-1} nitro blue tetrzolium chloride in AP buffer for 8 to 10 h at 22° C. After development, slides were washed in water and mounted with 50% (v/v) glycerol, 7% (w/v) gelatin, and 1% (v/v) phenol and covered with a cover slip.

C.4. Protein Extraction, *O*-Acetyltransferase Assays and Enzyme Kinetics

C.4.1. *O*-Acetyltransferase extraction from *Catharanthus roseus* tissue

Plant tissues were pulverized with a mortar and pestle in the presence of liquid nitrogen and the powder was treated with 2.5 mL of extraction buffer (100 mM HEPES pH 7.6, 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF) per gram fresh weight of tissue. After centrifugation at 10,000*g* for 15 min at 4° C, the supernatant desalted on

a PD-10 column as described previously for the recombinant proteins. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a protein standard.

C.4.2. Enzyme Assays and Reaction Product Identification

C.4.2.1. Enzyme Assays

O-Acetyltransferase activity assays were performed in a total reaction volume of 100 μL containing crude alkaloids (10 μL) extracted from either root, hairy root or leaf tissue, 0.05 μCi of [^{14}C]-acetyl CoA (sp. act. 51 mCi mmol^{-1}) (Amersham-Pharmacia) and 100 mM HEPES, pH 7.6 containing 2 mM ascorbate. DAT activity assays were carried out in a similar manner using 200 μM deacetylvindoline (DAV) as substrate, as previously described [Power *et al.* (1990)]. The reaction, which was initiated by the addition of protein, was incubated at 37° C for 20 min. The reaction was terminated with the addition of 25 μL 200 mM NaOH, shaken for 1 min and alkaloid reaction products were extracted in 500 μL ethyl acetate (EtOAc). The radioactivity of reaction products was quantified by liquid scintillation counting of 100 μL of the EtOAc fraction and the remaining organic phase was evaporated to dryness. Reaction products were dissolved in 10 μL of MeOH, and submitted to TLC on Si-Kieselgel F-254 plates (0.2 mm, E. Merck, Darmstadt). When available, co-chromatography of reference standards along with the reaction products was carried out. The plates were developed in methanol: ethyl acetate (1:9, v/v), viewed under UV light (λ = 254 and 365 nm, respectively) and exposed to X-ray film for autoradiography at -80° C.

C.4.2.2. Enzyme Kinetics

Assays were carried out at 37° C in a total volume of 100 μL containing either 0.20 μg of recombinant protein coresponding to clone *gDAT6* (rDAT6) or 6.0 μg of recombinant protein corresponding to clone *gDAT15* (rDAT15) for a linear reaction time of either 5 min or 20 min, respectively. The assays were terminated as previously described. Saturation curves for rDAT6 (rDAT) were generated at the following micromolar concentrations of [1-^{14}C]acetylcoenzyme A (sp. act. 51 mCi mmol^{-1}): 3.6, 4.6, 5.6, 6.5, 7.1, 8.1, 10.0, 10.8, 16.3, 16.7, 25.0, 32.5, and 50.0 in the presence of 200 μM DAV. Similarly, the following micromolar concentrations of DAV were used: 16.7, 21.4, 30.0, 37.5, 50.0, 75.0, 150.0 and 300.0 in the presence of 19.6 μM [1-^{14}C]acetylcoenzyme A. Assays were carried at each concentration for five separate determinations. For the generation of saturation curves for rDAT15 (rMAT), the following micromolar concentrations of [1-^{14}C]acetylcoenzyme A (sp. act. 51 mCi mmol^{-1}) were used: 2.0, 2.8, 3.6, 5.0, 6.3, 8.3, 12.5, 18.0 and 25.0 in the presence of 100 μM DAV. Similarly, the following micromolar concentrations of DAV were used: 136.4, 166.7, 214.3, 300.0, 375.0, 500.0, 750.0, 1000.0 and 1500.0 in the presence of 12.7 μM [1-^{14}C]acetylcoenzyme A. Assays were carried at each concentration for five separate determinations. The following micromolar concentrations of hörhammericine were used: 166.7, 214.3, 300.0, 500.0, 1000.0 and 1500.0 for generating saturation curves for rDAT15 in the presence of 12.7 μM [1-^{14}C]acetylcoenzyme A (sp. act. 59 mCi mmol^{-1}). Assays were carried at each concentration for five separate determinations. Saturation curves, Lineweaver-Burk reciprocal plots and apparent K_m and V_{max} values were calculated using the ENZFIT software (version 1.05, 1987, Elsevier, U.K.).

C.4.2.3. HPLC of *Catharanthus* Alkaloids and Quantification of Hörhammericine

Hörhammericine was quantified by HPLC with the photodiode array detector set to monitor the eluate at 254nm, 300nm and 329nm, respectively, based on the HPLC response factor of tabersonine [Rijhwani and Shanks (1998)].

HPLC analysis was performed using a WATERS 600 Multisolvent Delivery System fitted with a WATERS 991 Photodiode Array Detector (PDA), a 712 plus Autosampler (WATERS, Milford, MA) and a C18 (reverse phase) Nucleosil 100 (C18) 3U column (4.6 mm x 150 mm) (Alltech)(Mandel Scientific, Guelph, ONT) fitted with a guard column (Nucleosil C18; 7.5 x 4.6 mm, Alltech). The solvent system used was: (A) 0.2% (w/v) NH_4OAc in H_2O, pH *ca.* 7.6 and (B) MeCN. The samples were injected onto the pre-equilibrated column (55% (A)) at 1.0 ml min^{-1} followed by: a 30 min linear gradient to 30 % (A) at 1.0 ml min^{-1}, held at 30% (A) for 10 min and then increased to 100 % (B) in 1 min. Injections of a 5mM tabersonine/MeOH solution (20μL) were carried out in triplicate and the corresponding peak areas were determined with the use of the WATER'S PDA software including Stand-alone and Run & Report. Volumes of 10, 20 and 40μL, respectively of a *ca.* 2 mg mL^{-1} hörhammericine/MeOH solution were injected into the HPLC and their corresponding peak areas determined as described above for the tabersonine sample.

C.4.2.4. Re-activation of *O*-acetyltransferase Activity

Re-activation of rDAT15 was necessary for generating the saturation curves for rDAT15 with hörhammericine, as the activity was quite low. Re-activation was carried out by incubating an aliquot of protein with 1mM DTT, on ice, for 60 min, prior to assaying.

C.4.2.5. Chemical Modification of *O*-Acetyltransferases

Diethylpyrocarbonate (DEPC) was diluted in anhydrous ethanol, and the concentration was determined spectrophotometrically [Miles (1977)]. Inactivation of pQE::DAT6 (DAT) and pQE::DAT15 (MAT), respectively, was carried out by incubating desalted, crude recombinant protein (8.5 μg DAT protein and 137.0 μg MAT protein, respectively, in 100 μL of 100 mM HEPES-NaOH pH 7.0, 2 mM ascorbic acid) with a 0.5 μL aliquot of various concentrations of ethanol-diluted DEPC for 5, 10, and 15 min, respectively, at 23° C. After incubating, 15 μL aliquots were withdrawn, quenched with 1 μL 200 mM imidazole and assayed for *O*-acetyltransferase activity in a final volume of 100 μL. For the substrate-protection experiments, the enzymes were incubated for 10 min prior to the addition of 2 mM DEPC with various concentrations of substrates (DAV or acetyl CoA). Experiments were done in triplicate.

C.5. Light Studies on DAT Enzyme Activity

C.5.1. Seedling Treatments

C.5.1.1. Light Treatments

Catharanthus roseus (L.) G. Don cv. Little Delicata seeds were germinated as previously described (see section on: C*atharanthus roseus* seedlings and hairy root cultures) in growth chambers (model CMP 3023, Conviron, Asheville, NC) under controlled conditions (25° C, 70% relative humidity). Five day-old etiolated seedlings were subjected to either a further 3d of white light from 60-W cool-white fluorescent tubes (General Electric-Sylvania) and 60-W incandescent bulbs (Phillips Royale, Scarborough, Ontario, Canada) (18 h light: 6 h dark period; photon fluence rate: *ca.* 80 μmol·m^{-2}·s^{-1}) or complete darkness for a similar period of time. Photon fluence rate was calibrated using a Li-Cor photometer (model Li-189, Lincoln, NE). Batches of 50 seedlings were either sectioned into their respective cotyledons and hypocotyl/radicle segments or harvested whole depending on their purpose, frozen in liquid nitrogen and maintained at –80° C until ready for analysis. The collection of seedlings was carried out under a dim-green (25 W) safelight (Deco-Color, General Electric).

C.5.1.2. Red/Far-red Light Treatment

Five day-old etiolated seedlings were exposed to white light filtered through a Roscolux #19 filter (Location Michel Trudel Inc., Mtl., QC.) to provide red light of wavelength ≥ 660 nm (photon fluence rate: *ca.* 20 μmol·m^{-2}·s^{-1}) for various periods of time and then returned to darkness for a further 24 h prior to sectioning and

harvesting as described above. This filter transmitted only wavelengths longer than 555 nm, and greater than 80% of the irradiance at wavelengths longer than 620 nm. Treating seedlings with far-red light was carried out by filtering white light through a combination of Roscolux filters # 19, #83, and #89 to provide far-red light of wavelength ≥ 720 nm. This combination of filters transmitted only wavelengths longer than 700 nm, and greater than 50% of the irradiance at wavelengths longer than 761 nm. The spectral quality of light transmitted through the filters was verified using a spectrophotometer (model DU 7400, Beckman).

C.5.1.3. Chemical Treatment

In order to gain some understanding as to the putative secondary messengers which may be involved in the red-light activation of DAT enzyme activity, 5d-old etiolated seedlings were incubated in the presence of various concentrations of phytohormones and protein kinase inhibitors.

C.5.1.3.1. Phytohormones

Batches of 50 seedlings were transferred, under a dim-green safelight, to a 100 mm petri dish containing one layer of sterile Whatman #1 filter paper imbibed with 3-4 mL of the respective solution: brassinazole (BRZ; gift from Dr. T. Asami, RIKEN, Saitama, Japan), an inhibitor of brassinosteroid biosynthesis [Asami and Yoshida. (1999)], and N^6-(*meta*-hydroxybenzyl)adenine (*meta*-topolin, mT) (OlChemIm Ltd., Olomouc, Czech. Rep.), a cytokinin isolated from poplar [Strnad *et al.* (1997)]. The seedlings were vacuum infiltrated for 1 min prior to returning them

to complete darkness for a further 24 h and harvesting as previously described. Seedlings treated with mT (prepared in 1.4% EtOH, v/v) were returned to complete darkness for 24 and 48 h, respectively, prior to harvesting and seedlings treated with BRZ (prepared in 1% EtOH, v/v) were incubated in the dark for 24 h before harvesting.

Harvested seedlings or cotyledons were either freeze-dried and extracted for their alkaloid content for TLC and CAS revelation, as described for TLC of enzyme-catalyzed reaction products. In addition, HPLC analysis of vindoline and tabersonine profiles was carried out as described for the quantitation of hörhammercine, using vindoline and tabersonine standards. Alternatively, seedlings or their organs were extracted for their protein for DAT and MAT enzyme activity assays.

C.5.1.3.2. Protein Kinase and Calcium/Calmodulin inhibitors

Five day-old seedlings were treated, as described above, with various concentrations of staurosporine (ST; Sigma-Aldrich Canada Ltd.) prepared in 1% (v/v) DMSO, genistein (gift from Dr. N. Brisson, U de Mtl.) and trifluoperazine (TFP; gift from Dr. N. Brisson, U de Mtl.), respectively, prepared in sterile type I H_20. Following a 30 min pre-incubation period in the dark, the seedlings were exposed to 30 min of red light (*ca.* 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and returned to the dark for a further 24 hr. The seedlings were collected as described above and analyzed for DAT and MAT enzyme activities.

D. Results

D.1. Isolation of *O*-Acetyltransferase Genes

The purification and characterization of DAT from *C. roseus* was previously described [Power (1989); Power *et al.* (1990); Fahn and Stöckigt (1990)]. SDS-PAGE of pure DAT protein revealed it to be a dimer composed of 33 kDa and 21 kDa subunits [Power (1989); Power *et al.* (1990)]. After electrophoretic transfer of each subunit to nitrocellulose sheets, they were digested with trypsin and the peptides purified by HPLC [Alarco (1994)]. Three and 7 peptides were sequenced from the small and large subunits, respectively (Table II). The first and last five amino acids of peptide 54, belonging to the small subunit, were used to design four degenerate primers (Table II; Fig. 6). Since the distance between their cognate sequences was known, the primer combination and RT-PCR conditions were selected for amplification of a 73 bp product from leaf RNA. Only one combination of primers (54-4 and 54-6) yielded a PCR product encoding the complete sequence of peptide 54 (Fig. 6).

The 3'end of a DAT transcript was cloned by 3'RACE (Fig. 7) with two specific primers, DAT54-8 and DAT54-9 (Table I), designed from the internal sequence of the 73 bp PCR product. Clone W7-1 encoded a putative 75 amino acid sequence, which also contained a second tryptic fragment belonging to the small subunit of DAT (peptide 27; Fig. 8). Screening of a seedling cDNA library with clone W7-1 as a probe yielded a partial cDNA clone, A-3, which was partially sequenced and contained a distinct 123 amino acid ORF that was 81% identical to the sequence of W7-1 (Fig. 9). Since it did not correspond to W7-1, it was not sequenced further.

Table II. Partial peptide sequences obtained from the microsequencing of the purified DAT protein following trypsin digestion of the respective 33 kDa and the 21 kDa subunits as purified and isolated by SDS-PAGE.

33 kDa subunit	Sequence
peptide 14[a]	ISVETELSK
peptide 21[a]	TLIKPSSPTPQSLSR
peptide 24[a]	AVEFGIEKPTR
peptide 30[a]	SDNIIEQFPTKVE
peptide 50[b]	GLGDADQVLAYFAVSK
peptide 43[a]	TLVSYYPFAGK
peptide 55[a]	VEVLTAFLSR
21 kDa subunit	
peptide 27[b]	MSAFEKNEQLLQFVSN
peptide 36[a,b]	EKLTYVAQMEEFVK
peptide 54[a]	EFDISNFLDIDAYLSDSWC

[a]Peptide sequences obtained from the work carried out by [Alarco (1994)].

[b]Peptide sequences obtained from the work carried out by I. Roewer (post-doctoral fellow).

A)

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  1 GCT TAC CTT TCT GAT AGT TGG TGT AGA TTT CCC TTT TAC GAC GTT  45
  1 ala tyr leu ser asp ser trp cys arg phe pro phe tyr asp val  15

 46 GAT TTT GGA TGG GGA AAG CCG ATA TGG GTA TGC TTG TTT CAA CCC  90
 16 asp phe gly trp gly lys pro ile trp val cys leu phe gln pro  30

 91 TAT ATA AAA AAT TGT GTT GTT ATG ATG GAT TAT CCA TTT GGA GAT 135
 31 tyr ile lys asn cys val val met met asp tyr pro phe gly asp  45

136 GAT TAT GGA ATC GAA GCA ATA GTT TCC TTT GAA CAA GAA AAG ATG 180
 46 asp tyr gly ile glu ala ile val ser phe glu gln glu lys met  60

181 TCT GCC TTT GAG AAG AAC GAA CAG CTA CTT CAA TTT GTT TCT AAT 225
 61 ser ala phe glu lys asn glu gln leu leu gln phe val ser asn  75

226 TAA
    stop
```

B)

peptide 27 MSAFEKNEQLLQFVSN

peptide 54 EFDISNFLDIDAYLSDSWC

Figure 8. A) Nucleic acid and deduced amino acid sequence of the cloned W7-1 peptide obtained by 3'RACE. **B)** Partial peptide sequences obtained from micro-sequencing of the purified DAT protein. Highlighted regions of the cloned W7-1 peptide correspond to the highlighted regions in each of the peptides in **B)**.

Clone W7-1 was also used to screen *ca.* 420,000 plaques from a *C. roseus* genomic library and 4 genomic clones were isolated. Restriction map analysis combined with hybridization studies revealed that one genomic clone (*gDAT15*) contained the A-3 gene product, whereas the other three genomic clones (*gDAT4*, *gDAT6* and *gDAT16*) contained the W7-1 gene product (Fig. 10). Clone *gDAT6*, which was analysed further, contained no introns within a 439 amino acid ORF encoding 9 out of 10 sequences found in the 33 and 21 kD DAT subunits (Fig. 11; Table II). The predicted M_r of 49,890 Da is in good agreement with the SDS PAGE estimated M_r of 54,000 Da for the sum of the two purified DAT subunits [Power (1989); Power *et al.* (1990)] and the estimated M_r of 45,000 Da of the native protein determined by gel filtration chromatography [De Luca *et al.* (1985)]. As a result of its different restriction pattern, as observed by Southern blotting (Fig. 10), clone *gDAT15* was also characterized. The nucleic acid sequences obtained for the three *Eco* RI fragments from clone *gDAT15* were aligned together and resulted in a sequence of 1382 nucleic acids containing a 443 amino acid ORF (Fig. 12). This ORF was also without introns and its predicted M_r of 49,884 Da is in good agreement with the estimated M_r's for DAT.

D.1.1. Expression of *O*-Acetyltransferases in *E.coli*

The 439 aa ORF encoded by clone *gDAT6* and the 443 aa ORF encoded by clone *gDAT15* were inserted separately into pQE30 vectors for expression in *E. coli* with a $His_{(6)}$ N-terminal extension. Only bacteria transformed with the pQE vector harbouring clone *gDAT6*'s ORF contained high levels of DAT activity which could be induced with IPTG treatment (Table III). This result clearly demonstrated that

```
TTT TTT TTT GGT GAG CTA AGG AAA GCA AAG GAT AAA CTG AAG AAT
Phe Phe Phe Gly Glu Leu Arg Lys Ala Lys Asp Lys Leu Lys Asn

CTT TCC CAA GAA AAG CTT AAT TAT GTT GCA CGA ATG CAA GAT TTC
Leu Ser Gln Glu Lys Leu Asn Tyr Val Ala Arg Met Gln Asp Phe

GCA AAT TGC TTG AAA GAG CTT GAC ATT AGC AGC TTT TTC GAT ATG
Ala Asn Cys Leu Lys Glu Leu Asp Ile Ser Ser Phe Phe Asp Met

GAA AAC GTT GAC ATC GAT GCT TAC TTG TTC AGT AGC TGG TGT AGA
Glu Asn Val Asp Ile Asp Ala Tyr Leu Phe Ser Ser Trp Cys Arg

TTT CCC TTT TAT GAC ATT GAT --- TGA TGG --- AAG CCG ATA TGG
Phe Pro Phe Met Thr Ile Asp --- Ter Trp --- Lys Pro Ile Trp

GTA TGC ATG TTT CAA CCC TAT TTA AAA TGT ATT ATT ATG ATG GAT
Val Cys Met Phe Gln Pro Tyr Leu Lys Cys Ile Ile Met Met Asp

TAT CCA TTG GGA GAT GAT ATG --- ATC GAA GCA TTA ATC ACT CTT
Tyr Pro Leu Gly Asp Asp Met --- Ile Glu Ala Leu Ile Thr Leu

GAA CAA GAA AAA ATG CCA GCA TTT GAG AAC AAC GAG CTG CTC CTT
Glu Gln Glu Lys Met Pro Ala Phe Glu Asn Asn Glu Leu Leu Leu

CAT TGC --- TCT AAT TAA AAGATGATGATATGCCAAAATAAACAGCCAAATAA
His Cys --- Ser Asn Ter

ACAGCCAAAGCTGAAGCAAAGG
```

Figure 9. Partial DNA and deduced amino acid sequences of the A3 cDNA clone. Highlighted region represents compression within the sequence and therefore the nucleic acids could not be clearly distinguished. **---** represents gaps introduced in the DNA and the deduced amino acid sequences in order for the nucleic acid sequence to be in frame with that of DAT. The nucleic acid sequence was aligned in frame with that of DAT in order to obtain the deduced amino acid sequence. The partial A3 cDNA clone was not sequenced in both orientations.

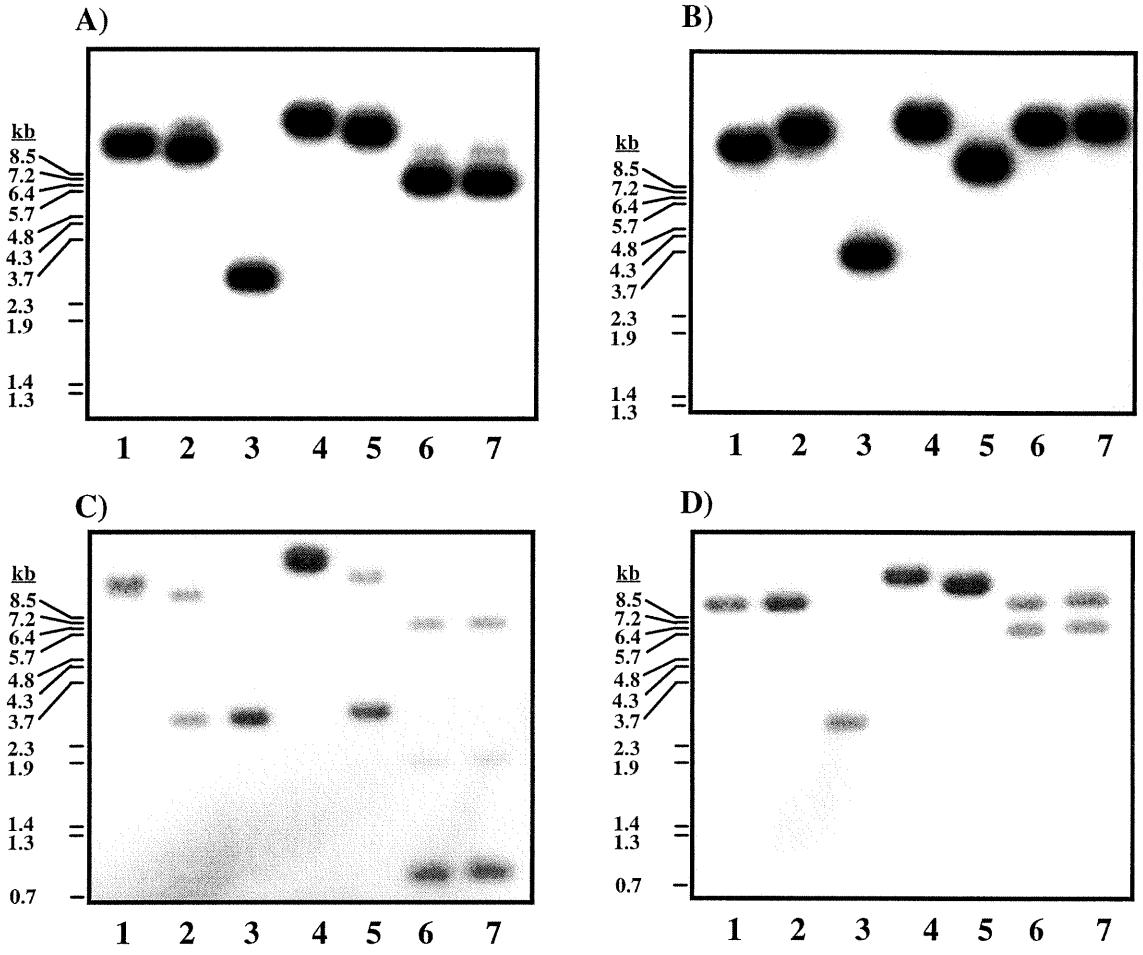

Figure 10. Southern blots of restriction digested genomic clones isolated from screening *ca.* 420,000 plaques from a *Catharanthus roseus* genomic library. Hybridization with W7-1 was carried out under high stringency conditions following electrophoresis on a 0.8% (w/v) agarose gel. **A**) clone #4, *gDAT*4; **B**) clone #6, *gDAT*6; **C**) clone #15, *gDAT*15; **D**) clone #16, *gDAT*16. Digestions were carried out as outlined in material and methods using the following endonucleases: lane 1— *Sal*I, lane 2— *Eco*RI, lane 3— *Eco*RI/*Sal*I double digestion, lane 4— *Bam*HI, lane 5— *Xba*I, lane 6— *Hind*III, lane 7— *Hind*III/*Sal*I double digestion. λ DNA/*Bst*EII markers' mol. wt. shown in kb to the left of each figure.

