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Molecular regulation of desacetoxyvindoline 4-hydroxylase. A 2-oxoglutarate-dependent dioxygenase involved in the biosynthesis of vindoline in *Catharanthus roseus*

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Molecular regulation of desacetoxyvindoline 4-hydroxylase. A 2-oxoglutarate-dependent dioxygenase involved in the biosynthesis of vindoline in *Catharanthus roseus*

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Abstract.

The second to last step in vindoline biosynthesis is catalyzed by deacetoxyvindoline 4-hydroxylase (D4H) which converts desacetoxyvindoline into deacetylvindoline. This enzyme belongs to the family of 2-oxoglutarate dependent dioxygenases which require 2-oxoglutarate, ascorbate, ferrous ions, molecular oxygen and alkaloid substrate for activity.

Sequencing of D4H cDNA and genomic clones revealed that D4H shares up to 30% amino acid identity with other dioxygenases of plant and fungal origin. These include ethylene forming enzyme from tomato, hyoscyamine 6ß-hydroxylase from *Hyoscyamus niger* and isopenicillin *N* synthase from *Aspergillus niger*, among others. The similarity between D4H and other dioxygenases was also noticed at the level of gene structure. The *d4h* gene contains two introns located at identical positions as those of two other plant dioxygenase genes and suggests they may have evolved from a common ancestral gene. Southern blot analysis showed that the *d4h* gene occurs as a single copy in the *Catharanthus* genome.

Tissue-specific expression of D4H appears to be transcriptionally regulated since the levels of D4H transcripts and enzyme activity strictly correlate with vindoline accumulating tissues. The highest levels of D4H expression occurred in leaves, with much lower levels present in stems and fruits, whereas there was no expression of D4H in roots.

Expression of D4H in developing seedlings was affected by light- and development-specific controls which are regulated by transcriptional, post-transcriptional and post-translational factors. Low levels of D4H enzyme activity were detected in etiolated seedlings, even when important amounts of the *d4h* transcript were detected. Upon illumination enzyme activity was increased 4- to 8-fold depending on the

developmental stage at which the seedlings were exposed. Light-mediated enzyme activation was more pronounced with 5 day old etiolated seedlings than with seedlings at older developmental stages. These results correlated with a noticeable increase of D4H immunoreactive protein with light treatment of 5 day old etiolated seedlings, rather than with clear changes in the level of *d4h* transcripts. In contrast, light treatment of older etiolated seedlings resulted in a less pronounced accumulation of D4H protein and a clear accumulation of *d4h* transcripts. Further studies showed that exposure of *Catharanthus* seedlings to light promoted the appearance of a new and slightly more acidic isoform of the D4H protein (pI 4.6), and the dissapearence of the more basic D4H isoform (pI 4.7) found in etiolated seedlings. Furthermore, the light effects on D4H appear to involve the phytochrome photoreceptor, since a 30 min red light pulse was sufficient to produce the same response as a 24 h period of continuous white light illumination and the response could be abolished with a far red light treatment.

The external application of methyl-jasmonate, a known chemical inducer of secondary metabolism, caused an 80% increase of the levels of D4H enzyme activity in light-grown *Catharanthus* seedlings. However, no induction of this activity could be observed in etiolated seedlings exposed to jasmonate, suggesting that this chemical inducer could not replace the light requirement for D4H activation and for vindoline biosynthesis. Furthermore, application of this inducer did not alter the developmental regulation of D4H in relation to earlier stages of vindoline biosynthesis.

Studies concerning the distribution of tryptophan decarboxylase (TDC) and D4H transcripts, protein and enzyme activity throughout different tissues of mature plants, revealed that young and actively growing tissues are the main sites of vindoline and general indole alkaloid biosynthesis. In addition, studies within the leaf tissues clearly showed that TDC and D4H are preferentially expressed at the base of the leaf and decrease progressively towards the leaf tip.

Immunocytochemical studies were used to identify which cells in *Catharanthus* leaves were involved in indole alkaloid biosynthesis. TDC was exclusively localized in the upper and lower epidermis of leaves and in the epidermis of stems, whereas it was spread throughout cortical cells near the root tip. In constrast, D4H was clearly localized only in laticifers and idioblasts of leaves and stems, whereas it was not detected in any root cells. A similar cellular distribution for both TDC and D4H was also found in cotyledons of developing seedlings and no differences in the cellular distribution of D4H were found in cotyledons of dark or light grown seedlings.

Key words: 2-oxoglutarate-dependent dioxygenase, alkaloid metabolism, *Catharanthus roseus*, cell-specialization, desacetoxyvindoline 4-hydroxylase, developmental regulation, light regulation, secondary metabolism, tryptophan decarboxylase, vindoline biosynthesis

Resumé.