```
   1  CAT TAT AGG TGT TCT TCC CGA CGT TAT ATA TTA GAC ATA AAA TAA GAA ATA TAT AAA TGT TAT GAA TTT GAT GAA
  76  GCG CGT CAT AAT TGA ATA TTT AGA ATA ATA ATA CAA TAT AAG GTA GAA TAA TAA AGT GAA GAG AAC GAA GTA GAG
 151  AGA AAG TAA AAC TAA AAA ATC AAA TCT GGA TCA TAT TAT TAA TAT TTT TCG TAA GAC ATA TTT ATA GAT TTA AGA
 226  TAC AAT AGA TTA CAA AAC CAT AAA AGA AAT GTA ATT TGT CAT TAG AAG GAC AAA ATC CAA TTA GAA TTG AAC TTT
 301  GAG AGT TCT AGT TCC CTT TAA ATA ATA TAT TTG AAC ATC TAT TAT GTG TAC ATT AAT TCA ATA ACA TTC CCT TTT
 376  TGT ATT TAT TAT GCT AAT TTC TTG CTG TGA ATG TTC CTT TTT TCT TTA TGA GTT TAA CAA AGA TTT AGA TAA TTA
 451  TGA GAG ATT TTA AAG TAT TTT TTC AAT TTT TAA CAT TGA AAT AAT TTA GAA ATG AAA TAT AAT TAT TAG TAA TTC
 526  TCA CAC TTT GAA GAG AAT ATT TTT ATT GAT TTA TGT AAA ATT TAT TTC CAA TAC TTT CTC GAA CTA ATA TTC ATT
 591  ACA TGA CTA CAC ATC TAC TTT ATC TTT GAC TTT TAG TTA TTA TAT ATA TAT ATA TAT ATA TTT AAT ACT CTT CAA
 666  AAG AAA AAA CAA CTG ATT TAT GTC ATA GGA ACT AAA ACC AAA ACC ATT CTG GCC ACA AAA AAA ATG AAA GTA TCA
 741  TAA GAA CGT TGG GTG GGA TGG GCA ACT GGA TAG CCT ATA TAT AAG TGT TGT GTG TTG GGG TCT GAA TAT ATA ACA

 816  GCA AGC AAA ATG GAG TCA GGA AAA ATA TCG GTT GAG ACA GAG ACA CTC TCC AAA ACG TTG ATC AAA CCC TCT TCT
               Met Glu Ser Gly Lys Ile Ser Val Glu Thr Glu Thr Leu Ser Lys Thr Leu Ile Lys Pro Ser Ser      22

 891  CCA ACC CCT CAA TCC CTC AGC CGC TAT AAC CTC TCC TAC AAT GAT CAA AAC ATA TAC CAA ACG TGC GTA TCC GTT
      Pro Thr Pro Gln Ser Leu Ser Arg Tyr Asn Leu Ser Tyr Asn Asp Gln Asn Ile Tyr Gln Thr Cys Val Ser Val      47

 966  GGT TTC TTT TAC GAA AAT CCC GAC GGC ATT GAA ATT TCT ACA ATT CGT GAG CAG CTA CAA AAT TCG CTC TCA AAA
      Gly Phe Phe Tyr Glu Asn Pro Asp Gly Ile Glu Ile Ser Thr Ile Arg Glu Gln Leu Gln Asn Ser Leu Ser Lys      72

1041  ACT CTT GTT TCT TAT TAT CCA TTT GCT GGA AAA GTT GTT AAA AAT GAC TAT ATC CAT TGC AAT GAC GAT GGA ATT
      Thr Leu Val Ser Tyr Tyr Pro Phe Ala Gly Lys Val Val Lys Asn Asp Tyr Ile His Cys Asn Asp Asp Gly Ile      97

1116  GAG TTC GTG GAA GTT CGA ATT CGT TGC CGC ATG AAT GAT ATT CTA AAG TAT GAA TTA AGA TCA TAT GCC AGA GAC
      Glu Phe Val Glu Val Arg Ile Arg Cys Arg Met Asn Asp Ile Leu Lys Tyr Glu Leu Arg Ser Tyr Ala Arg Asp     122

1191  CTA GTC CTT CCC AAA CGG GTA ACT GAA GGC AGT GAA GAT ACT ACA GCT ATT GTT CAA CTA AGT CAT TTT GAT TGC
      Leu Val Leu Pro Lys Arg Val Thr Glu Gly Ser Glu Asp Thr Thr Ala Ile Val Gln Leu Ser His Phe Asp Cys     147

1266  GGA GGT TTG GCG GTT GCT TTT GGC ATA TCT CAC AAA GTT GCT GAT GGA GGT ACA ATT GCT TCG TTT ATG AAG GAT
      Gly Gly Leu Ala Val Ala Phe Gly Ile Ser His Lys Val Ala Asp Gly Gly Thr Ile Ala Ser Phe Met Lys Asp     172

1341  TGG GCT GCT TCT GCA TGC TAT TTA TCT TCT TCT CAT CAC GTA CCA ACT CCG TTA TTG GTT TCT GAT TCC ATA TTT
      Trp Ala Ala Ser Ala Cys Tyr Leu Ser Ser Ser His His Val Pro Thr Pro Leu Leu Val Ser Asp Ser Ile Phe     197

1416  CCA CGT CAA GAT AAT ATA ATT TGT GAA CAA TTT CCT ACC TCC AAA AAT TGT GTG GAA AAG ACA TTT ATC TTC CCT
      Pro Arg Gln Asp Asn Ile Ile Cys Glu Gln Phe Pro Thr Ser Lys Asn Cys Val Glu Lys Thr Phe Ile Phe Pro     222

1491  CCG GAA GCC ATA GAA  AAG TTA AAA TCT AAG GCC GTT GAG TTT GGT ATT GAG AAA CCT ACT CGA GTA GAG GTT TTG
      Pro Glu Ala Ile Glu  Lys Leu Lys Ser Lys Ala Val Glu Phe Gly Ile Glu Lys Pro Thr Arg Val Glu Val Leu     247

1566  ACT GCT TTT CTC AGT CGA TGT GCC ACG GTA GCA GGG AAA TCA GCA GCC AAG AAT AAT AAT TGT GGC CAA TCT TTG
      Thr Ala Phe Leu Ser Arg Cys Ala Thr Val Ala Gly Lys Ser Ala Ala Lys Asn Asn Asn Cys Gly Gln Ser Leu     272

1641  CCA TTT CCA GTA CTC CAA GCT ATT AAT TTA CGT CCA ATT TTG GAA CTC CCA CAA AAT TCT GTG GGA AAT TTG GTT
      Pro Phe Pro Val Leu Gln Ala Ile Asn Leu Arg Pro Ile Leu Glu Leu Pro Gln Asn Ser Val Gly Asn Leu Val     297

1716  TCA ATT TAT TTC TCA CGT ACA ATT AAA GAA AAT GAT TAT CTC AAT GAA AAG GAA TAT ACA AAA CTA GTG ATT AAT
      Ser Ile Tyr Phe Ser Arg Thr Ile Lys Glu Asn Asp Tyr Leu Asn Glu Lys Glu Tyr Thr Lys Leu Val Ile Asn     322

1791  GAG CTA AGG AAA GAA AAA CAA AAG ATA AAG AAT CTT TCC CGA GAA AAG CTT ACT TAT GTA GCA CAA ATG GAA GAG
      Glu Leu Arg Lys Glu Lys Gln Lys Ile Lys Asn Leu Ser Arg Glu Lys Leu Thr Tyr Val Ala Gln Met Glu Glu     347

1866  TTC GTA AAA AGC TTG AAA GAG TTT GAC ATT AGC AAC TTT TTG GAT ATC GAT GCT TAC CTT TCT GAT AGT TGG TGT
      Phe Val Lys Ser Leu Lys Glu Phe Asp Ile Ser Asn Phe Leu Asp Ile Asp Ala Tyr Leu Ser Asp Ser Trp Cys     372

1941  AGA TTT CCC TTT TAC GAC GTT GAT TTT GGA TGG GGA AAG CCG ATA TGG GTA TGC TTG TTT CAA CCC TAT ATA AAA
      Arg Phe Pro Phe Tyr Asp Val Asp Phe Gly Trp Gly Lys Pro Ile Trp Val Cys Leu Phe Gln Pro Tyr Ile Lys     397

2016  AAT TGT GTT GTT ATG ATG GAT TAT CCA TTT GGA GAT GAT TAT GGA ATC GAA GCA ATA GTT TCC TTT GAA CAA GAA
      Asn Cys Val Val Met Met Asp Tyr Pro Phe Gly Asp Asp Tyr Gly Ile Glu Ala Ile Val Ser Phe Glu Gln Glu     422

2091  AAG ATG TCT GCC TTT GAG AAG AAC GAA CAG CTA CTT CAA TTT GTT TCT AAT TAA GAA GTT TAC TGC CCA AAT AAA
      Lys Met Ser Ala Phe Glu Lys Asn Glu Gln Leu Leu Gln Phe Val Ser Asn TER                              439

2166  AAG TGC CAA GAG TTT GGA TTC TTG ACC TTC TGC GTT ATT GTC TCT ATT AAG GTG TGA TGG TTA TTG TGT TTG ACT
2241  GTT ATT ATT CAA AAT AAG GAT TCA ACA TCT CCA AGT CAA TTT GTT TTT CAA AAT TTA ACG CCA AAA TAT GCT TTT
2316  GGA ATA TGA CTA TTA TTG TTC CTA TGT TAT TGT TCT TTG ATT TTT TTT AAT TCA TTT TAA GGA AGA GGT T
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Figure 11. DNA sequence and deduced amino acid sequence of the *DAT6* gene. Putative **CAAT** and **TATA** boxes, the translation start site (**ATG**) and the termination codon (**TAA**) are shown in bold-type. Highlighted regions correspond to the peptide sequences of DAT as determined from amino acid sequencing of trypsin-digested purified protein and as listed in Table II. Regions highlighted in black correspond to peptides of the 33 kDa subunit, while grey highlighted regions correspond to peptides of the 21 kDa subunit.

```
   1  TTACTTGGAAAGCAGCATATTGAGCAAA ATG GAC TCA ATA ACA ATG GTT GAA ACC GAG ACA CTC TCC
                                   Met Asp Ser Ile Thr Met Val Glu Thr Glu Thr Leu Ser     13

  68   AAA ACG TTG ATC AAA CCC TCT TCT CCA ACC CCT CAA TCA CTC AGC CAC TAC AAT CTC TCC
       Lys Thr Leu Ile Lys Pro Ser Ser Pro Thr Pro Gln Ser Leu Ser His Tyr Asn Leu Ser     33

 128   TAC AAT GAT CAA AAC ATA TAT CCA GAG TAT ATA TTC GCT GGT TTC TTT TAC TCA AAT CCC
       Tyr Asn Asp Gln Asn Ile Tyr Pro Glu Tyr Ile Phe Ala Gly Phe Phe Tyr Ser Asn Pro     53

 188   GAC GGC CAT GAA ATT TCC ACA ATT CGT GAG CAG CTC CAA AAT TCT CTT TCA AAA ACT CTT
       Asp Gly His Glu Ile Ser Thr Ile Arg Glu Gln Leu Gln Asn Ser Leu Ser Lys Thr Leu     73

 248   GTT TCT TAC TAT CCA TTT GCT GGA AAA GTT GTT AAA AAT GAC TAT ATC CAT TGC AAT GAC
       Val Ser Tyr Tyr Pro Phe Ala Gly Lys Val Val Lys Asn Asp Tyr Ile His Cys Asn Asp     93

 308   GAT GGA ATT GAA TTT GTA GAT GTT CGA ATT CAC TGC CGC ATG AAT GAT ATT CTA AAG CCT
       Asp Gly Ile Glu Phe Val Asp Val Arg Ile His Cys Arg Met Asn Asp Ile Leu Lys Pro    113

 368   GAA TTA AGA TCA TAT GCC AGT GAA CTA ATC CGT CCC AAT CGG TCA ACT GTC GGC AGT GAA
       Glu Leu Arg Ser Tyr Ala Ser Glu Leu Ile Arg Pro Asn Arg Ser Thr Val Gly Ser Glu    133

 428   GAT AGT ACA GCA TTA GTT CAG TTA AGT CAT TTT GAC TGC GGA GGA GTT GCC GTT GCT TTT
       Asp Ser Thr Ala Leu Val Gln Leu Ser His Phe Asp Cys Gly Gly Val Ala Val Ala Phe    153

 488   GGC ATA TCT CAC AAA GTT GCT GAT GCA GCT ACA ATT CTT TCG TTT ATT AAG GAT TGG GCT
       Gly Ile Ser His Lys Val Ala Asp Ala Ala Thr Ile Leu Ser Phe Ile Lys Asp Trp Ala    173

 548   GCT TCT ACA TGC GAT TTA TCT TCG TCT CAT GAT GTA TCA ACT CCA GTA TTG GTT TCT GAT
       Ala Ser Thr Cys Asp Leu Ser Ser Ser His Asp Val Ser Thr Pro Val Leu Val Ser Asp    193

 608   TCC ATA TTT CCA CGT CAA GAT AAT ATA ATT TGT GGA CAA TTT CCT GCC TCC CCA AAT TGT
       Ser Ile Phe Pro Arg Gln Asp Asn Ile Ile Cys Gly Gln Phe Pro Ala Ser Pro Asn Cys    213

 668   GTG AGA AAG AGA TTT CTC TTC TCT CCG GAA GCC ATA GAA AGG TTA AAA TCG AAG GCC ATT
       Val Arg Lys Arg Phe Leu Phe Ser Pro Glu Ala Ile Glu Arg Leu Lys Ser Lys Ala Ile    233

 728   GAG TTT GGT ATT GAG AAA CCT ACC CGG GTA GAA GTT TTG ACT GCT TTT CTC TGT CGA TGT
       Glu Phe Gly Ile Glu Lys Pro Thr Arg Val Glu Val Leu Thr Ala Phe Leu Cys Arg Cys    253

 788   GCT ACG GTA GCA GGG AAA TCA GCA GCC AAG AAT AAC AAT TGT GGC CAA TCT TTG CCT TTC
       Ala Thr Val Ala Gly Lys Ser Ala Ala Lys Asn Asn Asn Cys Gly Gln Ser Leu Pro Phe    273

 848   GCC GTA ATC CAA GCT GTT AAT TTA CGT CCA CTT TTG GAA CTG CCA AAG AAT TCC GTG GGA
       Ala Val Ile Gln Ala Val Asn Leu Arg Pro Leu Leu Glu Leu Pro Lys Asn Ser Val Gly    293

 908   AAT TTG ATT TCA ATT TAT TTC TCT ACA ATT AAA GAA AAT GAT ACT GTC AAT ATC GAA CAG
       Asn Leu Ile Ser Ile Tyr Phe Ser Thr Ile Lys Glu Asn Asp Thr Val Asn Ile Glu Gln    313

 968   GAA TTT ACA AAA CTA GTT ATT GGT GAG CTA AGG AAA GCA AAG GAT AAA CTG AAG AAT CTT
       Glu Phe Thr Lys Leu Val Ile Gly Glu Leu Arg Lys Ala Lys Asp Lys Leu Lys Asn Leu    333

1028   TCC CAA GAA AAG CTT AAT TAT GTT GCA CGA ATG CAA GAT TTC GCA AAT TGC TTG AAA GAG
       Ser Gln Glu Lys Leu Asn Tyr Val Ala Arg Met Gln Asp Phe Ala Asn Cys Leu Lys Glu    353

1088   CTT GAC ATT AGC AGC TTT TTC GAT ATG GAA AAC GTT GAC ATC GAT GCT TAC TTG TTC AGT
       Leu Asp Ile Ser Ser Phe Phe Asp Met Glu Asn Val Asp Ile Asp Ala Tyr Leu Phe Ser    373

1148   AGC TGG TGT AGA TTT CCC TTT TAT GAC ATT GAT TTT GGA TTG GGA AAG CCG ATA TGG GTA
       Ser Trp Cys Arg Phe Pro Phe Tyr Asp Ile Asp Phe Gly Leu Gly Lys Pro Ile Trp Val    393

1208   TGC ATG TTT CAA CCC CAT TTT AAA AAT TGT ATT ATT TTG ATG GAT TAT CCA TTT GGA GAT
       Cys Met Phe Gln Pro His Phe Lys Asn Cys Ile Ile Leu Met Asp Tyr Pro Phe Gly Asp    413

1268   GAT TAT GGA ATC GAA GCA TTA ATC ACT CTT GAA CAA GAA AAA ATG CCA GCA TTT GAG AAC
       Asp Tyr Gly Ile Glu Ala Leu Ile Thr Leu Glu Gln Glu Lys Met Pro Ala Phe Glu Asn    433

1328   AAC GAG CTG CTC CTT TCA TTT GCT TCT AAT TAA GAAGATGATGATATGCCAAAAT
       Asn Glu Leu Leu Leu Ser Phe Ala Ser Asn TER                                       443
```

Figure 12. DNA and deduced amino acid sequences of the *gDAT*15 gene.

clone *gDAT6* harboured the DAT gene. On the other hand, DAT activity in bacteria transformed with the pQE vector harbouring the ORF from clone *gDAT15* was also induced with IPTG treatment (Table III). This enzyme expressed *O*-acetyltransferase activity with a broader substrate specificity as it exhibited higher activity for minovincinine (Fig. 13), a root-specific, 19-hydroxylated monoterpene indole alkaloid (Fig. 5) initially isolated from the roots of *Catharanthus trichophyllus* [Cordell and Farnsworth (1976)] than it did for DAV (see Tables IV and V). As a result of its greater affinity for minovincinine than DAV and its kinetic characterization, clone *gDAT15* was tentatively identified as minovincinine-*O*-acetyltransferase (MAT).

D.2. Substrate Specificity and Kinetic Parameters

Recombinant MAT (rMAT) and DAT (rDAT) were partially purified (see Material and Methods and Appendix I) and used in enzyme assays with radioalabeled acetyl coenzyme A to compare their substrate specificities. The reaction products obtained from radioactive rMAT assays were analyzed by Si-Kieselgel TLC and autoradiography (Fig. 13). No reaction product was produced with lochnericine as substrate (Fig. 13, lane 1), whereas hörhammericine was converted to radioactive 19-*O*-acetyl-hörhammericine (R_f = 0.66). Crude *Catharanthus* extracts of root or hairy root alkaloids also contained a substrate that was *O*-acetylated by rMAT (Fig. 13, lane 3). Incubation of these extracts with rMAT protein produced an intense radioactive spot (R_f = 0.62) on the autoradiogram that co-migrated with an

Table III. Recombinant *O*-acetyltransferase activities[a].

Induction with IPTG (hour)	pQE-DAT6 (pkatal mg^{-1} protein)	pQE-DAT15 (pkatal mg^{-1} protein)	pQE30 (pkatal mg^{-1} protein)
0	74.7	0.6	0.08
1	252.5	3.9	0.06
2	546.7	5.3	0.05
3	526.0	5.6	0.09

[a]DAT activity was determined with DAV as substrate using crude extract from *E.coli* transformants containing either pQE-DAT6, pQE-DAT15, or the pQE30 vector as control. Induction of cultures with IPTG was carried out in triplicate and the values for the respective *O*-AT activity represents the mean of three trials.

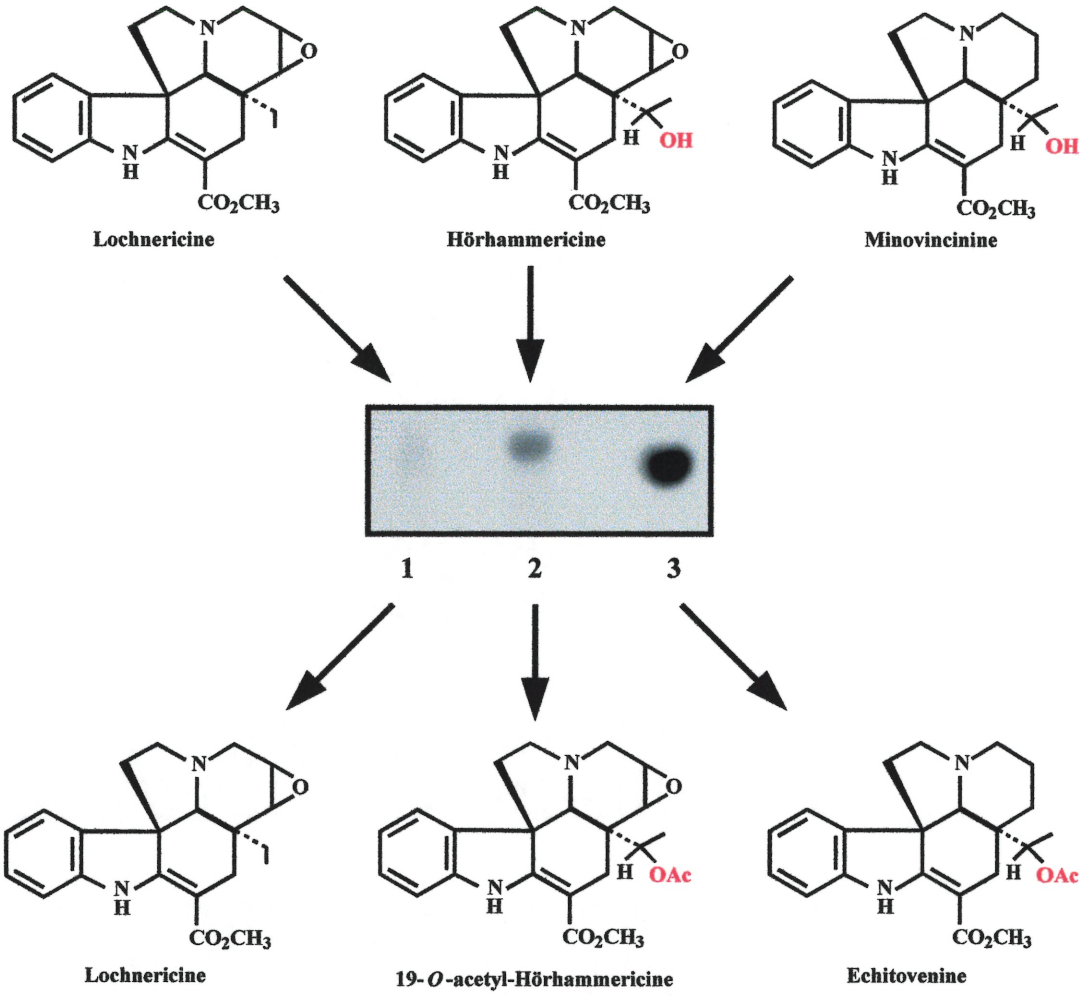


Figure 13. Autoradiogram of reaction products obtained from pQE-DAT15-catalyzed reactions. Authentic lochnericine (lane 1) and hörhammericine (lane 2) or a minovincinine-containing root extract (lane 3) were used, as substrates. The reaction products were extracted and chromatographed as described in material and methods. The structures of the respective substrates and their *O*-acetylated products are shown. Although no product was observed when the supplied substrate was lochnericine, its structure is included to show the requirement for the hydroxyl group at position 19 (shown in red) for enzyme activity.

echitovenine standard (Fig. 13, lane 3). These results suggest that *Catharanthus* roots may contain sufficient minovincinine to produce radioactive echitovenine. Attempts to purify this substrate from crude root extracts proved unsuccessful, which suggests it is present at very low levels.

The alkaloid substrate specificities of rDAT and rMAT were strikingly different (Table IV). Minovincinine-containing hairy root extract and hörhammericine were substrates for rMAT activity, whereas they were not accepted as substrates by rDAT (Table IV). In contrast, deacetylvindoline (DAV), which is the true substrate of rDAT, was also acetylated by rMAT. The apparent K_m of rMAT for DAV (Fig. 14B) was over 8-fold larger than that for rDAT (Fig. 15B; Table V) and DAT purified from *C. roseus* leaves [Power (1989); Power *et al.* (1990)]. The apparent specific activity of the partially purified rDAT (38.1 pkat μg^{-1}) (Table V) was similar to that found previously for DAT purified from *C. roseus* leaves (36 pkat μg^{-1})[Power (1989); Power *et al.* (1990)]. The specificity constant of rDAT for acetyl CoA and DAV was *ca.* 240 and *ca.* 10,000 fold greater, respectively, compared to that of rMAT (V_{max}/K_m, Table V). This low specificity constant is maintained for rMAT with hörhammericine as substrate (Table V) and confirms the low efficiency of this enzyme compared to DAT.

D.3. Expression Patterns of DAT and MAT in *Catharanthus roseus*

D.3.1. Tissue-Specific Expression of the MAT and DAT Genes

RNA gel blot analysis revealed that *MAT* is expressed differently than *DAT* in various *Catharanthus* tissues. The same RNA blot was first probed with the *DAT* ORF fragment and after stripping was re-probed with a 423 bp *Hind*III

Table IV. Substrate specificities of recombinant forms of DAT and MAT, as determined from TLC scrapings of the major radioactive bands.

	% Activity[a]	
Substrate[b]	**rDAT**	**rMAT**
Deacetylvindoline	100	48
Crude Hairy Root Alkaloid	NA[c]	100
Tabersonine	NA	NA
16-MeOH-Tabersonine	NA	NA
3-OH-Tabersonine	NA	NA
***N*-CH_3-3-OH-Tabersonine**	NA	NA
2,3-dihydro-Tabersonine	NA	NA
6,7-dihydro-3-OH-Tabersonine	NA	NA
Hörhammericine	NA	40
Lochnericine	NA	NA

[a]100 % activity refers to a total radioactivity count of 36,367 dpm for rDAT and 2233 dpm for re-activated rMAT reaction products, respectively.

[b]Assays were carried out as described in experimental procedures, containing either 0.17 μg of rDAT or 6.0 μg of rMAT, respectively, along with : 20 μM deacetylvindoline, or 20 μL of crude hairy root alkaloid extract, or *ca.* 10 μg of each respective alkaloid or 20 μg of hörhammericine or lochnericine, respectively, as substrate in a final assay vol. of 100 μL containing 1% (v/v) DMSO (final conc.) as the substrate solvent.

[c]NA= not accepted as a substrate

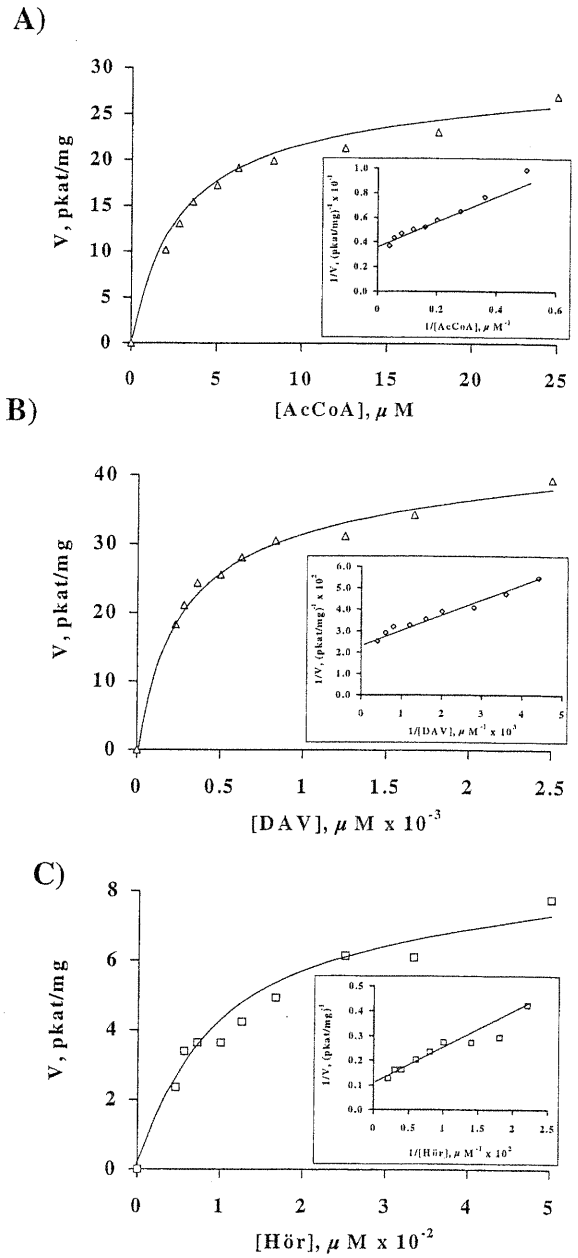


Figure 14. Saturation curves for rMAT using **A)** various concentrations of acetyl coenzyme A (AcCoA) and a fixed concentration of deacetylvindoline, **B)** various concentrations of deacetylvindoline (DAV) and a fixed concentration of AcCoA, and **C)** various concentrations of hörhammericine (Hör) and a fixed concentration of AcCoA. Insets are of reciprocal plots of **A)** 1/V vs. 1/[AcCoA], **B)** 1/V vs. 1/[DAV], and **C)** 1/V vs. 1/[Hör], respectively. Assays were carried out as described in material and methods.

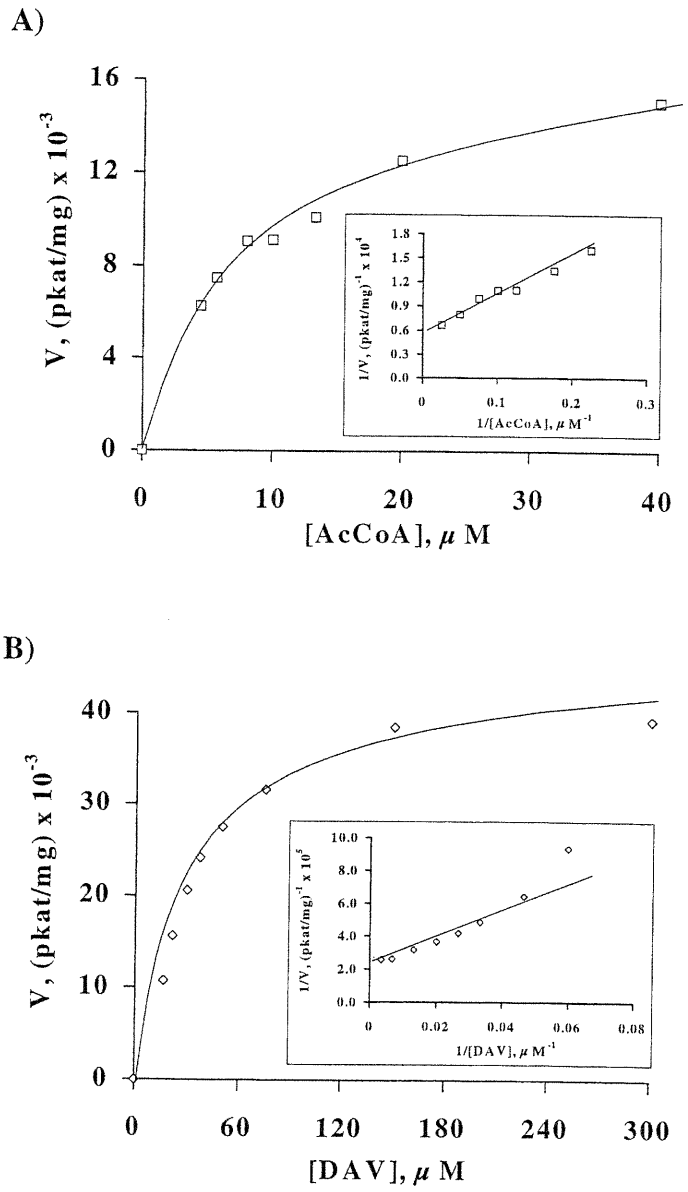
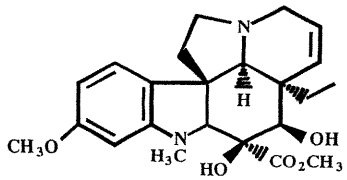
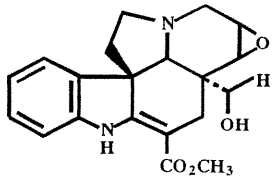


Figure 15. Saturation curves for rDAT using **A)** various concentrations of acetyl coenzyme A (AcCoA) and a fixed concentration of deacetylvindoline, and **B)** various concentrations of deacetylvindoline (DAV) and a fixed concentration of AcCoA. Insets are reciprocal plots of **A)** 1/V vs. 1/[AcCoA], and **B)** 1/V vs. 1/[DAV], respectively. Assays were carried out as described in material and methods.

Table V. Kinetic parameters of rMAT and rDAT with respect to various substrates. Estimated V_{max} and K_m values represent means ± SE of five separate determinations and were calculated by using the ENZFIT software (version 1.05).

Substrate	rMAT			rDAT		
	K_m (μM)	V_{max} (pkat mg^{-1})	V_{max}/K_m (pkat mg^{-1} μM^{-1})	K_m (μM)	V_{max} x 10^4 (pkat mg^{-1})	V_{max}/K_m (pkat mg^{-1} μM^{-1})
AcCoA[b]	3.0(±0.5)	25(±5)	8.3	9.5(±0.5)	1.90	2000
 Deacetylvindoline	250(±25)	31(±5)	0.12	30(±5)	3.81	1270
 Hörhammericine	120(±20)	10(±5)	0.08	NA[a]	NA	NA

[a]**NA** = not accepted as substrate

[b]Kinetic parameters of rMAT for AcCoA were estimated using DAV as the substrate.

MAT::pBluescript fragment (Fig. 16) under high stringency conditions (Fig. 17). *MAT* transcripts were detected only in 5d-old etiolated seedlings and to a lower level in 14d-old hairy roots, whereas *DAT* transcripts were detected predominantly in leaf tissue, flower petals and were faintly detected in stems.

Further studies showed that the MAT gene was already active in 3d-old etiolated seedlings (Fig. 18A, 3D) and that light was not required to activate its expression (Fig. 18A, 6L). The expression of *MAT*, which was mostly restricted to roots, was also observed at low levels in hypocotyls, but not in cotyledons (Fig. 18B). The level of the *MAT* transcript that was most abundant in 3d-old etiolated seedlings, decreased significantly after 5 days of growth and was virtually non-detectable in 6d-old seedlings (Fig. 18A). These results were consistent with the appearance of MAT enzyme activity during etiolated seedling development, where maximum enzyme activity was found in young 4d- to 5d-old radicles, repectively (Fig. 18C). To further locate the site of MAT gene expression within *Catharanthus* roots, 14d-old lateral hairy roots were divided into sections and were analysed for the abundance of MAT transcripts (Fig. 19A). These were most abundant within the first full centimeter from the root tip and decreased rapidly in developmentally older hairy root sections. These results were in agreement with RNA *in situ* hybridization studies, which located MAT gene expression within the cortex and epidermis of tissues near the root tip (Fig. 19B).

```
  1  AAGCTTAATTATGTTGCACGAATGCAAGATTTCGCAAATTGCTTG  45
 46  AAAGAGCTTGACATTAGCAGCTTTTTCGATATGGAAAACGTTGAC  90
 91  ATCGATGCTTACTTGTTCAGTAGCTGGTGTAGATTTCCCTTTTAT 135
136  GACATTGATTTTGGATTGGGAAAGCCGATATGGGTATGCATGTTT 180
181  CAACCCCATTTTAAAAATTGTATTATTTTGATGGATTATCCATTT 225
226  GGAGATGATTATGGAATCGAAGCATTAATCACTCTTGAACAAGAA 270
271  AAAATGCCAGCATTTGAGAACAACGAGCTGCTCCTTTCATTTGCT 315
316  TCTAATTAAGAAGATGATGATATGCCAAAATAAACAGCCAAAGCT 360
361  GAAGAAAGGGCCCAAGAGTTTCAGCATGATTGTCTCTCTGCAGCA 405
406  GGAATTCGATATCAAGCTT
```

Figure 16. This 423 bp *Hind*III MAT : :pBluescript fragment was used as a specific probe for the MAT Southern and Northern blots in Figs. 17, 18, and 19. The MAT ORF was subcloned into the *Bam*HI, *Pst*I sites of pBluescript. The highlighted region corresponds to the portion of DNA belonging to pBluescript. The underlined nucleic acids represent the *Hind*III restriction sites and arrowhead indicates the *Hind*III cleavage sites. **TAA** = the stop codon in the MAT ORF.

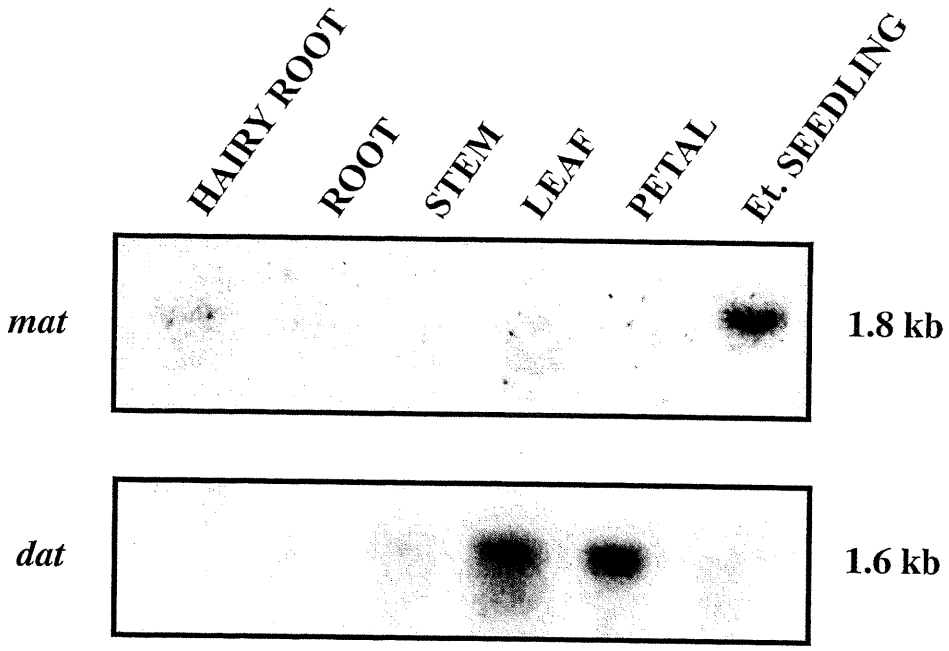


Figure 17. Northern blots of total RNA isolated from *C. roseus* hairy roots, roots, stems, leaves, flower petals or etiolated seedlings. Blots were probed with the *DAT* ORF fragment and a 423 bp *Hind* III fragment of *MAT*::pBluescript, under high stringency conditions as described in material and methods. RNA was quantified by ethidium bromide (EthBr) staining and 20 μg of total RNA per sample was electrophoresed on a 7.7% (v/v) formaldehyde/ 1% (w/v) agarose gel. RNA was transferred to nitrocellulose for hybridization as described in material and methods.

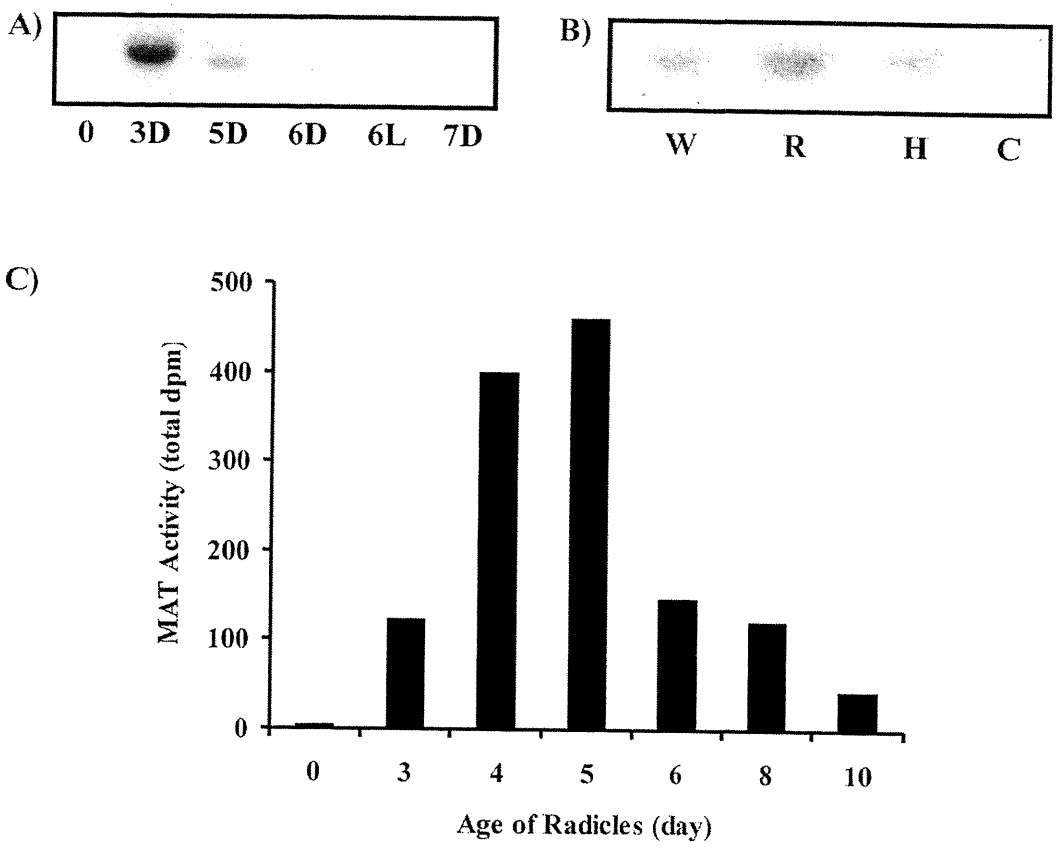


Figure 18. Northern blots of total RNA isolated from **A)** Zero to 7d-old etiolated (D) seedlings, or 5d-old etiolated seedlings treated with light (L) for 24 h (6L) and **B)** 5d-old : whole etiolated seedlings (W), roots (R), hypocotyls (H) and cotyledons (C). Hybridization was carried out under high stringency conditions using a 423 *Hind* III fragment of *MAT*::pBluescript. RNA was quantified by ethidium bromide (EthBr) staining and 20 μg of total RNA was electrophoresed on a 7.7% (v/v) formaldehyde/ 1% (w/v) agarose gel and transferred to nitrocellulose for hybridization. The blots in **A)** and **B)** were each exposed for 3 days. **C).** Distribution of MAT activity in 0 to 10d-old radicles isolated from dark-grown seedlings. MAT activity is reported as total dpm and corresponds to the formation of ^{14}C-labelled echitovenine after isolation by TLC (see Material and Methods), as determined by liquid scintillation counting of isolated radioactive bands. Data presented is the average of two trials.

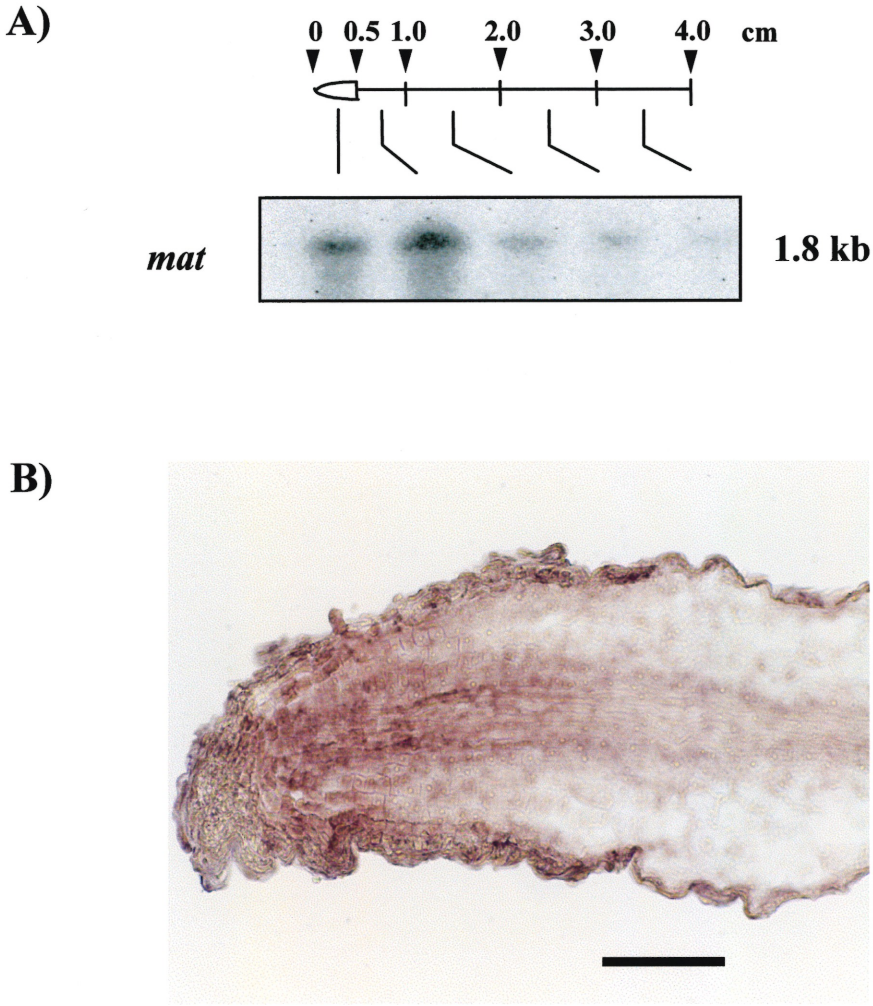


Figure 19. Tissue- and cell-specific localization of *mat*. **A)**. Northern blot of total RNA isolated from 0.5 cm sections of lateral hairy root tissue (as shown in the schematic). Hybridization with a 423 bp *Hind* III fragment of *MAT*::pBluescipt, was carried out under high stringency conditions. **B)**. Localization of *mat* mRNA by *in situ* RNA hybridization in hairy roots. The longitudinal section of a 14d-old lateral hairy root apex was hybridized with antisense RNA for *mat* as described in Material and Methods section C.3.3. Magnification = 250X. Bar = 100 μm.

D.3.2. Gene Copy Number of MAT and DAT

Blots containing *Eco*RI, *Hind*III, *Xba*I or *Eco*RV digested *Catharanthus* genomic DNA were probed with either the *MAT* ORF fragment (Fig. 20A, MAT) or the *DAT* ORF fragment (Fig. 20A, DAT) at high stringency. The strongly hybridizing bands in the MAT-probed blot of *ca.* 3.0, 1.3, 3.5 and 8.0 kb correspond to the respective fragments obtained from *Eco*RI, *Hind*III, *Xba*I and *Eco*RV restriction digestion whereas the DAT-probed blot revealed strongly hybridizing bands of *ca.* 5.7, 4.4, 8.3 and 2.3 kb for the same digestions. The different hybridization patterns indicate the structural differences between MAT and DAT (Fig. 20B) and revealed that *MAT* and *DAT* occur as single copies in the *Catharanthus* diploid genome.

D.4. Comparison of MAT and DAT

Sequence analysis of *MAT* and *DAT* revealed ORFs for putative 443 and 439 amino acid MAT and DAT proteins, respectively (Fig. 21). Sequence comparison of *MAT* and *DAT* showed a 63% nucleic acid identity (data not shown) between these two genes and a 78% amino acid identity between the putative ORFs (Fig. 21).