La vinblastine est un alcaloïde cytotoxique qui est isolée des feuilles de la pervenche de Madagascar (*Catharanthus roseus*). Cet alcaloïde indole dimérique est formé par le couplage oxidatif de deux monomères, la catharanthine et la vindoline (Svoboda et Blake, 1975). La vinblastine ainsi qu'un alcaloïde apparenté, la vincristine, sont utilisées pour le traitement de la maladie de Hodgkin, et pour certaines formes de leucémie, respectivement (Johnson et coll., 1960). Étant donné l'importance pharmaceutique et la valeur marchande élevée de ces alcaloïdes dimériques, les technologies de cultures in vitro de suspension cellulaire de *Catharanthus* ont été développées afin de fournir des sources alternatives de production de ces substances naturelles. Certaines lignées cellulaires produisant des niveaux élevés de catharanthine ont été obtenues, cependant, aucune des suspensions cellulaires ne produit la vindoline (Moreno et coll., 1995; van der Heijden et coll., 1989).

La vindoline est l'alcaloïde majeur des feuilles des plantes matures ainsi que des cotylédons des plantules de *Catharanthus roseus*. Cet alcaloïde est élaboré par une séquence de réactions enzymatiques longues et complexes à partir de précurseurs provenant des voies de l'acide shikimique et de l'acide mévalonique.

La phase initiale de la biosynthèse de la vindoline comprend la formation de tryptamine à partir du tryptophane, et la condensation de la tryptamine avec un glycoside iridoïde, la sécologanine, qui produit l'intermédiaire central, la strictosidine. Ces réactions sont catalysées respectivement par les enzymes tryptophane décarboxylase (TDC) et strictosidine synthétase (SS), respectivement. Des études avec des plantules en développement ont toutefois indiqué que l'apparition des activités TDC et SS ne coïncide pas avec la phase d'accumulation de la vindoline (Alvarez-Fernandez et coll., 1989; De Luca et coll., 1986). En fait, ces étapes biosynthétiques initiales

précèdent l'apparition, au cours du développement des plantules, de la phase terminale de la biosynthèse.

La phase finale de la biosynthèse de la vindoline inclu une séquence ordonnée de six réactions enzymatiques qui sont responsables de la transformation du précurseur tardif, la tabersonine. Cette séquence enzymatique comprend l'hydroxylation aromatique en C-16, la 16-*O*-méthylation, l'hydration de la double liaison C2-C3, la *N*(1)-méthylation, l'hydroxylation en C-4, et la 4-*O*-acétylation (Fig. 1; De Luca et coll., 1986). La première réaction est catalysée par la tabersonine-16-hydroxylase (T16H), une monooxygénase dépendante du cytochrome P-450 et associée avec la fraction microsomale. Par la suite, une *O*-méthylation qui semble de nature cytosolique est catalysée par l'AdoMet:16hydroxytabersonine O-méthyltransférase (16-OMT; St-Pierre et De Luca, 1995). Après l'hydration de la double liaison C2-C3, la réaction suivante est catalysée par l'AdoMet:2,3-dihydro-3-hydroxytabersonine-*N*-méthyltransférase (NMT) associé aux membranes des thylacoïdes (De Luca et coll., 1987; Dethier et De Luca, 1993), tandis que la 4-hydroxylation est catalysée par une dioxygénase soluble dépendante du 2-oxoglutarate, la désacetoxyvindoline 4-hydroxylase (D4H; De Carolis et coll., 1990; De Carolis et De Luca, 1993). Enfin, la dernière étape de la biosynthèse de la vindoline est catalysée dans le cytosol par l'acétyl-CoA: déacetylvindoline *O*-acétyltransferase (DAT; De Luca et coll., 1985; Power et coll., 1990).

Les cultures de cellules en suspension possèdent les activités enzymatiques T16H et 16-OMT (St-Pierre et De Luca, 1995), mais sont déficientes en enzymes qui catalysent les trois dernières étapes de la biosynthèse de la vindoline (De Carolis et coll., 1990; De Luca et coll., 1987). Cela explique l'absence dans les cultures cellulaires de la vindoline et de ces précurseurs immédiats, la désacetoxyvindoline et la déacetylvindoline (Kurz et coll., 1980; Kutney et coll., 1980).

De manière similaire, les plantules de *C. roseus* cultivées à l'obscurité accumulent des niveaux élevés de tabersonine et des traces d'autres intermédiaires

ultérieurs à la tabersonine ainsi que de faibles quantités de vindoline (Balsevich et coll., 1986; De Luca et coll., 1986). Par contre, l'exposition de plantules étiolées à la lumière induit une accumulation des activités enzymatiques de la D4H et de la DAT dans les cotylédons (De Carolis et coll., 1990; De Luca et coll., 1986; De Luca et coll., 1989), et l'apparition de ces enzymes entraîne une conversion quantitative de la tabersonine et de ces métabolites en vindoline (Balsevich et coll., 1986; De Luca et coll., 1986).
L' induction de l'activité enzymatique D4H (De Carolis, 1994) et la conversion quantitative de la tabersonine et de ses métabolites en vindoline sont aussi observées dans les plantules étiolées soumises à une brève exposition à la lumière rouge. Le rôle du photorécepteur phytochrome dans ce processus fût démontré lorsque des traitements à la lumière rouge sombre ont renversé l'effet de la lumière rouge. De plus, le jasmonate de méthyle (Aerts et coll., 1994) et les régulateurs de croissance auxiniques (Aerts et coll, 1992) altèrent le niveau de plusieurs enzymes de la voie de biosynthèse de la vindoline ainsi que l' accumulation de la vindoline et de ses intermédiaires dans les plantules.