A protein database derived from the three-dimensional structure Brookhaven protein data bank (BLAST) was previously searched [Altschul *et al.* (1990)] with the DAT sequence and two short blocks of similarity to the catalytic domain of dihydrolipoyl *S*-acetyltransferase (DHLAAT) were identified. DHLAAT is a component of the pyruvate dehydrogenase complex that catalyses acetyltransfer from the bound dihydrolipoamide cofactor to coenzyme A [see: St-Pierre *et al.* (1998)]. One region of similarity lies in the active center of DHLAAT [Mattevi *et al.* (1992)],

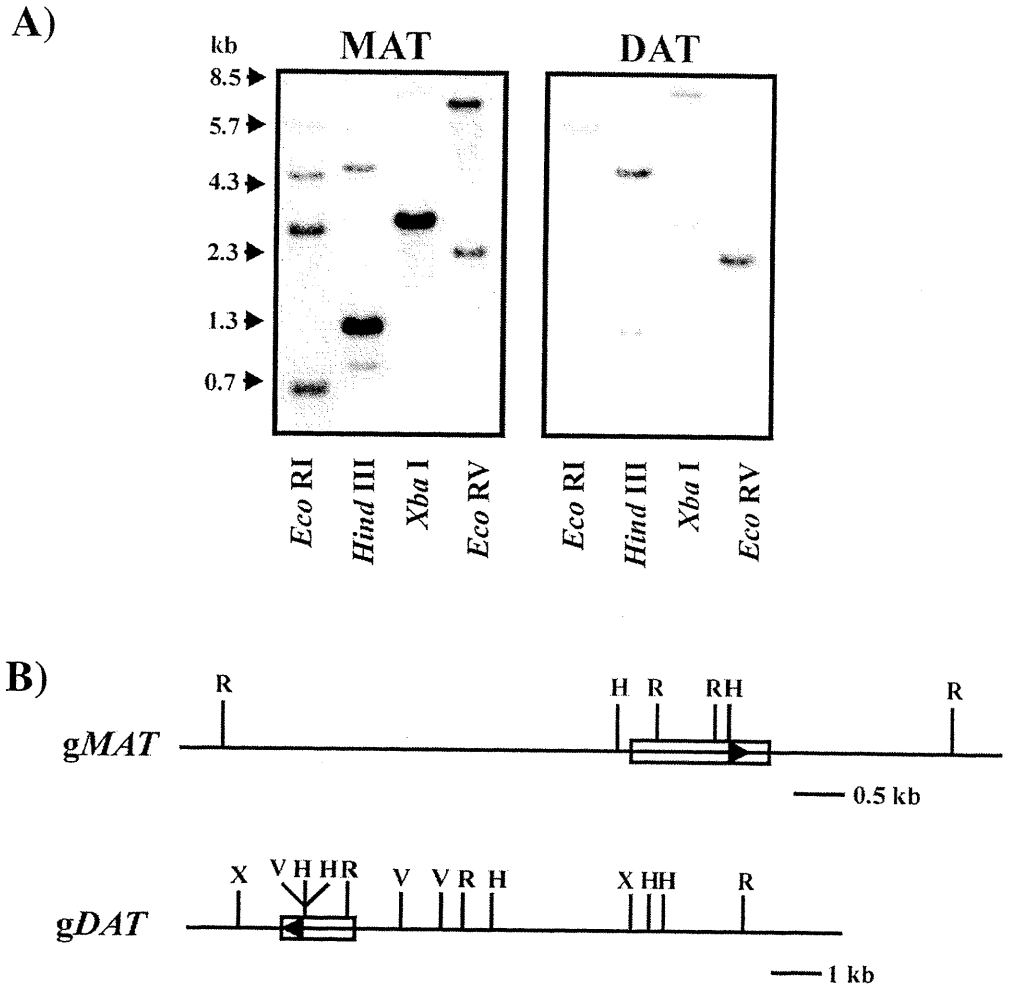


Figure 20. Southern blot analysis of the *MAT* and *DAT* genes in *C. roseus*. **A)** Genomic DNA (*g*DNA) isolated from *Catharanthus* leaves was restriction digested with *Eco*RI (R), *Hind* III (H), *Xba* I (X) and *Eco* RV (V). Approx. 12 μg of *g*DNA was electrophoresed per lane, in duplicate, to allow for hybridization under high stringency conditions with the *DAT* and *MAT* ORF fragments. Numbers to the left of the figure correspond to λDNA/*Bst*EII markers' mol. wt., in kb. **B)** Restriction map of the respective genomic clones, *gDAT* (*gDAT*6) and *gMAT* (*gDAT*15), as deduced from the restriction digest patterns of the genomic Southerns. The ORF segments are illustrated as boxes, with arrows indicating the orientation of the ORF in the genomic clones.

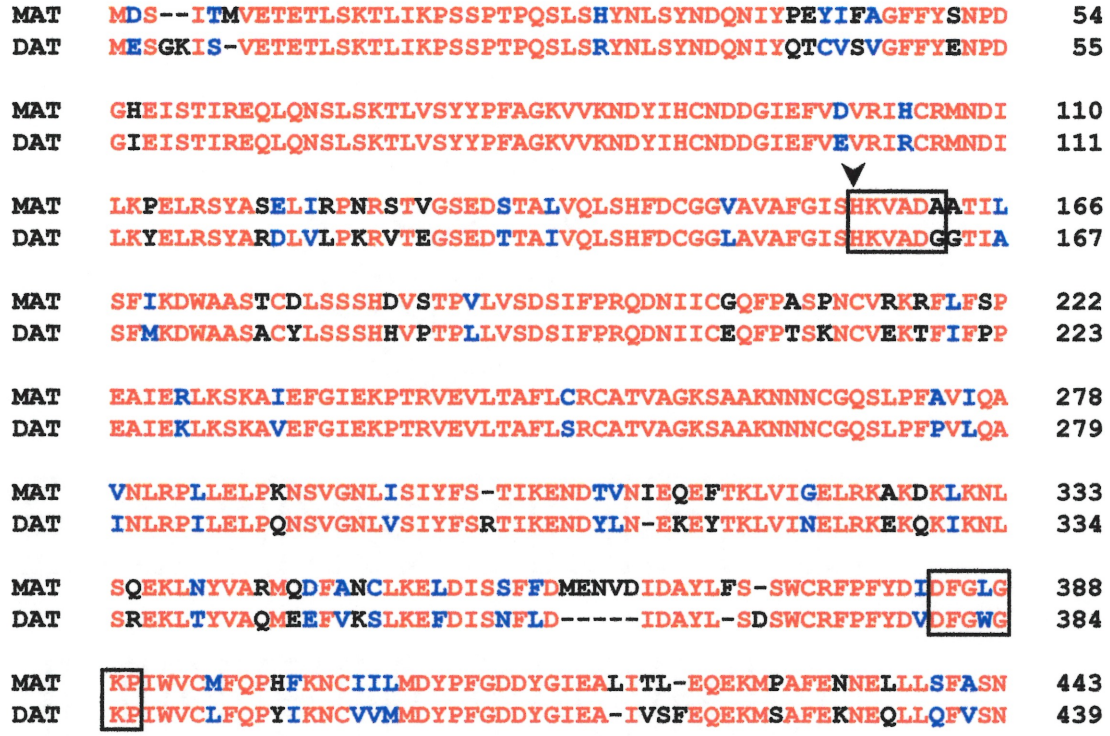


Figure 21. Amino acid alignment of rMAT and rDAT gene products. Identical amino acids are shown in red, conserved amino acids are in blue and differing amino acids are black. The boxed residues highlight the conserved HXXXDG active site and DFGWGKP motif, while the arrow identifies the active site histidine residue.

which contains the HXXXDG motif, that is conserved throughout the DHLAAT [Reed and Hackert (1990)] and chloramphenicol *O*-acetyltransferase (CAT) gene families [Shaw and Leslie (1991)]. With the high degree of similarity between the deduced amino acid sequences of both DAT and MAT (Fig. 21), both conserved domains were also found within the MAT sequence (Fig. 21, Fig. 22).

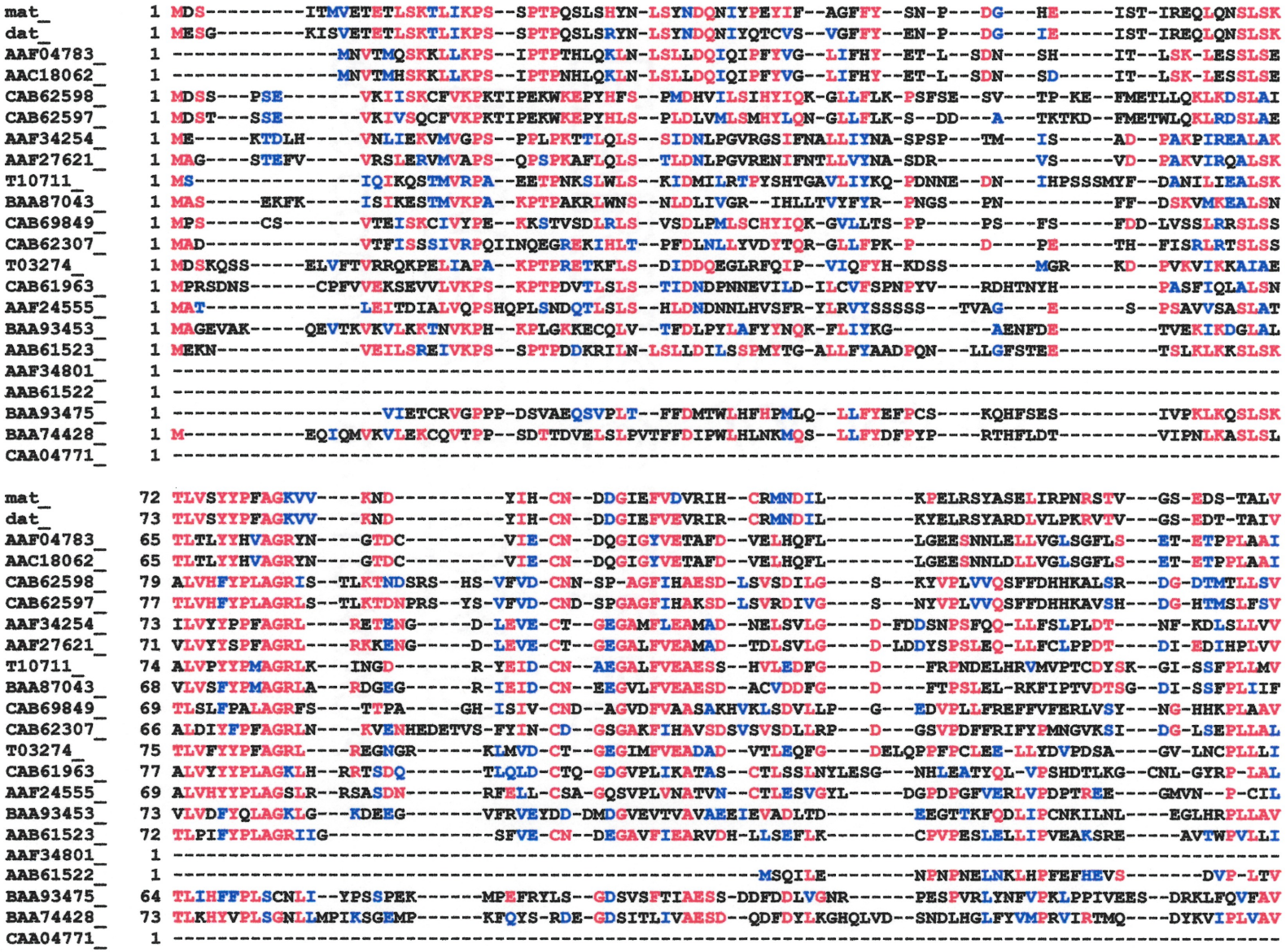
A BLAST search with the deduced amino acid sequence of the MAT ORF revealed similarities to twenty-one plant proteins (Fig. 22).

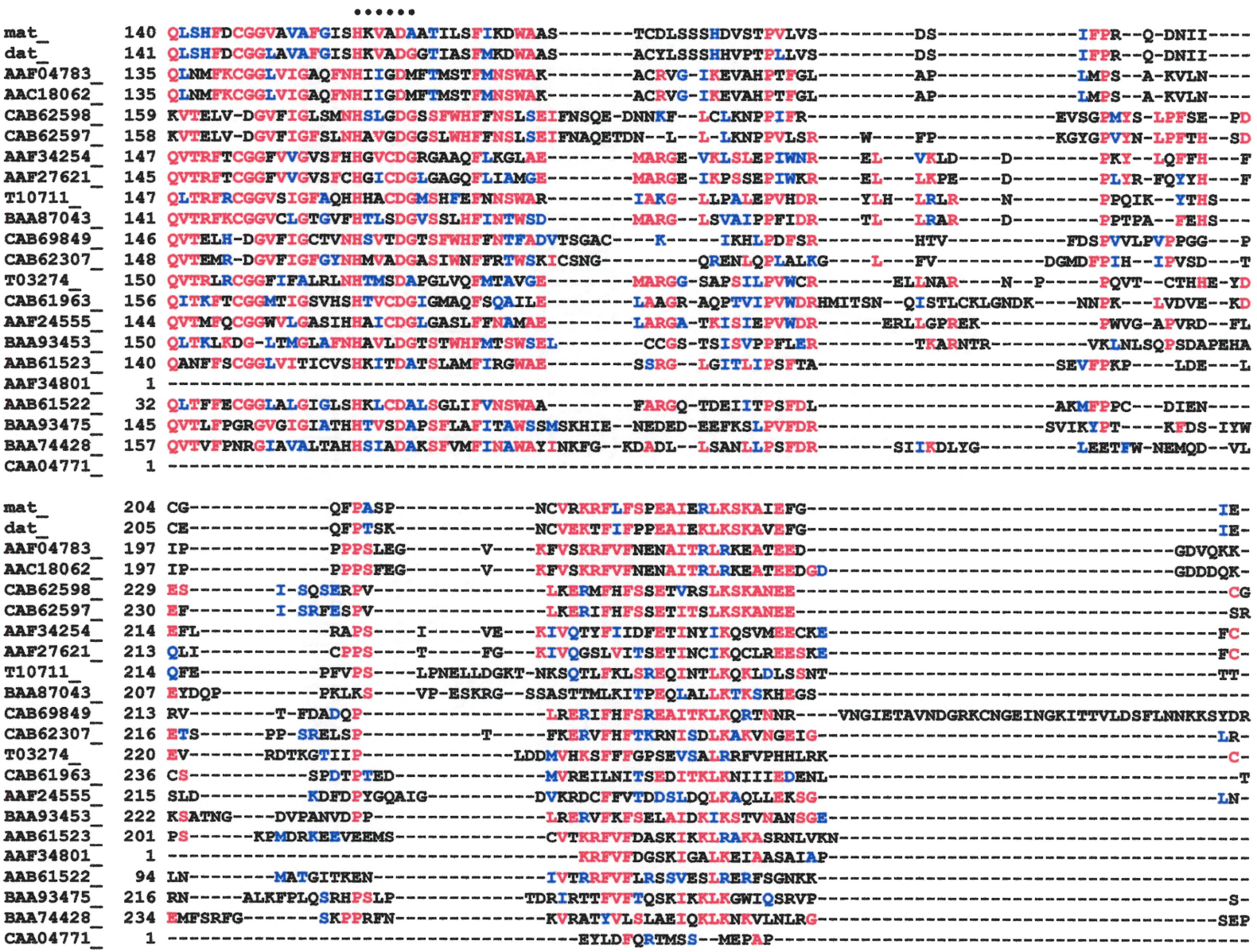
D.4.1. Chemical Modification of MAT and DAT

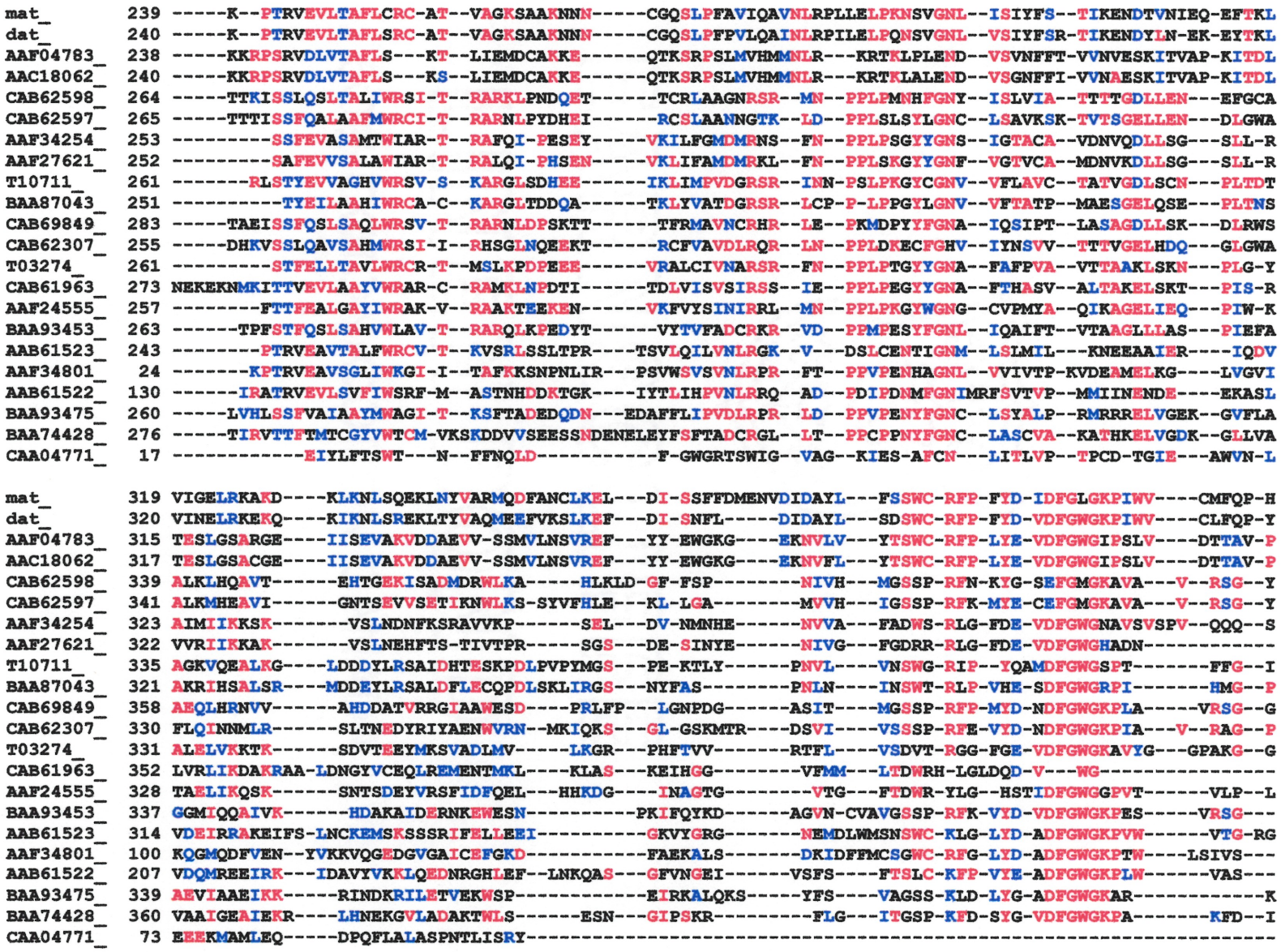
CAT [Lewendon *et al.* (1994)], DHLAAT [Hendle *et al.* (1995)], choline acetyltransferase [Carbini and Hersh (1993)], and carnitine palmitoyltransferase II [Brown *et al.* (1994)] have a conserved HXXXDG motif whose histidine residue was shown by site specific mutation or by chemical modification to be essential for catalytic activity. The presence of this motif in both MAT and DAT (Fig. 21) suggests that histidine may function as a general base in catalyzing acetyl transfer between acetyl CoA and deacetylvindoline (in the case of DAT), as proposed by Shaw and Leslie (1991) for CAT.

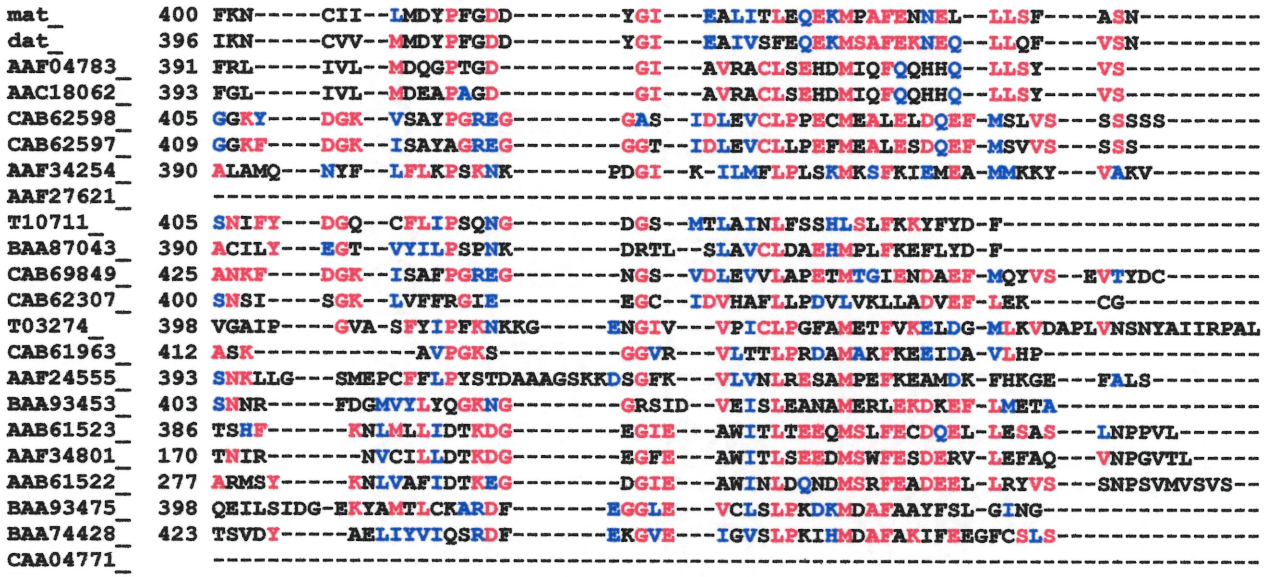
Incubation of DAT and MAT, respectively, with diethylpyrocarbonate (DEPC), which is known to react with a considerable degree of specificity towards histidine [Miles (1977)], resulted in a concentration dependent loss of both DAT and MAT activity (Fig. 23A and B, respectively). At 2 mM DEPC, *ca.* 75 - 85% of the initial activities were lost after a 5 min incubation suggesting that DEPC-sensitive residues are required for DAT and MAT activities. Preincubation of DAT with 5 or

Figure 22. (pages 80-83). Alignments of MAT and DAT with 20 related sequences of plant origin. The deduced amino acid sequnces of MAT and DAT were aligned to those of acetyl-CoA :benzylalcohol acetyltransferase of *Clarkia concinna* (GenBank acc. : **AAF04783**), acetyl-CoA :benzylalcohol acetyltransferase; BEAT of *Clarkia breweri* (GenBank acc. : **AAC18062**), *N*-hydroxycinnamoyl/benzoyltransferase-like protein of *Arabidopsis thaliana* (GenBank acc. : **CAB62598**), proan-thranilate *N*-benzoyltransferase-like protein of *Arabidopsis thaliana* (GenBank acc. : **CAB62597**), taxadienol acetyltransferase of *Taxus cuspidata* (GenBank acc. : **AAF27621**), 10-deactylbaccatin III-10-*O*-acetyltransferase of *Taxus cuspidata* (GenBank acc. : **AAF27621**), anthranilate *N*-benzoyltransferase (EC 2.3.1.144) of *Dianthus caryophyllus* (GenBank acc. : **T10711**), *N*-hydroxycinnamoyl/benzoyltrans-ferase of *Ipomoea batatas* (GenBank acc. : **BAA87043**), anthranilate *N*-benzoyltransferase-like protein of *Arabidopsis thaliana* (GenBank acc. : **CAB69849**), anthranilate *N*-hydroxycinnamoyl/benzoyltransferase-like protein of *Arabidopsis thaliana* (GenBank acc. : **CAB62307**), hypersensitivity-related hsr201 protein of *Nicotiana tabacum* (GenBank acc. : **T03274**), F1K23.10 of *Arabidopsis thaliana* (GenBank acc. : **AAF24555**), acyltransferase homolog of *Petunia x hybrida* (GenBank acc. : **BAA93453**), residues 1 to 219 of F21J9.20-like protein from *Euphorbia esula* (GenBank acc. : **AAF34801**), residues 1 to 331 of F21J9.19 of *Arabidopsis thaliana* (GenBank acc. : **AAB61522**), anthocyanin acyltransferase of *Perilla frutescens* (GenBank acc. : **BAA93475**), anthocyanin 5-aromatic acyltransferase of *Gentiana triflora* (GenBank acc. : **BAA74428**), and a ripening-induced protein of *Fragaria vesca* (Genbank acc. : **CAA04771**). Identical residues are highlighted in black and similar residues are highlighted in grey. The conserved HXXXDG sequence is indicated by dots. Dashes were introduced for proper alignment of sequences.


mat_ 1 MDS---------ITMVETETLSKTLIKPS--SPTPQSLSHYN-LSYNDQNIYPEYIF--AGFFY--SN-P---DG---HE-----IST-IREQLQNSLSK
dat_ 1 MESG--------KISVETETLSKTLIKPS--SPTPQSLSRYN-LSYNDQNIYQTCVS--VGFFY--EN-P---DG---IE-----IST-IREQLQNSLSK
AAF04783_ 1 ---------------MNVTMQSKKLLKPS--IPTPTHLQKLN-LSLLDQIQIPFYVG--LIFHY--ET-L--SDN---SH-----IT--LSK-LESSLSE
AAC18062_ 1 ---------------MNVTMHSKKLLKPS--IPTPNHLQKLN-LSLLDQIQIPFYVG--LIFHY--ET-L--SDN---SD-----IT--LSK-LESSLSE
CAB62598_ 1 MDSS---PSE-------VKIISKCFVKPKTIPEKWKEPYHFS--PMDHVILSIHYIQK-GLLFLK-PSFSE--SV---TP-KE--FMETLLQKLKDSLAI
CAB62597_ 1 MDST---SSE-------VKIVSQCFVKPKTIPEKWKEPYHLS--PLDLVMLSMHYLQN-GLLFLK-S--DD---A---TKTKD--FMETWLQKLRDSLAE
AAF34254_ 1 ME-----KTDLH-----VNLIEKVMVGPS--PPLPKTTLQLS--SIDNLPGVRGSIFNALLIYNA-SPSP---TM---IS-----AD--PAKPIREALAK
AAF27621_ 1 MAG----STEFV-----VRSLERVMVAPS--QPSPKAFLQLS--TLDNLPGVRENIFNTLLVYNA-SDR---------VS-----VD--PAKVIRQALSK
T10711_ 1 MS---------------IQIKQSTMVRPA--EETPNKSLWLS--KIDMILRTPYSHTGAVLIYKQ-PDNNE--DN---IHPSSSMYF--DANILIEALSK
BAA87043_ 1 MAS-----EKFK-----ISIKESTMVKPA--KPTPAKRLWNS--NLDLIVGR--IHLLTVYFYR--PNGS---PN----------FF--DSKVMKEALSN
CAB69849_ 1 MPS-----CS-------VTEISKCIVYPE--KKSTVSDLRLS--VSDLPMLSCHYIQK-GVLLTS-PP-----PS---FS-----FDD-LVSSLRRSLSS
CAB62307_ 1 MAD--------------VTFISSSIVRPQIINQEGREKIHLT--PFDLNLLYVDYTQR-GLLFPK-P------D-----PE-----TH--FISRLRTSLSS
T03274_ 1 MDSKQSS-----ELVFTVRRQKPELIAPA--KPTPRETKFLS--DIDDQEGLRFQIP--VIQFYH-KDSS--------MGR----KD--PVKVIKKAIAE
CAB61963_ 1 MPRSDNS------CPFVVEKSEVVLVKPS--KPTPDVTLSLS--TIDNDPNNEVILD-ILCVFSPNPYV----RDHTNYH---------PASFIQLALSN
AAF24555_ 1 MAT--------------LEITDIALVQPSHQPLSNDQTLSLS--HLDNDNNLHVSFR-YLRVYSSSSS---TVAG----E------S--PSAVVSASLAT
BAA93453_ 1 MAGEVAK-----QEVTKVKVLKKTNVKPH--KPLGKKECQLV--TFDLPYLAFYYNQK-FLIYKG---------AENFDE---------TVEKIKDGLAL
AAB61523_ 1 MEKN-------------VEILSREIVKPS--SPTPDDKRILN-LSLLDILSSPMYTG-ALLFYAADPQN---LLGFSTEE---------TSLKLKKSLSK
AAF34801_ 1
AAB61522_ 1
BAA93475_ 1 --------------------VIETCRVGPPP-DSVAEQSVPLT--FFDMTWLHFHPMLQ--LLFYEFPCS----KQHFSES---------IVPKLKQSLSK
BAA74428_ 1 M-----------EQIQMVKVLEKCQVTPP--SDTTDVELSLPVTFFDIPWLHLNKMQS--LLFYDFPYP----RTHFLDT---------VIPNLKASLSL
CAA04771_ 1
mat_ 72 TLVSYYPFAGKVV----KND----------YIH-CN--DDGIEFVDVRIH--CRMNDIL--------KPELRSYASELIRPNRSTV---GS-EDS-TALV
dat_ 73 TLVSYYPFAGKVV----KND----------YIH-CN--DDGIEFVEVRIR--CRMNDIL--------KYELRSYARDLVLPKRVTV---GS-EDT-TAIV
AAF04783_ 65 TLTLYYHVAGRYN----GTDC---------VIE-CN--DQGIGYVETAFD--VELHQFL--------LGEESNNLELLVGLSGFLS---ET-ETPPLAAI
AAC18062_ 65 TLTLYYHVAGRYN----GTDC---------VIE-CN--DQGIGYVETAFD--VELHQFL--------LGEESNNLDLLVGLSGFLS---ET-ETPPLAAI
CAB62598_ 79 ALVHFYPLAGRIS--TLKTNDSRS--HS-VFVD-CNN-SP-AGFIHAESD-LSVSDILG----S---KYVPLVVQSFFDHHKALSR---DG-DTMTLLSV
CAB62597_ 77 TLVHFYPLAGRLS--TLKTDNPRS--YS-VFVD-CND-SPGAGFIHAKSD-LSVRDIVG----S---NYVPLVVQSFFDHHKAVSH---DG-HTMSLFSV
AAF34254_ 73 ILVYYPPFAGRL----RETENG-----D-LEVE-CT--GEGAMFLEAMAD--NELSVLG----D-FDDSNPSFQQ-LLFSLPLDT----NF-KDLSLLVV
AAF27621_ 71 VLVYYSPFAGRL----RKKENG-----D-LEVE-CT--GEGALFVEAMAD--TDLSVLG----D-LDDYSPSLEQ-LLFCLPPDT----DI-EDIHPLVV
T10711_ 74 ALVPYYPMAGRLK---INGD-------R-YEID-CN--AEGALFVEAESS--HVLEDFG----D----FRPNDELHRVMVPTCDYSK--GI-SSFPLLMV
BAA87043_ 68 VLVSFYPMAGRLA---RDGEG------R-IEID-CN--EEGVLFVEAESD--ACVDDFG----D----FTPSLEL-RKFIPTVDTSG--DI-SSFPLIIF
CAB69849_ 69 TLSLFPALAGRFS----TTPA-----GH-ISIV-CND--AGVDFVAASAKHVKLSDVLLP---G---EDVPLLFREFFVFERLVSY---NG-HHKPLAAV
CAB62307_ 66 ALDIYFPFAGRLN----KVENHEDETVS-FYIN-CD--GSGAKFIHAVSDSVSVSDLLRP---D---GSVPDFFRIFYPMNGVKSI---DG-LSEPLLAL
T03274_ 75 TLVFYYPFAGRL----REGNGR------KLMVD-CT--GEGIMFVEADAD--VTLEQFG----DELQPPFPCLEE-LLYDVPDSA----GV-LNCPLLLI
CAB61963_ 77 ALVYYYPLAGKLH--RRTSDQ-------TLQLD-CTQ-GDGVPLIKATAS--CTLSSLNYLESG---NHLEATYQL-VPSHDTLKG--CNL-GYRP-LAL
AAF24555_ 69 ALVHYYPLAGSLR--RSASDN-------RFELL-CSA-GQSVPLVNATVN--CTLESVGYL-----DGPDPGFVERLVPDPTREE----GMVN--P-CIL
BAA93453_ 73 VLVDFYQLAGKLG---KDEEG-------VFRVEYDD-DMDGVEVTVAVAEEIEVADLTD--------EEGTTKFQDLIPCNKILNL----EGLHRPLLAV
AAB61523_ 72 TLPIFYPLAGRIIG---------------SFVE-CN--DEGAVFIEARVDH-LLSEFLK--------CPVPESLELLIPVEAKSRE-----AVTWPVLLI
AAF34801_ 1
AAB61522_ 1 --MSQILE--------NPNPNELNKLHPFEFHEVS-------DVP-LTV
BAA93475_ 64 TLIHFFPLSCNLI--YPSSPEK------MPEFRYLS--GDSVSFTIAESS-DDFDDLVGNR------PESPVRLYNFVPKLPPIVEES--DRKLFQVFAV
BAA74428_ 73 TLKHYVPLSGNLLMPIKSGEMP------KFQYS-RDE-GDSITLIVAESD--QDFDYLKGHQLVD--SNDLHGLFYVMPRVIRTMQ----DYKVIPLVAV
CAA04771_ 1


mat_ 140 QLSHFDCGGVAVAFGISHKVADAATILSFIKDWAAS-------TCDLSSSHDVSTPVLVS---------DS-------------IFPR--Q-DNII----
dat_ 141 QLSHFDCGGLAVAFGISHKVADGGTIASFMKDWAAS-------ACYLSSSHHVPTPLLVS---------DS-------------IFPR--Q-DNII----
AAF04783_ 135 QLNMFKCGGLVIGAQFNHIIGDMFTMSTFMNSWAK--------ACRVG-IKEVAHPTFGL---------AP-------------LMPS--A-KVLN----
AAC18062_ 135 QLNMFKCGGLVIGAQFNHIIGDMFTMSTFMNSWAK--------ACRVG-IKEVAHPTFGL---------AP-------------LMPS--A-KVLN----
CAB62598_ 159 KVTELV-DGVFIGLSMNHSLGDGSSFWHFFNSLSEIFNSQE-DNNKF--LCLKNPPIFR-----------------------EVSGPMYS-LPFSE--PD
CAB62597_ 158 KVTELV-DGVFIGFSLNHAVGDGGSLWHFFNSLSEIFNAQETDN--L--L-LKNPPVLSR----W----FP----------KGYGPVYN-LPFTH--SD
AAF34254_ 147 QVTRFTCGGFVVGVSFHHGVCDGRGAAQFLKGLAE--------MARGE-VKLSLEPIWNR----EL---VKLD----D---------PKY--LQFFH---F
AAF27621_ 145 QVTRFTCGGFVVGVSFCHGICDGLGAGQFLIAMGE--------MARGE-IKPSSEPIWKR----EL---LKPE----D---------PLYR-FQYYH---F
T10711_ 147 QLTRFRCGGVSIGFAQHHHACDGMSHFEFNNSWAR--------IAKG--LLPALEPVHDR----YLH--LRLR----N---------PPQIK--YTHS---
BAA87043_ 141 QVTRFKCGGVCLGTGVFHTLSDGVSSLHFINTWSD--------MARG--LSVAIPPFIDR----TL---LRAR----D---------PPTPA--FEHS---
CAB69849_ 146 QVTELH-DGVFIGCTVNHSVTDGTSFWHFFNTFADVTSGAC----K-----IKHLPDFSR---------HTV-----------FDSPVVLPVPPGG---P
CAB62307_ 148 QVTEMR-DGVFIGFGYNHMVADGASIWNFFRTWSKICSNG---------QRENLQPLALKG----L---FV----------DGMDFPIH--IPVSD---T
T03274_ 150 QVTRLRCGGFIFALRLNHTMSDAPGLVQFMTAVGE--------MARGG-SAPSILPVWCR-------ELLNAR----N--P-----PQVT--CTHHE-YD
CAB61963_ 156 QITKFTCGGMTIGSVHSHTVCDGIGMAQFSQAILE--------LAAGR-AQPTVIPVWDRHMITSN--QISTLCKLGNDK----NNPK---LVDVE--KD
AAF24555_ 144 QVTMFQCGGWVLGASIHHAICDGLGASLFFNAMAE--------LARGA-TKISIEPVWDR------ERLLGPREK----------PWVG-APVRD--FL
BAA93453_ 150 QLTKLKDG-LTMGLAFNHAVLDGTSTWHFMTSWSEL--------CCGS-TSISVPPFLER---------TKARNTR---------VKLNLSQPSDAPEHA
AAB61523_ 140 QANFFSCGGLVITICVSHKITDATSLAMFIRGWAE--------SSRG--LGITLIPSFTA----------------------SEVFPKP----LDE---L
AAF34801_ 1 ----------
AAB61522_ 32 QLTFFECGGLALGIGLSHKLCDALSGLIFVNSWAA--------FARGQ-TDEIITPSFDL----------------------AKMFPPC---DIEN----
BAA93475_ 145 QVTLFPGRGVGIGIATHHTVSDAPSFLAFITAWSSMSKHIE--NEDED-EEFKSLPVFDR----------------------SVIKYPT----KFDS-IYW
BAA74428_ 157 QVTVFPNRGIAVALTAHHSIADAKSFVMFINAWAYINKFG--KDADL--LSANLLPSFDR-------SIIKDLYG---------LEETFW-NEMQD--VL
CAA04771_ 1 ----------
mat_ 204 CG-------------QFPASP-------------NCVRKRFLFSPEAIERLKSKAIEFG-----------------------------------IE-
dat_ 205 CE-------------QFPTSK-------------NCVEKTFIFPPEAIEKLKSKAVEFG-----------------------------------IE-
AAF04783_ 197 IP-------------PPPSLEG--------V----KFVSKRFVFNENAITRLRKEATEED-----------------------------GDVQKK-
AAC18062_ 197 IP-------------PPPSFEG--------V----KFVSKRFVFNENAITRLRKEATEEDGD---------------------------GDDDQK-
CAB62598_ 229 ES---------I-SQSERPV-----------------LKERMFHFSSETVRSLKSKANEE-----------------------------------CG
CAB62597_ 230 EF---------I-SRFESPV-----------------LKERIFHFSSETITSLKSKANEE-----------------------------------SR
AAF34254_ 214 EFL------------RAPS-----I------VE---KIVQTYFIIDFETINYIKQSVMEECKE---------------------------------FC-
AAF27621_ 213 QLI------------CPPS-----T------FG---KIVQGSLVITSETINCIKQCLREESKE---------------------------------FC-
T10711_ 214 QFE------------PFVPS-----LPNELLDGKT-NKSQTLFKLSREQINTLKQKLDLSSNT---------------------------------TT-
BAA87043_ 207 EYDQP----------PKLKS-----VP-ESKRG--SSASTTMLKITPEQLALLKTKSKHEGS---------------------------------
CAB69849_ 213 RV---------T-FDADQP-------------------LRERIFHFSREAITKLKQRTNNR----VNGIETAVNDGRKCNGEINGKITTVLDSFLNNKKSYDR
CAB62307_ 216 ETS------PP-SRELSP------------T------FKERVFHFTKRNISDLKAKVNGEIG-----------------------------------LR-
T03274_ 220 EV-------RDTKGTIIP--------------LDDMVHKSFFFGPSEVSALRRFVPHHLRK---------------------------------C-
CAB61963_ 236 CS-----------SPDTPTED-------------MVREILNITSEDITKLKNIIIEDENL-----------------------------------T
AAF24555_ 215 SLD----------KDFDPYGQAIG----------DVKRDCFFVTDDSLDQLKAQLLEKSG---------------------------------LN-
BAA93453_ 222 KSATNG----DVPANVDPP----------------LRERVFKFSELAIDKIKSTVNANSGE---------------------------------
AAB61523_ 201 PS------KPMDRKEEVEEMS--------------CVTKRFVFDASKIKKLRAKASRNLVKN---------------------------------
AAF34801_ 1 ----------KRFVFDGSKIGALKEIAASAIAP---------------------------------
AAB61522_ 94 LN--------MATGITKEN----------------IVTRRFVFLRSSVESLRERFSGNKK---------------------------------
BAA93475_ 216 RN-----ALKFPLQSRHPSLP-----------TDRIRTTFVFTQSKIKKLKGWIQSRVP---------------------------------S-
BAA74428_ 234 EMFSRFG-------SKPPRFN--------------KVRATYVLSLAEIQKLKNKVLNLRG---------------------------------SEP
CAA04771_ 1 ----------EYLDFQRTMSS--MEPAP---------------------------------


mat_ 239 -----K--PTRVEVLTAFLCRC-AT--VAGKSAAKNNN-----CGQSLPFAVIQAVNLRPLLELPKNSVGNL--ISIYFS--TIKENDTVNIEQ-EFTKL
dat_ 240 -----K--PTRVEVLTAFLSRC-AT--VAGKSAAKNNN-----CGQSLPFPVLQAINLRPILELPQNSVGNL--VSIYFSR-TIKENDYLN-EK-EYTKL
AAF04783_ 238 -----KKRPSRVDLVTAFLS---KT--LIEMDCAKKE------QTKSRPSLMVHMMNLR---KRTKLPLEND--VSVNFFT-VVNVESKITVAP-KITDL
AAC18062_ 240 -----KKRPSRVDLVTAFLS---KS--LIEMDCAKKE------QTKSRPSLMVHMMNLR---KRTKLALEND--VSGNFFI-VVNAESKITVAP-KITDL
CAB62598_ 264 -----TTKISSLQSLTALIWRSI-T--RARKLPNDQET------TCRLAAGNRSR--MN--PPLPMNHFGNY--ISLVIA--TTTTGDLLEN---EFGCA
CAB62597_ 265 -----TTTISSFQALAAFMWRCI-T--RARNLPYDHEI------RCSLAANNGTK--LD--PPLSLSYLGNC--LSAVKSK-TVTSGELLEN---DLGWA
AAF34254_ 253 ---------SSFEVASAMTWIAR-T--RAFQI-PESEY-----VKILFGMDMRNS--FN--PPLPSGYYGNS--IGTACA--VDNVQDLLSG---SLL-R
AAF27621_ 252 ---------SAFEVVSALAWIAR-T--RALQI-PHSEN-----VKLIFAMDMRKL--FN--PPLSKGYYGNF--VGTVCA--MDNVKDLLSG---SLL-R
T10711_ 261 -------RLSTYEVVAGHVWRSV-S--KARGLSDHEE------IKLIMPVDGRSR--INN-PSLPKGYCGNV--VFLAVC--TATVGDLSCN---PLTDT
BAA87043_ 251 ----------TYEILAAHIWRCA-C--KARGLTDDQA------TKLYVATDGRSR--LCP-P-LPPGYLGNV--VFTATP--MAESGELQSE---PLTNS
CAB69849_ 283 -----TAEISSFQSLSAQLWRSV-T--RARNLDPSKTT------TFRMAVNCRHR--LE--PKMDPYYFGNA--IQSIPT--LASAGDLLSK---DLRWS
CAB62307_ 255 -----DHKVSSLQAVSAHMWRSI-I--RHSGLNQEEKT------RCFVAVDLRQ--LN--PPLDKECFGHV--IYNSVV--TTTVGELHDQ---GLGWA
T03274_ 261 ---------STFELLTAVLWRCR-T--MSLKPDPEEE------VRALCIVNARSR--FN--PPLPTGYYGNA--FAFPVA--VTTAAKLSKN---PLG-Y
CAB61963_ 273 NEKEKNMKITTVEVLAAYVWRAR-C--RAMKLNPDTI------TDLVISVSIRSS--IE--PPLPEGYYGNA--FTHASV--ALTAKELSKT---PIS-R
AAF24555_ 257 --------FTTFEALGAYIWRAK-V--RAAKTEEKEN------VKFVYSINIRRL--MN--PPLPKGYWGNG--CVPMYA--QIKAGELIEQ---PIW-K
BAA93453_ 263 ------TPFSTFQSLSAHVWLAV-T--RARQLKPEDYT------VYTVFADCRKR--VD--PPMPESYFGNL--IQAIFT--VTAAGLLLAS---PIEFA
AAB61523_ 243 --------PTRVEAVTALFWRCV-T--KVSRLSSLTPR----TSVLQILVNLRGK--V---DSLCENTIGNM--LSLMIL---KNEEAAIER----IQDV
AAF34801_ 24 -------KPTRVEAVSGLIWKGI-I--TAFKKSNPNLIR---PSVWSVSVNLRPR--FT--PPVPENHAGNL--VVIVTP-KVDEAMELKG----LVGVI
AAB61522_ 130 ------IRATRVEVLSVFIWSRF-M--ASTNHDDKTGK----IYTLIHPVNLRRQ--AD--PDIPDNMFGNIMRFSVTVP--MMIINENDE----EKASL
BAA93475_ 260 -----LVHLSSFVAIAAYMWAGI-T--KSFTADEDQDN---EDAFFLIPVDLRPR--LD--PPVPENYFGNC--LSYALP--RMRRRELVGEK--GVFLA
BAA74428_ 276 -----TIRVTTFTMTCGYVWTCM-VKSKDDVVSEESSNDENELEYFSFTADCRGL--LT--PPCPPNYFGNC--LASCVA--KATHKELVGDK--GLLVA
CAA04771_ 17 ------------EIYLFTSWT---N--FFNQLD----------F-GWGRTSWIG--VAG--KIES-AFCN---LITLVP--TPCD-TGIE---AWVN-L
mat_ 319 VIGELRKAKD----KLKNLSQEKLNYVARMQDFANCLKEL---DI-SSFFDMENVDIDAYL---FSSWC-RFP-FYD-IDFGLGKPIWV----CMFQP-H
dat_ 320 VINELRKEKQ----KIKNLSREKLTYVAQMEEFVKSLKEF---DI-SNFL-----DIDAYL---SDSWC-RFP-FYD-VDFGWGKPIWV----CLFQP-Y
AAF04783_ 315 TESLGSARGE----IISEVAKVDDAEVV-SSMVLNSVREF---YY-EWGKG----EKNVLV---YTSWC-RFP-LYE-VDFGWGIPSLV----DTTAV-P
AAC18062_ 317 TESLGSACGE----IISEVAKVDDAEVV-SSMVLNSVREF---YY-EWGKG----EKNVFL---YTSWC-RFP-LYE-VDFGWGIPSLV----DTTAV-P
CAB62598_ 339 ALKLHQAVT------EHTGEKISADMDRWLKA-----HLKLD-GF-FSP--------NIVH---MGSSP-RFN-KYG-SEFGMGKAVA---V--RSG--Y
CAB62597_ 341 ALKMHEAVI------GNTSEVVSETIKNWLKS-SYVFHLE---KL-LGA--------MVVH---IGSSP-RFK-MYE-CEFGMGKAVA---V--RSG--Y
AAF34254_ 323 AIMIIKKSK-------VSLNDNFKSRAVVKP------SEL---DV-NMNHE------NVVA---FADWS-RLG-FDE-VDFGWGNAVSVSPV--QQQ--S
AAF27621_ 322 VVRIIKKAK-------VSLNEHFTS-TIVTPR-----SGS---DE-SINYE------NIVG---FGDRR-RLG-FDE-VDFGWGHADN----------
T10711_ 335 AGKVQEALKG----LDDDYLRSAIDHTESKPDLPVPYMGS---PE-KTLY------PNVL----VNSWG-RIP--YQAMDFGWGSPT-------FFG--I
BAA87043_ 321 AKRIHSALSR----MDDEYLRSALDFLECQPDLSKLIRGS---NYFAS--------PNLN----INSWT-RLP-VHE-SDFGWGRPI-------HMG--P
CAB69849_ 358 AEQLHRNVV------AHDDATVRRGIAAWESD----PRLFP---LGNPDG------ASIT----MGSSP-RFP-MYD-NDFGWGKPLA-----VRSG--G
CAB62307_ 330 FLQINNMLR------SLTNEDYRIYAENWVRN--MKIQKS---GL-GSKMTR----DSVI----VSSSP-RFE-VYD-NDFGWGKPIA---V--RAG--P
T03274_ 331 ALELVKKTK------SDVTEEYMKSVADLMV-----LKGR---PHFTVV-------RTFL----VSDVT-RGG-FGE-VDFGWGKAVYG---GPAKG--G
CAB61963_ 352 LVRLIKDAKRAA-LDNGYVCEQLREMENTMKL----KLAS---KEIHGG--------VFMM---LTDWRH-LGLDQD-V---WG----------------
AAF24555_ 328 TAELIKQSK------SNTSDEYVRSFIDFQEL---HHKDG----INAGTG--------VTG---FTDWR-YLG--HSTIDFGWGGPVT------VLP--L
BAA93453_ 337 GGMIQQAIVK------HDAKAIDERNKEWESN----------PKIFQYKD------AGVN-CVAVGSSP-RFK-VYD-VDFGWGKPES-----VRSG---
AAB61523_ 314 VDEIRRAKEIFS-LNCKEMSKSSSRIFELLEEI----------GKVYGRG-------NEMDLWMSNSWC-KLG-LYD-ADFGWGKPVW------VTG-RG
AAF34801_ 100 KQGMQDFVEN--YVKKVQGEDGVGAICEFGKD----------FAEKALS-------DKIDFFMCSGWC-RFG-LYD-ADFGWGKPTW----LSIVS---
AAB61522_ 207 VDQMREEIRK---IDAVYVKKLQEDNRGHLEF--LNKQAS---GFVNGEI------VSFS----FTSLC-KFP-VYE-ADFGWGKPLW------VAS---
BAA93475_ 339 AEVIAAEIKK-----RINDKRILETVEKWSP----------EIRKALQKS-----YFS----VAGSS-KLD-LYG-ADFGWGKAR----------K
BAA74428_ 360 VAAIGEAIEKR----LHNEKGVLADAKTWLS------ESN---GIPSKR---------FLG---ITGSP-KFD-SYG-VDFGWGKPA-------KFD--I
CAA04771_ 73 EEEKMAMLEQ-----DPQFLALASPNTLISRY----------


mat_ 400 FKN-----CII--LMDYPFGDD--------YGI---EALITLEQEKMPAFENNEL--LLSF----ASN---------
dat_ 396 IKN-----CVV--MMDYPFGDD--------YGI---EAIVSFEQEKMSAFEKNEQ--LLQF----VSN---------
AAF04783_ 391 FRL-----IVL--MDQGPTGD----------GI---AVRACLSEHDMIQFQQHHQ--LLSY----VS----------
AAC18062_ 393 FGL-----IVL--MDEAPAGD----------GI---AVRACLSEHDMIQFQQHHQ--LLSY----VS----------
CAB62598_ 405 GGKY----DGK--VSAYPGREG--------GAS--IDLEVCLPPECMEALELDQEF-MSLVS---SSSSS-------
CAB62597_ 409 GGKF----DGK--ISAYAGREG--------GGT--IDLEVCLLPEFMEALESDQEF-MSVVS---SSS---------
AAF34254_ 390 ALAMQ---NYF--LFLKPSKNK-------PDGI--K-ILMFLPLSKMKSFKIEMEA-MMKKY---VAKV--------
AAF27621_ ----------
T10711_ 405 SNIFY---DGQ--CFLIPSQNG--------DGS--MTLAINLFSSHLSLFKKYFYD-F-------------------
BAA87043_ 390 ACILY---EGT--VYILPSPNK--------DRTL--SLAVCLDAEHMPLFKEFLYD-F-------------------
CAB69849_ 425 ANKF----DGK--ISAFPGREG--------NGS--VDLEVVLAPETMTGIENDAEF-MQYVS--EVTYDC-------
CAB62307_ 400 SNSI----SGK--LVFFRGIE---------EGC--IDVHAFLLPDVLVKLLADVEF-LEK-----CG----------
T03274_ 398 VGAIP----GVA-SFYIPFKNKKG-----ENGIV---VPICLPGFAMETFVKELDG-MLKVDAPLVNSNYAIIRPAL
CAB61963_ 412 ASK------------AVPGKS---------GGVR---VLTTLPRDAMAKFKEEIDA-VLHP----------------
AAF24555_ 393 SNKLLG---SMEPCFFLPYSTDAAAGSKKDSGFK---VLVNLRESAMPEFKEAMDK-FHKGE---FALS--------
BAA93453_ 403 SNNR-----FDGMVYLYQGKNG--------GRSID--VEISLEANAMERLEKDKEF-LMETA---------------
AAB61523_ 386 TSHF------KNLMLLIDTKDG--------EGIE---AWITLTEEQMSLFECDQEL-LESAS---LNPPVL------
AAF34801_ 170 TNIR-------NVCILLDTKDG--------EGFE---AWITLSEEDMSWFESDERV-LEFAQ---VNPGVTL-----
AAB61522_ 277 ARMSY-----KNLVAFIDTKEG--------DGIE---AWINLDQNDMSRFEADEEL-LRYVS---SNPSVMVSVS--
BAA93475_ 398 QEILSIDG-EKYAMTLCKARDF-------EGGLE---VCLSLPKDKMDAFAAYFSL-GING----------------
BAA74428_ 423 TSVDY-----AELIYVIQSRDF-------EKGVE---IGVSLPKIHMDAFAKIFEEGFCSLS---------------
CAA04771_ ----------

25 μM AcCoA, substantially reduced the DEPC based inactivation of DAT (Fig. 23C), whereas 20 μM DAV did not protect the enzyme (Fig. 23C). In a similar set of experiments, preincubation of MAT with 25 μM AcCoA, reduced the inactivation by DEPC, as approx. 55% of the initial MAT activity was retained (Fig. 23D). However, as was found with DAT, 20 μM DAV was ineffective in protecting MAT from inactivation (Fig. 23D).

D.5. Light Regulation of DAT

D.5.1. White Light

Previous studies [De Luca *et al.* (1988)] showed that exposure of 5d-old dark-grown *Catharanthus* seedlings to white light, increased DAT activity approx. 10-fold within 72 h of treatment. These studies were repeated and similar results were obtained (Fig. 24). When 5 day old etiolated seedlings were exposed to continuous white light, DAT activity increased to a maximum after 96 h of exposure, compared to dark grown seedlings. In contrast, transfer of 72 h light treated seedlings to darkness, caused DAT activity to decline, rather than increase to the 96 h levels (Fig. 24). The activation of DAT and vindoline biosynthesis by light, prompted further experiments to probe the biological properties of this process.

D.5.2. Red (R) Light

Previous studies have shown that tabersonine and later intermediates in vindoline biosynthesis are rapidly converted to vindoline upon light exposure of dark-grown *Catharanthus* seedlings [Balsevich *et al.* (1986); De Luca *et al.* (1986)]. Other

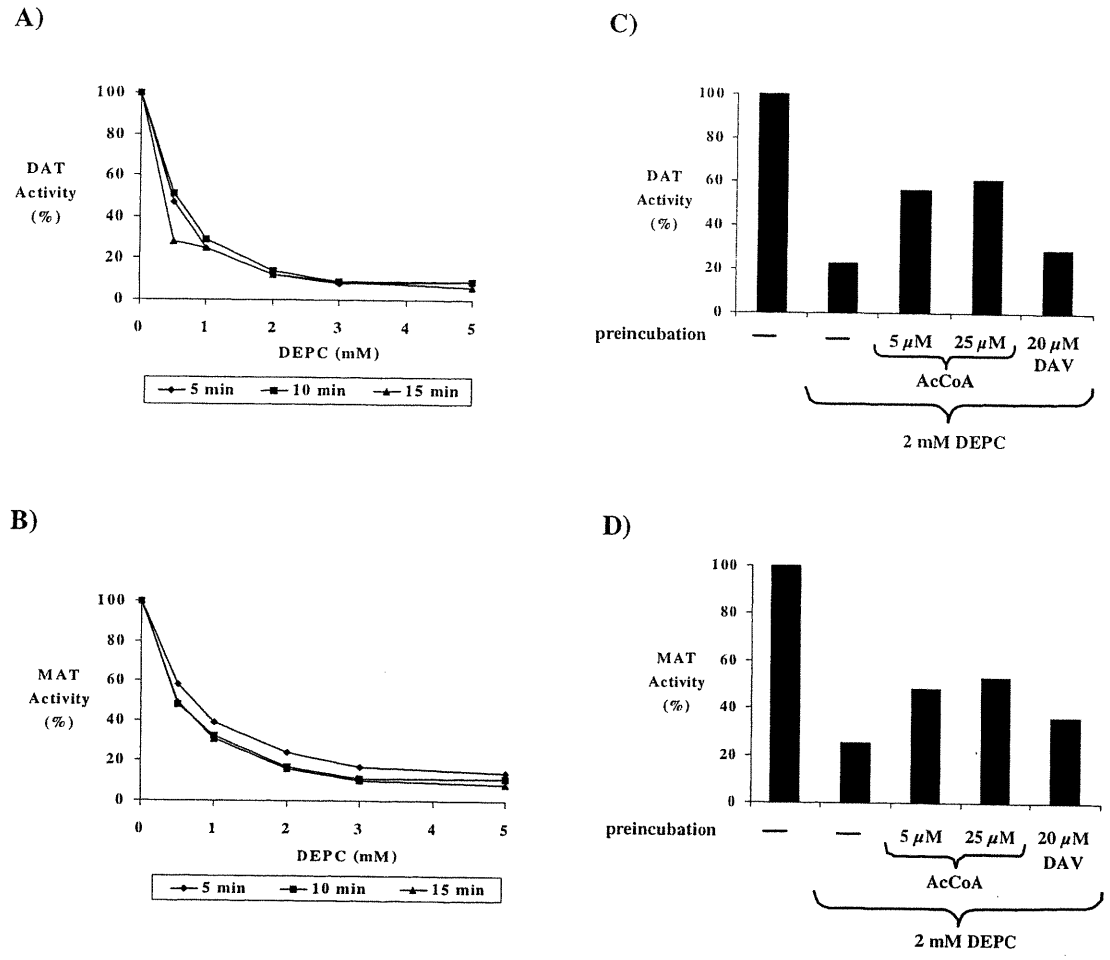


Figure 23. Effect of diethylpyrocarbonate (DEPC) on DAT and MAT activity, and substrate protection against DEPC inactivation. Inactivation of **A)** DAT and **B)** MAT by increasing concentrations of DEPC, after 5, 10, and 15 min incubation at 23° C. Effect of 10 min preincubation with or without acetyl coenzyme A (AcCoA) and deacetylvindoline (DAV) on **C)** DAT and **D)** MAT inactivation by DEPC. Crude recombinant protein extracted from *E. coli* was used, as described in Material and Methods section C.4.2.5. The data represents the average values of three independent experiments.

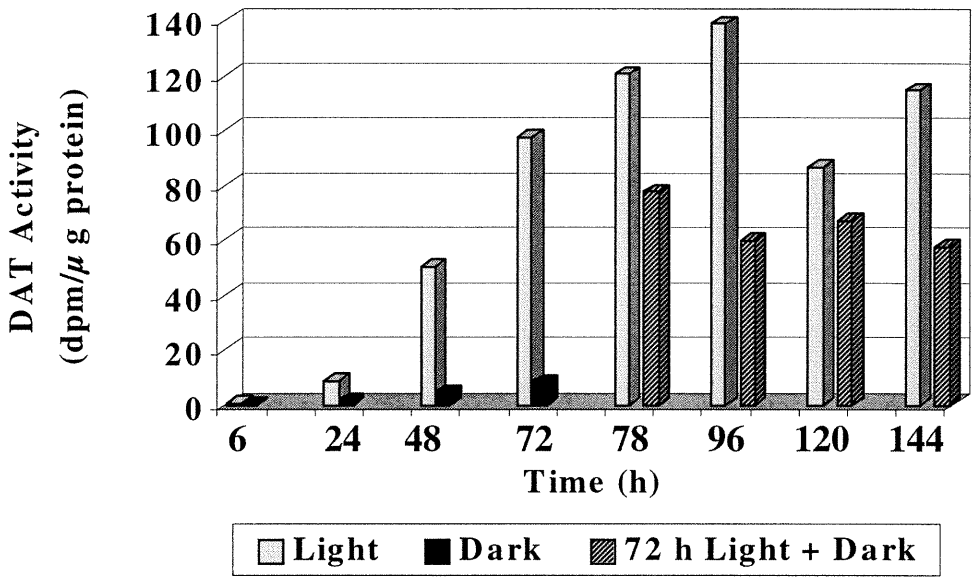


Figure 24. Time course of DAT activity in 5d-old, dark-grown *Catharanthus roseus* seedlings exposed to white light (grey bars) or complete darkness (black bars) for 72 h. The seedlings were returned to darkness (hatched bars) following the initial 72 h exposure to white light, or exposed to white light for a further 72 h (totaling 144 h).

studies [Aerts and De Luca (1992)], which showed that R light treatment of dark-grown *Catharanthus* seedlings results in a *ca.* 2.25-fold increase in DAT activity compared to dark-grown controls, illustrate that the phytochrome system may be involved in the process.

The accumulation of vindoline in R light exposed 4, 5 and 6 day old etiolated *C. roseus* seedlings could be qualitatively monitored by TLC after spraying with CAS to reveal differently colored alkaloids (Fig. 25). The appearance of pink colored vindoline could be easily followed in this TLC system. The results show that this system was not useful for monitoring differences in vindoline accumulation as a result of R light treatment, since low levels of vindoline can accumulate during later stages of seedling development (compare Fig. 25 A-C) in the absence of light (Fig. 25, lanes 9, 19 and 29). HPLC analyses of these samples suggested that 4d-old, dark-grown seedlings exposed to 30 min R light caused a *ca.* 3-fold increase in the level of vindoline, as compared to seedlings which had not been exposed to R light or kept in continuous darkness for 24 h (Fig. 26A). Exposing 5d- and 7d-old seedlings to R light for various times did not produce any significant increases in vindoline levels as compared to 24 h dark-treated (D) controls (Fig. 26A). Similar analyses showed few apparent differences in tabersonine levels of R light treated and dark-exposed 4d-, 5d-, and 7d-old seedlings (Fig. 26B). Only a slight difference was observed in the level of vindoline in 5d-old dark-grown seedlings exposed to 72 h of white light as compared to dark controls, while a noticeable difference in tabersonine was observed (Fig. 26C). The latter results do not correlate with those obtained in previous studies [Balsevich *et al.* (1986); De Luca *et al.* (1986)], which showed that light treatment

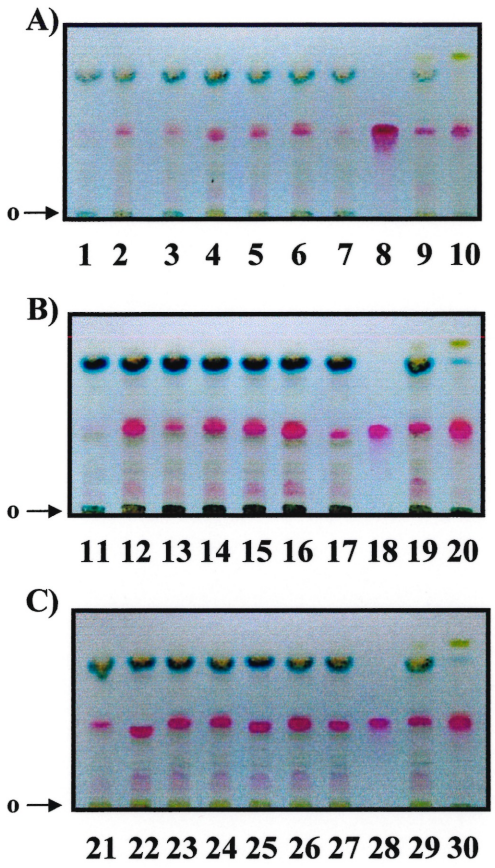


Figure 25. Thin-layer chromatography and ceric ammonium sulphate (CAS) revelation of alkaloids isolated from **A**) 4d-, **B**) 5d-, and **C**) 6d-old etiolated *C. roseus* seedlings exposed to : lanes 1, 11, 21— 0 min of R (T= 0); lanes 2, 12, 22— 30 min R (*ca.* 20 μmol·m^{-2}·s^{-1})(T= 30); lanes 3, 13, 23— 45 min R (T= 45); lanes 4, 14, 24— 60 min R (T= 60); in lanes 5, 15, 25— 90 min red light (T= 90); lanes 6, 16, 26— 120 min R (T= 120); and lanes 7, 17, 27— 24 hr of darkness. Alkaloid samples in lanes 9, 19 and 29 were isolated from 5d-old etiolated seedlings which were exposed to 72 h of darkness, while alkaloid samples in lanes 10, 20 and 30 are from 5d-old etiolated seedlings exposed to 72 h of white light (as described in Material and Methods). Lanes 8, 18 and 28 contain 5 μg of the vindoline reference standard. Alkaloid samples equivalent to the volume of 5 seedlings was applied to the TLC plate. **o** denotes the origin of sample application.

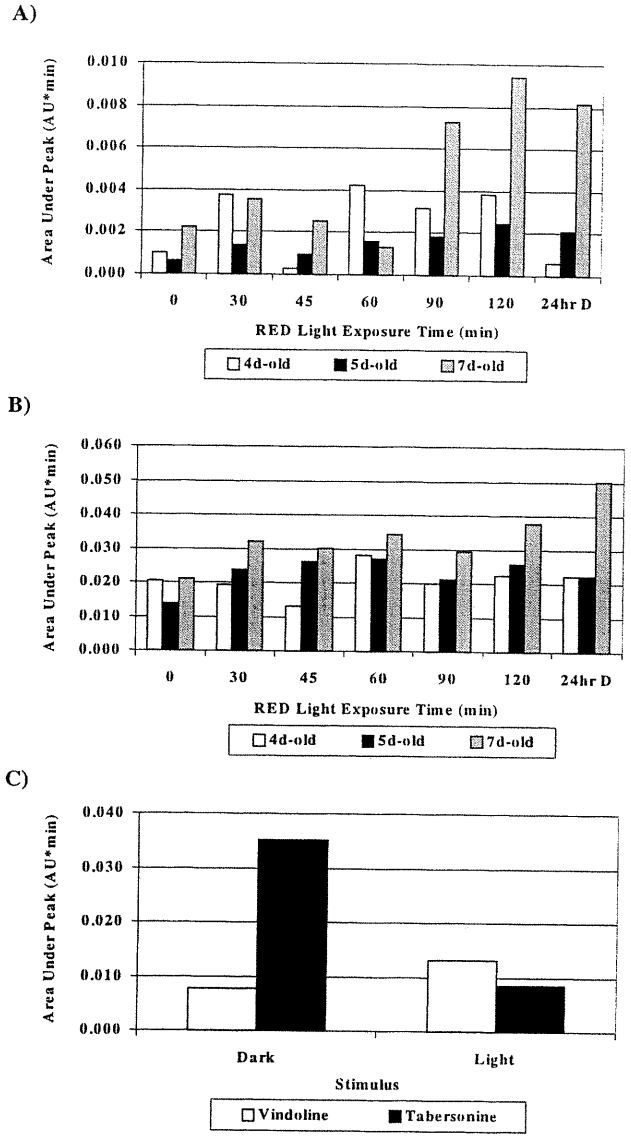


Figure 26. A) Vindoline and **B)** tabersonine profiles obtained from HPLC analyses of alkaloids extracted from *C. roseus* seedlings aged 4, 5, and 7 days old, exposed to red light (*ca.* 20 μmol·mol^{-2}·s^{-1}) as indicated. **C)** Vindoline and tabersonine profiles from 5d-old etiolated seedlings exposed to either 72 h of white light (*ca.* 60 μmol·mol^{-2}·s^{-1}) or complete darkness. The area under the peaks corresponding to vindoline and tabersonine was calculated from HPLC chromatograms, based on the retention times of vindoline and tabersonine reference standards. Injections of 50 μL of each sample (total sample vol= 1 mL) were subjected to HPLC.

led to the quantitative conversion of tabersonine and later intermediates into vindoline.

These equivocal results led to a re-evaluation of the expression DAT activity during R light treatment compared to dark-exposed seedlings. Treatment of 5d-old etiolated seedlings to a minimum 15 min R light pulse (*ca.* 20 μmol·mol^{-2}·s^{-1}) was sufficient to cause a *ca.* 2-fold increase in DAT activity (Fig. 27A), as had been previously observed by Aerts and De Luca (1992). The increase in DAT activity was optimal after a 24 h incubation in complete darkness (Fig. 27B), and far-red (FR) light was able to reverse the effect of R light on DAT activity following a 30 min exposure to R light (Fig. 27C). These observations reveal that the phytochrome system plays an active role in mediating the light response. Exposing 5d-old, dark-grown seedlings to 3d of white light, as compared to a similar exposure to continuous darkness, demonstrated a *ca.* 12-fold increase in DAT activity, in light-exposed seedlings (Fig. 27D), as had previously been observed (Fig. 24). These studies suggest that additional factors to the phytochrome system may be required to induce DAT activity.

While DAT activity was clearly affected by the presence of either R or W light (Fig. 27A-D), MAT activity remained relatively constant and unaffected by light exposure (Fig. 27D).

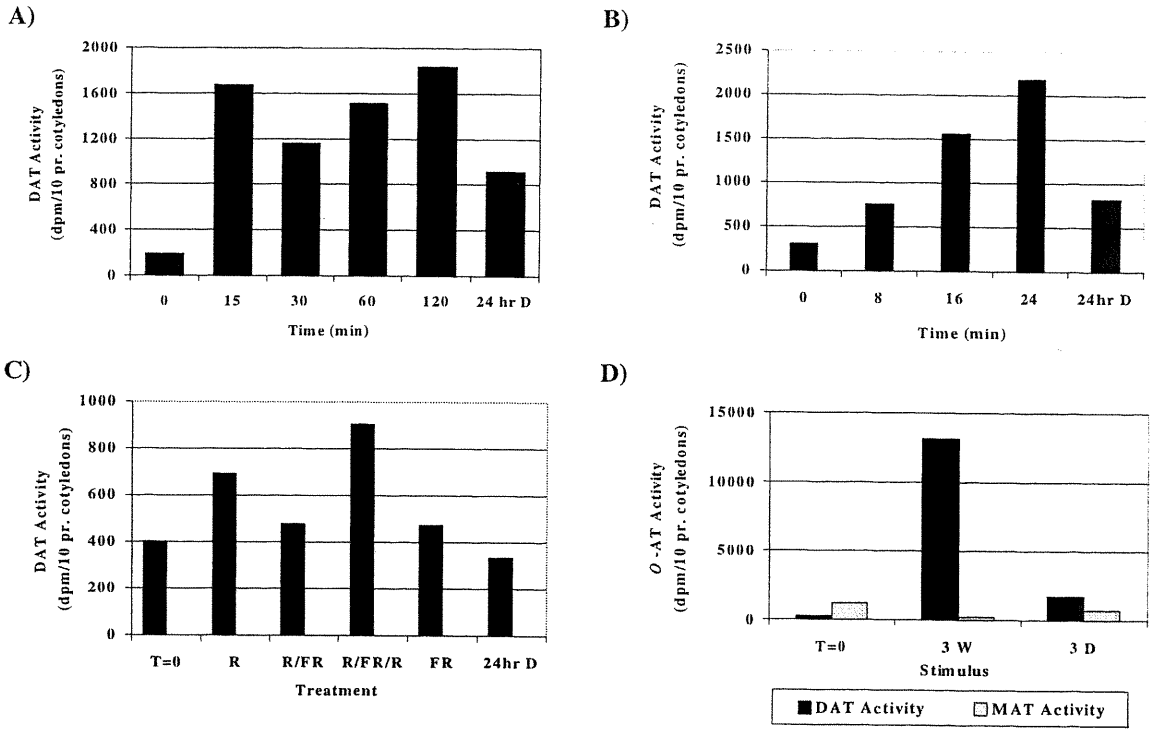


Figure 27. DAT activities in cotyledons of 5d-old etiolated seedlings exposed to red light (*ca.* 20 μmol·mol^{-2}·s^{-1}) for **A)** various times and returned to darkness for 24 h prior to harvesting and **B)** 30 min, returned to darkness and harvested at the indicated times. **C)** DAT activity in cotyledons of 5d-old etiolated seedlings exposed to 9 min of red light (R) (*ca.* 20 μmol·mol^{-2}·s^{-1}), far-red (FR), or R followed by an equivalent exposure to FR, respectively, and returned to darkness for 24 h prior to harvesting. **D)** DAT and MAT activities in cotyledons of 5d-old etiolated seedlings exposed to 3d of white (3W) light (*ca.* 60 μmol·mol^{-2}·s^{-1}) or 3d of continuous darkness (3D).

D.5.3. Light and Phytohormones

D.5.3.1. *meta*-Topolin (mT)

The recent discovery of a highly active aromatic cytokinin (CK) from poplar, N^6-(3-hydroxybenzyl)adenine (*meta*-topolin, mT) [Strnad *et al.* (1997)], prompted us to study its effect on dark-grown *Catharanthus* seedlings and DAT activity, as CKs have been reported to mimick the effect of R light on chloroplast development and gene expression [see review by : Thomas *et al.* (1997) and references therein]. It was therefore believed that mT-treated, dark-grown *Catharanthus* seedlings would result in expressing induced levels of DAT activity. Incubating 5d-old etiolated *Catharanthus* seedlings with varying concentrations of mT, followed by a 48 h incubation in complete darkness, did not cause any difference in DAT activity, as compared to a water-treated control (Fig. 28A). In fact, the water-treated control exhibited the maximum of DAT activity as compared to mT-treated seedlings (Fig. 28A).

D.5.3.2. Brassinazole (BRZ)

The recent discovery of a BR-specific inhibitor, brassinazole (BRZ) [Asami and Yoshida (1999); Min *et al.* (1999); Asami *et al.* (2000)], has made it possible to investigate the role of BRs in many wild-type plant species. *Catharanthus* seedlings were therefore incubated with varying concentrations of BRZ, in the dark, for 48 h prior to harvesting and assaying for DAT activity (Fig. 28B). It was possible that BRZ inhibition of BR biosynthesis in 5d-old, dark-grown *Catharanthus* seedlings would exhibit a light-grown phenotype and result in expression of elevated levels of

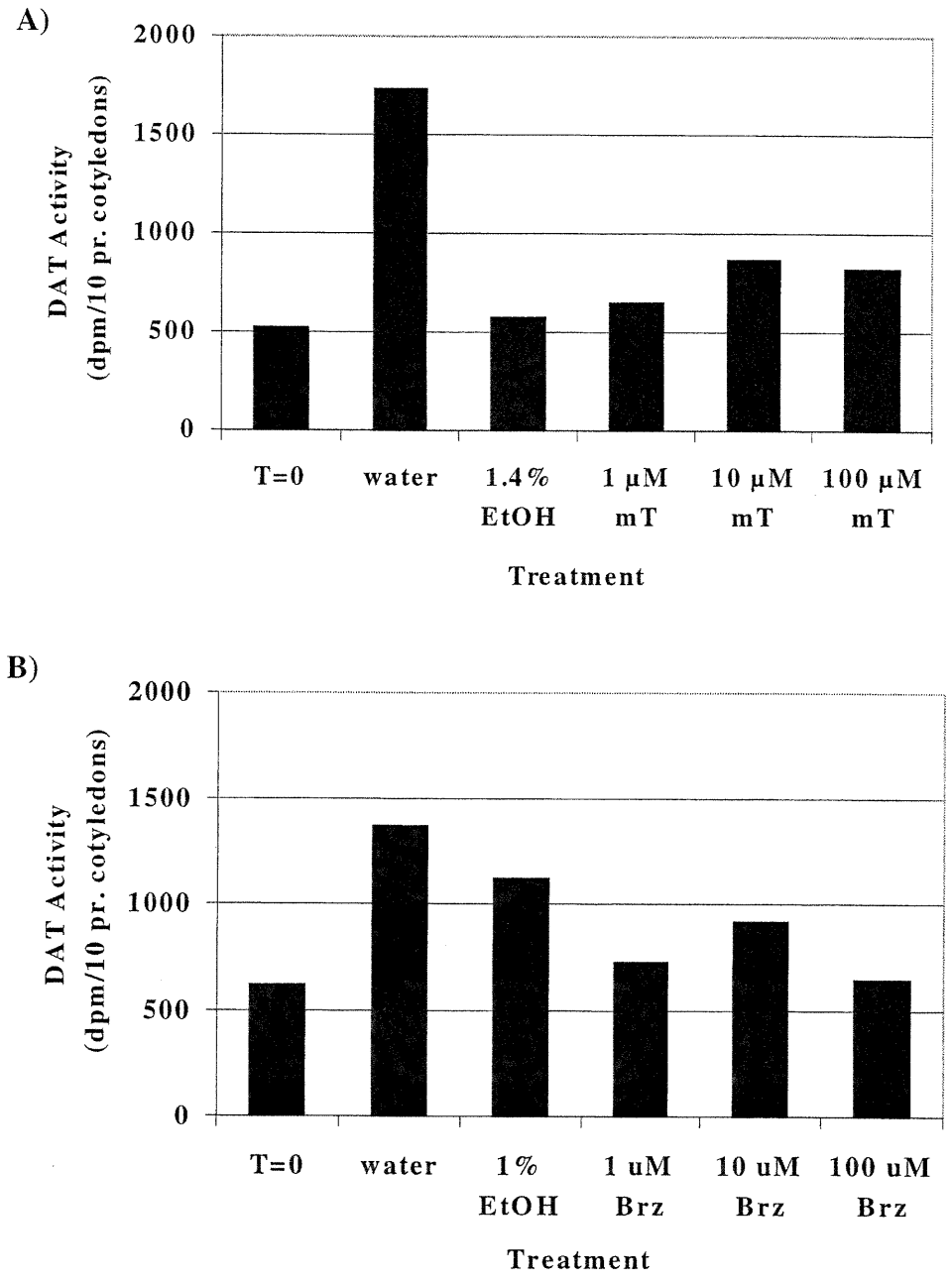


Figure 28. DAT activity in cotyledons of 5d-old etiolated seedlings incubated, in complete darkness, with various concentrations of **A)** the cytokinin, *meta*-topolin (mT) and **B)** the brassinosteroid biosynthesis inhibitor, brassinazole (Brz). Seedlings were incubated for 48 h prior to harvesting.

DAT activity. However, DAT activity was not induced by incubating etiolated seedlings in BRZ concentrations as high as 100 μM (Fig. 28B). Allowing BRZ-treated seedlings to continue growing in the dark for several more days did not result in any noticeable morphological differences as compared to control seedlings (data not shown).

D.5.3.3. Protein Kinase, Ca^{2+}/Calmodulin Inhibitors

D.5.3.3.1. Staurosporine (ST)

Young, 5d-old, etiolated *Catharanthus roseus* seedlings were vacuum infiltrated with staurosporine (ST), a broad spectrum protein kinase inhibitor, followed by a 2 h exposure to R light (*ca.* 20 μmol·mol^{-2}·s^{-1}) and a 24 h dark incubation in order to observe the effect of ST on the activation of DAT enzymatic activity as mediated by R light. Seedlings exposed to 10 and 100 nM ST showed a *ca.* 30% reduction in their DAT activity levels as compared to DMSO-treated controls (Fig. 29A). These preliminary results suggest that perhaps a (or several) protein kinase, which is a target of ST inhibition may act as a positive regulator of DAT activity in the signal transduction pathway involving R light and phytochrome.

D.5.3.3.2. Trifluoperazine (TFP)

Catharanthus roseus seedlings were incubated with trifluoperazine (TFP), a calcium/calmodulin (Ca^{2+} /CaM) antagonist, to observe whether Ca^{2+} is involved in the R light/phytochrome-mediated signalling pathway leading to the activation of DAT enzymatic activity. Treating 5d-old etiolated *Catharanthus* seedlings with

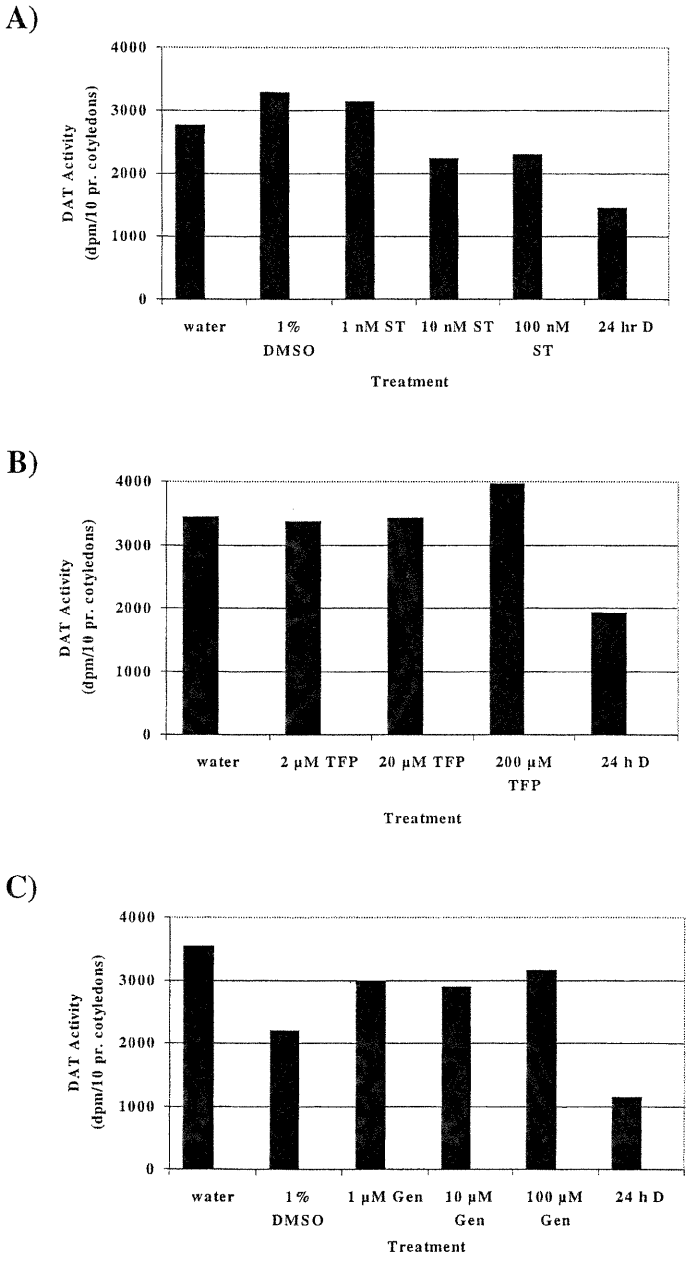


Figure 29. DAT activities in cotyledons of 5d-old etiolated seedlings incubated with various concentrations of the following protein kinase and Ca^{2+}/calmodulin inhibitors : **A)** staurosporine (ST), **B**) trifluoperazine (TFP), and **C**) genistein (Gen). Following vacuum infiltration, the seedlings were incubated for 30 min in complete darkness before being exposed to R (*ca.* 20 μmol·mol^{-2}·s^{-1}) for 120 min and returned to complete darkness for 24 h prior to sectioning and harvesting.

various concentrations of TFP resulted in maintaining a constant level of DAT activity at concentrations of 2 and 20 μM TFP, respectively, as compared to water-treated controls, while a *ca.* 15 % increase in DAT activity was observed with 200 μM TFP (Fig. 29B). These preliminary results suggest that TFP may possibly enhance DAT activity, by inhibiting a putative, Ca^{2+} /CaM-dependent negative regulator, as has previously been reported for light-mediated *CHS* gene expression in soybean cell cultures and tomato [Frohnmeyer *et al.* (1998)].

D.5.3.3.3. Genistein

Genistein, a tyrosine-histidine kinase inhibitor [Akiyama *et al.* (1987); Huang *et al.* (1992)], was tested for its effect on the R light/phytochrome-mediated induction of DAT activity in *Catharanthus* seedlings. Genistein-treated *Catharanthus* seedlings exhibited a *ca.* 44% increase in DAT activity, as opposed to DMSO-treated control seedlings (Fig. 29C), suggesting that DAT activity in *Catharanthus* may possibly be negatively regulated by a tyrosine-histidine kinase. It is interesting to note that DMSO inhibited DAT activity to some extent (Fig. 29C), whereas it stimulated DAT activity in the ST series of experiments (Fig. 29A).

E. Discussion

The Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) has been used extensively to study MIA chemistry and biochemistry. The initial studies of Noble *et al.* (1958), describing the presence of the anticancer drug VBL, paved the way for extensive efforts to describe the chemistry of *Catharanthus* and to synthesize these molecules in the laboratory. Several research groups also focused on the use of cell cultures to manufacture these important molecules. These studies resulted in a higher level of understanding of MIA biosynthesis, their metabolism and the factors which regulate this complex and elaborate pathway.

The inability of *Catharanthus* cell suspension cultures to accumulate the antineoplastic agents, VBL and VCR has been attributed to their inability to synthesize the vindoline component of these dimeric alkaloids [De Luca *et al.* (1985)]. Detailed biochemical studies have revealed that late stages of vindoline biosynthesis are regulated differently than those involved in the generation of the other major *C. roseus* alkaloids. For this reason, we have chosen to study the terminal deacetylvindoline 4-*O*-acetyltransferase (DAT) step in vindoline biosynthesis in developing seedling and plant tissues, in order to better understand the complex regulation of the late stages of this pathway.

E.1. Cloning and Characterization of DAT and MAT

Initial unsuccessful attempts to clone DAT involved screening a *C. roseus* cDNA expression library with a polyclonal antibody raised against the native DAT protein [Alarco (1994)]. Three families of genes were isolated which showed

homology to HSP70 and ferredoxin reductase while the third had no known homology. A second approach involved purification of DAT to homogeneity in order to obtain protein sequence information. Degenerate oligonucleotides were designed and synthesized based on the resulting tryptic peptide sequences obtained [Alarco (1994)] (Table II). A PCR-based approach to amplify DNA fragments and to screen *Catharanthus* cDNA and genomic librairies also proved unsuccessful, since no identifiable clones encoding DAT peptides were isolated [Alarco (1994)].

The successful cloning of DAT was finally achieved by using a combination of RT-PCR , 3'- and 5'-RACE (Figs. 6-7). This strategy, based on the method of Lingner *et al.* [1991], who successfully cloned a *Saccharomyces cerevisiae* poly (A) polymerase gene, involved PCR amplification of a DNA fragment known to encode a specific peptide sequence. In the case of DAT, primers based on the first and last 5 amino acids of peptide 54 yielded an expected 73 bp PCR product encoding the complete sequence of this peptide (Fig. 6). Two specific primers based on the internal sequence of the 73 bp PCR product were used in 3'RACE to isolate clone W7-1 which encoded a putative 75 amino acid sequence (Fig. 8A), containing the sequences to two DAT tryptic peptides (Fig. 8A, B) belonging to the carboxy terminal end of the DAT protein. A *Catharanthus roseus* cDNA library derived from poly(A) RNA isolated from 7 day old light grown seedlings, was screened with W7-1 to isolate an incomplete partial clone (A-3, Fig. 9), which was 81% identical to W7-1 (data not shown). These results suggested that clone A-3 was a homologue of DAT whose function remained to be characterized.

In order to isolate more clones, a *Catharanthus* genomic library was screened with W7-1 resulting in the isolation of four genomic clones (Fig. 10). Although the restriction digestions of the four clones were not entirely complete and the double digestions were not very efficient, Southern blotting and hybridization with W7-1 revealed that two (*gDAT*4 and *gDAT*16) of the four (*gDAT*4, *gDAT*6, *gDAT*15 and *gDAT*16) clones had similar restriction patterns (Fig. 10). Sequencing of the sub-cloned hybridizing fragments from the four genomic clones revealed that *gDAT*6 contained no introns within a 439 amino acid ORF and encoded 9 out of 10 peptide sequences found in the 33 and 21 kDa DAT subunits (Fig. 11; Table II). Clone *gDAT*15 contained an intronless 443 amino acid ORF which included the A-3 gene sequence (Fig. 12).

E.2. Biochemical and Kinetic Properties of Cloned Gene Products

The functional expression of clone *gDAT*6 in *E. coli* (Table II) established, unequivocally, that the expressed protein catalysed the terminal step in vindoline biosynthesis. In contrast, functional expression of clone *gDAT*15 in *E. coli* (Table II) established that the expressed protein probably catalyzed the *O*-acetylation of minovincinine to yield echitovenine (Fig. 13; Table IV), and was therefore named minovincinine-*O*-acetyltransferase (MAT).

DAT had previously been purified from *Catharanthus* leaves to yield a 33/21 kDa heterodimer [Power (1989); Power *et al.* (1990)], and a 26/20 kDa heterodimer [Fahn and Stöckigt (1990)] using a different purification scheme. It was therefore believed that the DAT protein was a heterodimer, as previously described for other

ATs such as isopenicillin *N* acyltransferase involved in penicillin G and V [Martín *et al.* (1994)] biosynthesis, and acetyl CoA:deacetylcephalosporin C-*O*-acetyltransferase which catalyzes the terminal reaction in the biosynthesis of cephalosporin C [Matsuda *et al.* (1992)]. However, the DAT gene encoded a single 439 amino acid protein with an estimated M_r of 50 kDa, which is in good agreement with the published M_r's of 54-56 kDa obtained by high performance gel filtration [Power (1989); Power *et al.* (1990); Fahn and Stöckigt (1990)]. This suggests that the large and small subunits of leaf-purified DAT could be derived from the cleavage of the N- and C-terminal end of the *DAT* gene product. The fact that the DAT antibody recognized a single mol. wt. band of *ca.* 50 kDa on SDS-PAGE confirmed that the cleavage of DAT, by endogenous proteases, was an artefact of the purification scheme [St-Pierre *et al.* (1998)].

The MAT gene shared 63% nucleic acid identity with the DAT gene and encoded a putative 443 amino acid protein with an estimated M_r of *ca.* 50 kDa, that shared 78% amino acid identity with DAT (Fig. 21). MAT-affinity purified DAT antibody also recognized a *ca.* 50 kDa protein, from root extracts, following SDS-PAGE and immunoblotting, as was reported earlier using DAT-affinity purified DAT antibody [see Fig. 7b in : St-Pierre *et al.* (1998)]. However, the results were difficult to reproduce, due to the low abundance of the MAT protein (data not shown).

Despite their high degree of amino acid identity, kinetic studies demonstrated the recombinant proteins (rMAT and rDAT) to be quite different with respect to their substrate specificities (Table IV) and catalytic efficiencies (V_{max}/K_m) (Table V). While rMAT appeared to be capable of catalyzing the *O*-acetylation of minovincinine and hörhammericine (Fig. 13) in addition to DAV (Table IV), rDAT only accepted

DAV as substrate (Table IV). Furthermore, the specificity constant of rDAT for acetyl CoA and DAV was approximately 240- and 10,000-fold greater than that of rMAT (Table V), demonstrating that rMAT is a poor acetyltransferase, particularly for alkaloid substrates related to minovincinine, as it also exhibited a low turnover rate with respect to hörhammericine (Table V). These findings clearly indicated the presence of two distinct tissue-specific acetyl CoA-dependent *O*-ATs in *C. roseus* that are responsible for the biosynthesis of a root-specific, 19-*O*-acetylated tabersonine derivative, echitovenine, and a leaf-specific 4-*O*-acetylated derivative, vindoline.

E.3. DAT and MAT are Regulated by Tissue-, Development- and Environment-Specific Controls

The distribution of DAT transcripts in leaves and petals (Fig. 17) corroborates earlier findings that DAT expression occurs only in above ground tissues, which also correlates with the distribution of DAT protein and enzymatic activity [St-Pierre *et al.* (1998)]. These results are also in agreement with earlier reports which demonstrated the leaf-specific distribution of enzymes responsible for catalyzing the conversion of tabersonine to vindoline [De Carolis *et al.* (1990); De Luca *et al.* (1985), (1987); St-Pierre and De Luca (1995); Vázquez-Flota *et al.* (1997)], which coincides with the sites of accumulation of these alkaloids [Balsevich *et al.* (1986); De Luca *et al.* (1986); De Luca *et al.* (1992)]. Taken together, these results clearly established that vindoline biosynthesis occurs within young leaf tissue of the Madagascar periwinkle. Alternatively, the localization of MAT transcripts and enzyme activity (Figs. 17-19) within young root tissue, hairy root tips, and radicles of etiolated seedlings, coincides with the accumulation of root-specific tabersonine derivatives such as lochnericine,

hörhammericine [Shanks *et al.* (1998)], echitovenine and minovincinine [Cordell and Farnsworth (1976)], and helps to explain how these alkaloids are made.

The localization and expression patterns of *TDC* [St-Pierre *et al.* (1999)], *STR*1 [St-Pierre *et al.* (1999)] and *MAT* within cortical cells near the root tip (Fig. 19), suggests that the whole pathway leading to tabersonine and its O-acetylated derivatives may be expressed in these cells. In contrast, it remains to be shown if the epidermis of above ground plant parts, which is the other primary site of *TDC* and *STR1* expression [St Pierre *et al.* (1999)], are also sites of tabersonine biosynthesis. These results raise the possibility that tabersonine or some modified derivative (16-methoxytabersonine) produced in cortical root cells is transported to above ground laticifers for the subsequent 6 or 4, respective enzymatic steps for vindoline formation. The recent cloning and characterization of tabersonine 16-hydroxylase from Madagascar periwinkle cell cultures [Schröder *et al.* (1999)] and the presence of a tabersonine 16-*O*-methyltransferase in cell cultures suggest that 16-methoxytabersonine could be produced in underground tissues. In addition, the presence of enzymes like MAT in the same root cortical cells, suggest that the amount of tabersonine transported to the laticifers may be limited by sequestration of this metabolite into lochnericine, hörhammericine, echitovenine and minovincinine. It is presently unclear why *Catharanthus* roots accumulate such alkaloids or if they have any biological role.

Catharanthus hairy root cultures, which appear to be more stable than cell cultures, have recently been investigated for their ability to produce indole alkaloids. Hairy roots accumulate tabersonine, lochnericine and hörhammericine (Fig. 5) in

addition to serpentine and ajmalicine [Rijhwani and Shanks (1998); Shanks *et al.* (1998)]. Roots isolated from the plant also accumulate the same types of corynanthe [El-Deeb *et al.* (1957)], iboga [Svoboda *et al.* (1963)] and aspidosperma [Nair and Pillay (1959)] alkaloids, but not vindoline. The possible presence of the tabersonine pathway, as well as MAT in roots may explain how alkaloids like 19-hydroxytabersonine [Kutney *et al.* (1980)], lochnericine, hörhammericine [Shanks *et al.* (1998)] and echitovenine [Cordell and Farnsworth (1976)] are made.

Low levels of vindoline were recently reported to accumulate in hairy root cultures transformed with *Agrobacterium rhizogenes* [O'Keefe *et al.* (1997)]. In addition, suspension cultures established after leaf disc transformation with either *Agrobacterium tumefasciens* or *A. rhizogenes*, accumulated catharanthine as well as low levels of vindoline and also showed a deacetylvindoline *O*-acetyltransferase activity, which catalyzes the last step in vindoline biosynthesis [Bhadra *et al.* (1993); O'Keefe *et al.* (1997)]. The hairy root cultures in our laboratory, which do not make vindoline, obtained from Vázquez-Flota *et al.* (1997), were shown to have MAT enzyme activity. However, the kinetic studies performed with rMAT show that this enzyme will catalyze the 4-*O*-acetylation of DAV and strongly suggests that the enzyme described in [Bhadra *et al.* (1993)] may be MAT rather than DAT. On the other hand, the reported DAT activity in transformed shooty teratoma cultures [O'Keefe *et al.* (1997)], may likely be due to an active vindoline pathway and DAT activity occurring specifically in the shooty component of the teratoma. *In situ* localization studies as performed by St. Pierre *et al.* (1999) might be useful to clarify which part of the teratoma expresses the DAT activity.

The existence of biosynthetic steps capable of transforming tabersonine in root tissue, may also suggest that all the tabersonine being produced in root cortical cells is converted into lochnericine, hörhammericine, echitovenine and minovincinine (Fig. 5), rather than being transported to above ground laticifers for the production of vindoline. The exclusive localization and expression of *TDC* and *STR*1 within above ground epidermal tissue [St-Pierre *et al.* (1999)], in addition to the specific localization of *D4H* and *DAT* within laticifers and idioblasts of leaves [St-Pierre *et al.* (1999)] suggests that the epidermis might be the alternative site of tabersonine biosynthesis. This intermediate would then be transported by an unknown mechanism to the idioblasts and laticifers for further elaboration into vindoline. Although fluorescence microscopy clearly displayed a network of laticifer cells associated with the vasculature within leaf tissue [St-Pierre *et al.* (1999)], supporting the idea of a root-to-leaf transport of tabersonine, it remains difficult to explain how tabersonine might be transported from epidermal cells to laticifers. However, leaf idioblasts, which are more closely associated with the epidermis might be connected in some undetermined manner to allow transport of the tabersonine intermediate to idioblasts for further elaboration to yield vindoline. In this context it would be highly informative to determine the alkaloid composition of epidermis, idioblasts, laticifers and other cells within the leaf, in order to address some of the above questions.

E.4. Regulation of *MAT* and *DAT* Gene Expression

The light induced gene expression of DAT along with the distribution of DAT protein and enzymatic activity within young leaf tissue, and more particularly within

laticifers and idioblasts of leaf tissue, clearly demonstrate the tissue- and development-specific controls that regulate vindoline biosynthesis [see results in: St-Pierre *et al.* (1999)]. Similarly, maximal expression of MAT enzyme activity occurred in 4d- to 5d-old radicles of etiolated seedlings (Fig. 18C), approximately 24 to 48 h after the optimal levels of transcript accumulation were achieved (Fig. 18A). These results, together with the cortical cell localization near the root tip of *mat* transcripts (Fig. 19) demonstrate that MAT gene expression is restricted to root tissue and is developmentally regulated.

The distribution patterns for DAT transcripts, protein and enzyme activity which closely reflect those found for D4H [Vazquez-Flota *et al.* (1997)], suggest that perhaps these two enzymes share similar mechanisms of regulation. The occurrence of laticifers containing immunoreactive D4H protein within young, etiolated cotyledons, has revealed that despite their presence, light is essential for inducing D4H activity [Vázquez-Flota *et al.* (2000)]. These reported findings suggest that in addition to being regulated developmentally, light is essential and is thought to interact with factors present within laticifers that activate D4H activity.

Unlike the root-specific pathways (Fig. 5), which occur in the dark, the late stages of vindoline biosynthesis in developing seedlings (Fig. 4) have been shown to be light-regulated [Aerts and De Luca (1992); De Carolis (1994); De Luca *et al.* (1986), (1988); St-Pierre and De Luca (1995); Vázquez-Flota *et al.* (1997), (2000); Vázquez-Flota and De Luca (1998a)]. The time-dependent accumulation of DAT transcripts, protein and enzyme activity, in light-exposed seedlings, suggests coordinate regulation of DAT [see Fig. 8 in St-Pierre *et al.* (1998)]. A previous report

has also shown that induction of DAT activity by light is mediated by phytochrome [Aerts and De Luca (1992)]. Exposing young etiolated seedlings to far-red light reversed the inducing effect of red light (Fig. 27C) [Aerts and De Luca (1992)], as had been demonstrated with D4H [Vázquez-Flota and De Luca (1998a); Vázquez-Flota *et al.* (2000)], suggesting that these two enzymes share similar, phytochrome-dependent, mechanisms of regulation. The presence of low levels of DAT activity observed in young etiolated seedlings (Fig. 27), before any exposure to light, suggests that the system is 'leaky'. Furthermore, the low levels of DAT activity observed as a result of red light exposure, as compared to white light-exposed seedlings (Fig. 27), suggests that other factors such as blue light and/or phytohormones, may be involved in the light-mediated induction of DAT activity.

Cytokinins (CKs) have been reported to be involved in the physiological processes associated with light-controlled plant/seedling development as well as light-induced gene expression [see review by: Thomas *et al.* (1997) and references therein]. In particluar, CKs have been shown to mimic the effect of red light on chloroplast development and gene expression [see review by : Thomas *et al.* (1997) and references therein]. In particluar, studies carried out with *Arabidopsis* seedlings have shown that small doses of CKs caused de-etiolation of dark-grown wild-type (WT) seedlings, a phenotype similar to that observed in the *det1* and *det2* mutants [Chory *et al.* (1994)]. The more recent cloning and characterization of a tomato 'high pigment' mutant (*hp-2*), a homolog to the *Arabidopsis det1* mutant, established that in tomato, CK can more precisely phenocopy an *hp* mutant rather than the *Arabidopsis det1* phenotype [Mustilli *et al.* (1999)]. Further evidence demonstrating

the effect of CKs on light-activated gene expression and light-induced development was obtained from the characterization of a high-CK *Arabidopsis* mutant, *amp1*, [Chaudhury *et al.* (1993)] displaying de-etiolated characteristics in the dark [Chin-Atkins *et al.* (1996)]. This study revealed the ability of CKs to mimic the *amp1* phenotype by growing WT seedlings, in the dark, on cytokinin-containing medium. In contrast, CKs may indirectly mediate the action of light on hypocotyl elongation, in *Arabidopsis*, as a result of CK-induced ethylene production [Cary *et al.* (1995)] and the effects of CKs and light on hypocotyl elongation may be independent and additive [Su and Howell (1995)].

Exposure of dark-grown *Amaranthus caudatus* cotyledon-hypocotyl explants to benzyladenine, zeatin or *meta*-topolin (mT) [Holub *et al.* (1998)] activated betacyanin synthesis. In contrast, exposing young etiolated *Catharanthus* seedlings to mT, a highly active aromatic cytokinin from poplar [Strnad *et al.* (1997); Holub *et al.* (1998)] did not substitute for the red- (Fig. 27A) or white light- (Fig 27D) mediated induction of DAT activity. However, the lack of a positive control makes these initial experiments inconclusive, as it is not clear if the cytokinins were perceived by the plant tissues.

In addition to CKs, brassinosteroids (BRs) are also involved in light-mediated plant development and gene expression. Direct evidence for this was obtained when *epi*-brassinolide (BL) rescued the dark grown *Arabidopsis det2* (de-etiolated) phenotype to re-establish etiolated type growth [Li *et al.* (1996)]. This gene encodes a 5α-reductase that is involved in the BR biosynthesis [Li *et al.* (1996); Fujioka *et al.* (1997)]. Furthermore, dark-grown *det2* mutants expressed increased levels of light-

induced chalcone synthase activity and accumulated anthocyanins [Chory *et al.* (1991)], illustrating the involvement of BRs in repressing some light-regulated processes.

Our preliminary studies indicate that BRs may not be involved in the light-mediated activation of DAT enzyme activity. *Catharanthus* seedlings treated with brassinazole (BRZ), a cytochrome P450 inhibitor of BR biosynthesis [Asami and Yoshida (1999); Min *et al.* (1999); Asami *et al.* (2000)], in the dark, did not result in any induction of DAT activity (Fig. 26B).

The inability to mimic the effect of light by mT and BRZ on inducing DAT activity in young etiolated *Catharanthus* seedlings (Fig. 28) should not be disregarded but rather pursued with greater intensity, as the complex mechanism regulating DAT activity depends on the developmental and environmental controls. Similarly, the effects of the broad-spectrum protein kinase inhibitor, staurosporine (ST) (Fig. 29A), the calcium/calmodulin inhibitor, trifluoperazine (TFP) (Fig. 29B), and the tyrosine-histidine kinase inhibitor, genistein (GEN) (Fig. 29C) activity were negative, but these experiments should be repeated taking the developmental stage of the seedling into consideration. Although the results are preliminary, the slight increase in DAT activity as a result of TFP and GEN treatment (Fig. 29B and C, respectively) suggests that perhaps DAT is negatively regulated by a calcium/calmodulin-dependent factor as well as a tyrosine/histidine kinase, respectively, requiring further investigation.

E.5. DAT and MAT Help Define a New Family of Acyltransferases in Plants

A protein database search (BLAST) with the deduced amino acid sequence of the MAT ORF revealed significant similarity to twenty-one plant proteins (Fig. 22).

Among the several proteins showing similarity to both MAT and DAT were : acetyl-CoA :benzylalcohol acetyltransferase (BEAT) which catalyzes the formation of benzylacetate, a major constituent of the floral scent from *Clarkia* [Dudareva *et al.* (1998)]; 10-deacetylbaccatin III-10-*O*-acetyltransferase responsible for catalyzing the formation of baccatin III, the last diterpene intermediate in taxol biosynthesis in *Taxus cuspidata* [Walker and Croteau (2000)]; anthranilate *N*-benzoyltransferase from carnation which is involved in the formation of the dianthramide phytoalexins [Yang *et al.* (1998)]; a hypersensitivity-related protein, Hsr 201, from tobacco, which is related to a tomato protein whose gene is expressed during fruit maturation [Czernic *et al.* (1996)]; and anthocyanin 5-aromatic acyltransferase from *Gentiana triflora* possibly involved in flower colour development [Fujiwara *et al.* (1998)]. All of the proteins outlined in figure 22 maintain a perfect conservation of histidine, aspartate, and to some extent glycine residues, within the HXXXDG domain, which is believed to compose the active sites of MAT and DAT. Regions with the highest conservation appear to be at the N-terminal portion of MAT and DAT, which also corresponds to the dihydrolipoyl *S*-acetyltransferase (DHLAAT) catalytic domain [Reed and Hackert (1990)]. There are also discrete regions of high similarity in the C terminal regions of all the plant proteins, particularly the phenylalanine, glycine and to some extent trytophan residues within the DFGWGKP motif (Fig. 22, residues 384-390 of MAT and residues 380-386 of DAT; and Fig. 21) which are highly conserved in most of these acyltransferases.

The MAT gene along with DAT therefore belong to a large family of acyltransferases with a putative active site related to chloramphenicol acetyl-

transferase (CAT) [Shaw and Leslie (1991)] and DHLAAT gene families [Reed and Hackert (1990)]. The recent cloning and characterization of a strawberry alcohol acyltransferase (SAAT), responsible for flavour biogenesis in ripening fruit, also contains the highly conserved HXXXDG and DFWGKP domains as well as a newly identified and highly conserved 13 amino acid domain (LSXTLXXXYXXXG) (corresponding to residues 70-82 in MAT and residues 69-81 in DAT; Fig. 21) [Aharoni *et al.* (2000)]. In addition, eight residues (LSETLTLY) of this third domain also occur in the *Chlorella vulgaris* carboxyl transferase β subunit of acetyl-CoA carboxylase (Genbank accession number : BAA57908). This enzyme, which catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate, suggests that this third domain may also be important in reactions using acetyl-CoA as cosubstrate. Members of these gene families which belong to the 'BAHD' superfamily of acyltransferases, appear to be particularly abundant in plants and display similar M_r's, as well as these 3 conserved amino acid domains (Fig. 22) [Reed and Hackert (1990); Shaw and Leslie (1991); also see review by : St-Pierre and De Luca (2000) and references therein].

The MAT protein sequence contains an extra five amino acids (MENVD) compared to DAT (Fig. 21), which we speculate may in part be responsible for the substrate specificity and kinetic property differences of these two enzymes. Site-specific mutagenesis and enzyme activity measurements could be useful to determine if these 5 amino acids play such roles. The HXXXDG active site motif contains a HIS residue (Fig. 21 and 22, respectively), that may bind acetyl CoA, according to studies carried out with a number of CAT-related ATs (see review by: St-Pierre and De Luca

(2000) and references therein). Partial inactivation of both MAT and DAT with diethylpyrocarbonate (DEPC) (Fig. 23A and 23B), a histidine modifying agent [Miles (1977)], and the subsequent partial protection from inactivation by acetyl CoA suggest that a histidine residue is involved in the catalytic activities of MAT (Fig 23D) and DAT (Fig. 23C) [St-Pierre *et al.* (1998)], as well as for other related *O*-ATs [see review by : St-Pierre and De Luca (2000) and references therein]. Moreover, these results support the hypothesis that the DEPC modified residue is at or near the active site of these enzymes. The reactivation of DAT [Power (1989)] and MAT (data not shown) enzyme activity with the sulfhydryl protecting reagent, DTT, suggests that reduced sulfhydryl groups are also required for activity.

F. Summary

The cloning and characterization of the DAT and MAT genes revealed the presence of two distinct acetyl CoA-dependent *O*-acetyltransferases in *Catharanthus roseus*, which shared a high degree of similarity in their nucleic and amino acid sequences. The developmentally regulated expression of DAT and MAT was shown to occur within particular cells in different tissues, while the light induction of DAT depended upon a phytochrome-influenced mechanism. DAT and MAT exhibited their greatest differences with respect to their location within the plant, their substrate specificities, and their kinetic parameters.

These studies, which improve our understanding of the complex regulation of vindoline biosynthesis in the Madagascar periwinkle, also provide the necessary tools to gain further understanding of indole alkaloid biosynthesis. The cloning of the DAT and MAT genes, as well as the TDC, STR1 and D4H genes has made it possible, through *in situ* localization studies (Fig. 19B) [St-Pierre *et al.* (1999); Vázquez-Flota and De Luca (2000)] to obtain detailed knowledge of cell biological processes required for vindoline biosynthesis.

The alignment of MAT and DAT with 21 related *O*-acyltransferases, obtained from the BLAST protein database, revealed the presence of two highly conserved motifs, HXXXDG and DFGWGKP (Figs. 21-22). The histidine residue contained in the former motif is thought to be involved in binding the acetyl-CoA co-substrate, as demonstrated by its ability to reverse the inactivation incurred by DEPC (Fig. 23C and D). Furthermore, the presence of these domains in MAT and DAT, demonstrates their relatedness to the chloramphenicol acetyltransferase and dihydrolipoyl *S*-

acetyltransferase gene families. In addition, the database results help define a new 'super' family of plant-derived 'BAHD' acyltransferases [St-Pierre *et al.* (2000)].

The availability of compounds such as the brassinosteroid biosynthesis inhibitor, brassinazole [Asami and Yoshida (1999)] should make it possible to gain further detailed knowledge regarding the possible involvement of brassinosteroids in regulating indole alkaloid biosynthesis. Furthermore, the studies carried out to identify the secondary messengers involved in the light-mediated regulation of anthocyanin biosynthesis, and more specifically chalcone synthase [Bowler *et al.* (1994); Frohnmeyer *et al.* (1998)] can serve as a basis for directing further studies towards deciphering the signal transduction pathways and the secondary messengers involved in the light/phytochrome-mediated regulation of the last two steps in vindoline biosynthesis.

G. References

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H. Appendix I. Summary of Purification of Recombinant DAT and MAT Proteins

Recombinant proteins were purified as described in Material and Methods sections C.2.3. and C.2.4. Fractions of 1 mL were collected and the highest DAT activities eluted in the second half of the gradient (fractions 16 to 30). A 500 mL bacterial suspension yielded 1.53 mg of *ca.* 80% pure recombinant DAT protein as determined by SDS-PAGE (Fig. 30). The recombinant DAT protein was purified 152-fold from PD-10 desalted crude extracts, with a specific activity of 88.65 pkat/mg protein (Table VI).

The purification of recombinant MAT protein was carried out in a similar manner, whereby 1 mL fractions were collected and the highest MAT activity-containing fractions eluted in the middle of the gradient (fractions 10 to 21). A 500 mL bacterial suspension yielded 6.89 mg of *ca.* 80% pure recombinant MAT protein as determined by SDS-PAGE (Fig. 31). The recombinant MAT protein was purified 24-fold from PD-10 desalted crude extracts, with a specific activity of 119.85 pkat/mg protein (Table VII).

The purified proteins were stored at -20° C in elution buffer until required for further analyses.

High percentage recoveries for both recombinant DAT and MAT proteins (Tables VI and VII) may possibly be due to: (i) strong activation of each protein as a result of purification, (ii) the elimination of an inhibitor which would account for the low activities in the crude extracts and/or (iii) the elimination of esterases, for example, which may compete with DAT and MAT for acetyl CoA.

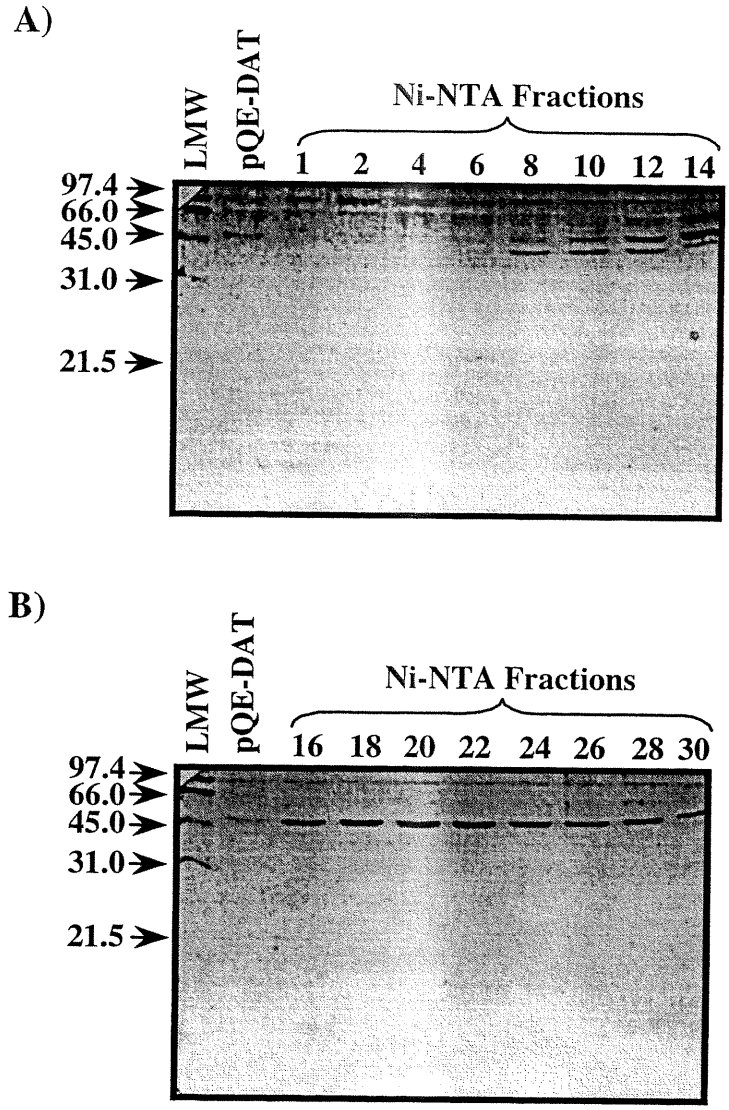

Figure 30. SDS-PAGE of purified recombinant DAT protein (pQE-DAT) using nickel-nitrilotriacetic acid metal affinity (Ni-NTA) chromatography. Proteins were stained with Coomassie blue R-250. The number to the left of the figures correspond to the molecular weights of the low molecular weight standards (**LMW**) in kDa. Samples of *ca.* 0.70 μg protein were loaded per lane, on a 10% (w/v) SDS gel. Sample **pQE-DAT** corresponds to the PD-10 desalted fraction before Ni-NTA chromatography. Numbers correspond to Ni-NTA-eluted fractions : **A)** fractions 1 to 14 and **B)** fractions 16 to 30. Fractions 16-30 were pooled and used for subtrate specificity and kinetic characterization studies of DAT.

Table VI. Summary of purification of recombinant DAT protein.

Purification Step	Total Protein (mg)	Specific Activity[c] (pkat/mg protein)	Total Activity (pkat)	Purification Fold	Percent Recovery (%)
PD-10[a]	21.50	0.58	12.53	1	100
Ni-NTA affinity[b]	1.53	88.65	135.23	152	1079

[a]As a standard procedure, protein was first assayed for enzyme activity following desalting on PD-10 columns, as opposed to prior to chromatography on PD-10.

[b]Ni-NTA affinity chromatography was carried out, as described in Material and Methods, section C.2.4.

[c]Enzyme assays were carried out as described in Material and Methods, section C.4.2.1., using *ca.* 215 μg desalted recombinant DAT protein and incubating for 20 min at 37° C. Recombinant DAT protein was assayed using 20 μM deacetylvindoline as substrate. Assays carried out using Ni-NTA affinity purified protein contained *ca.* 7.2 μg of recombinant DAT protein.

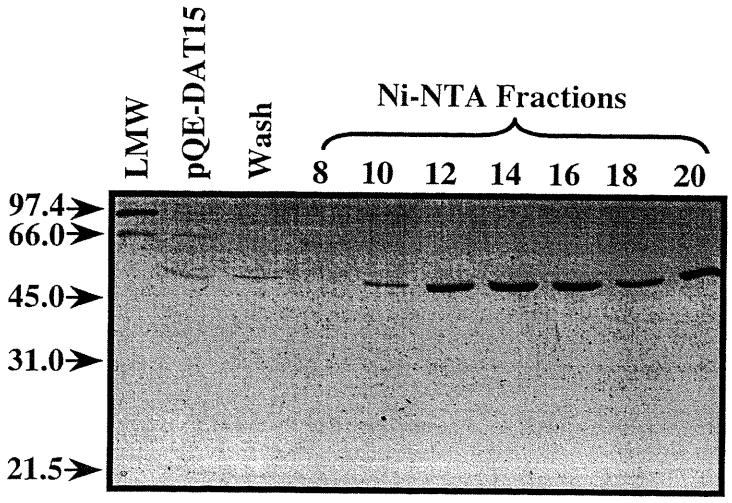

Figure 31. SDS-PAGE of purified recombinant MAT protein (pQE-DAT15) using nickel-nitrilotriacetic acid metal-affinity (Ni-NTA) chromatography. Proteins were stained with Coomassie blue R-250. The numbers to the left of the figure correspond to the molecular weights of the low molecular weight standards (**LMW**) in kDa. Samples of *ca.* 2.5 μg protein were loaded per lane, on a 10% (w/v) SDS gel. Sample **pQE-DAT15** corresponds to the PD-10 desalted fraction before Ni-NTA chromatography and the **wash** sample corresponds to the eluate fraction during a 5mM imidazole wash step following sample application on the column (see section C.2.4. of Material and Methods). Numbers correspond to Ni-NTA-eluted fractions. Fractions 10-21 were pooled and used for substrate specificity and kinetic characterization studies of MAT.

Table VII. Summary of purification of recombinant pQE-DAT15 protein.

Purification Step	Total Protein (mg)	Specific Activity[c] (pkat/mg protein)	Total Activity (pkat)	Purification Fold	Percent Recovery (%)
PD-10[a]	38.5	0.73	28.08	1	100
Ni-NTA affinity[b]	6.89	17.39	119.85	24	427

[a]As a standard procedure, protein was first assayed for enzyme activity following desalting on PD-10 columns, as opposed to prior to chromatography on PD-10.

[b]Ni-NTA affinity chromatography was carried out, as described in Material and Methods, section C.2.4.

[c]Enzyme assays were carried out as described in Material and Methods, section C.4.2.1., using *ca.* 385 μg desalted recombinant DAT15 protein and incubating for 20 min at 37° C. Recombinant DAT15 protein was assayed using 20 μL hairy root alkaloid as substrate. Assays carried out using Ni-NTA affinity purified protein contained *ca.* 32 μg of recombinant DAT15 protein.

H. Appendix II. TLC Solvent Systems Used to Resolve the Identity of the Reaction Product From a pQE-DAT15-Catalyzed Reaction

The reaction products from pQE-DAT15 (rMAT)-catalyzed reactions using root or hairy root alkaloid extracts as a source of substrate were isolated and submitted to TLC as described in Material and Methods section C.4.2.1. TLC was carried out using four solvent systems (see Table VIII), specific for resolving minovincinine [Cordell and Farnsworth (1976)], in order to resolve the identity of the MAT reaction product. Autoradiography on X-ray film was performed following TLC and in each solvent system, the major radioactive band co-chromatographed with the authentic echitovenine standard (Table VIII).

Table VIII. TLC and autoradiography of reaction products from a pQE-DAT15-catalyzed reaction.

	R_f value		
Solvent System[a]	**Vindoline Std.**	**Echitovenine Std.**	**Reaction Product[b]**
A	0.42	0.66	0.66
B	—	0.34	0.34
C	0.44	0.51	0.51
D	0.40	0.67	0.67

[a]A — ethyl acetate:methanol (9:1); B — 1-butanol:acetic acid:water (10:1:1); C — benzene:triethylamine (9:1); D — ethyl acetate:absolute ethanol (3:1)

[b]Reaction product obtained from pQE-DAT15-catalyzed reaction using either hairy root or root alkaloid extract as substrate. Reaction product was co-chromatographed with echitovenine reference standard.