Les études réalisées avec les cultures cellulaires et avec les plantules en croissance de *Catharanthus* illustrent que les mécanismes de régulation de la phase terminale de la biosynthèse de la vindoline sont dépendants de l'environnement, du stage de développement et du type de tissu. Cela suggère que les enzymes qui catalysent les deux dernières réactions jouent un rôle crucial dans la régulation de la biosynthèse de vindoline. Dans le cadre des études sur la régulation du métabolisme secondaire chez *Catharanthus roseus*, le projet de cette thèse porte plus particulièrement sur la régulation moléculaire de la désacetoxyvindoline 4-hydroxylase (EC.1.14.11.11). Cet enzyme fait partie de la famille des dioxygénases à 2-oxoglutarate puisque qu'il requiert, en plus de son substrat alcaloïde, le 2-oxoglutarate, l'ascorbate, l'ion ferreux, et l'oxygène moléculaire pour son activité (De Carolis et coll., 1990; De Carolis et De Luca, 1993). La régulation de la D4H a été caractérisée au niveau de l'activité enzymatique dans les plantules en développement (De Carolis et coll., 1990). Cet enzyme a aussi été purifié et sa cinétique caractérisée dans notre laboratoire (De

Carolis et De Luca, 1993). De plus, des clones d' ADNc ont été isolés et partiellement séquencés.

Ce projet a pour objectifs:

1) La caractérisation complète des clones d'ADNc de la D4H isolés par De Carolis (1994).
2) L'analyse moléculaire de la régulation de la D4H au cours du développement.
3) L'étude des effets des inducteurs chimiques du métabolisme secondaire sur la régulation de la D4H au cours du développement.
4) L'identification des types cellulaires impliqués dans la biosynthèse de la vindoline.

Résultats.

L'analyse complète des clones d'ADNc a indiqué que la séquence en acides aminés de la D4H partage 30% de similarité avec les autres dioxygénases de source végétale ou fongique tel que l'enzyme formant l'éthylène de la tomate, l'hyoscyamine 6-ß hydroxylase de l' *Hyoscyamus niger* et l'isopénicilline *N*-synthétase de l' *Aspergillus niger*. La similarité entre la D4H et les autres dioxygénases se prolonge aussi au niveau de la structure du gène. L'analyse de la séquence d'un clone d'ADN génomique de *Catharanthus* indique que le gène *d4h* est interrompu par deux introns insérés à des positions identiques à celle des introns retrouvés dans les autres gènes de dioxygénase. Ce degré de conservation suggère que la D4H, ainsi que les autres dioxygénases, pourrait avoir évolué d'un gène ancestral commun. L'analyse par transfert de type Southern démontre que le gène *d4h* est présent à une seule copie par génome haploïde de *Catharanthus*.

La régulation tissulaire spécifique de la D4H dans la plante était apparemment contrôlée au niveau de la transcription puisque les niveaux maximaux de l'activité

enzymatique et des transcrits sont observés dans les feuilles, tandis que des niveaux beaucoup plus faibles sont présents dans les tiges et les fruits, et qu'ils sont absents dans les racines.

Par contre dans les plantules, l'expression de la D4H est sujette à une régulation complexe, sous l'influence du développement et de la lumière, qui implique des mécanismes aux niveaux transcriptionnels, post-transcriptionnels et post-traductionnels. Dans les plantules étiolées, les niveaux d'activité enzymatique sont faibles même lorsque des quantités élevés de transcrit sont observées. Suite à une exposition à la lumière, l'activité enzymatique augmente de 4 à 8 fois selon le stade de développement des plantules au moment du traitement lumineux. La réponse est plus prononcée à des stades de développement hâtifs (plantules de 5 jours) que tardifs (plantules de 7 jours). L'augmentation induite par la lumière de l'activité enzymatique de la D4H dans les plantules de 5 jours s'accompagne d'une augmentation notable de la quantité de protéine immunoréactive à l'anticorps anti-D4H. Cependant, le niveau de transcrit n'augmentait pas suite à ce traitement. Avec des plantules de 7 jours, l'augmentation plus faible de l'activité enzymatique induite par la lumière s'accompagne aussi d'une augmentation plus modeste du niveau de protéine immunoréactive que dans les plantules de 5 jours. Toutefois, le niveau de transcrit dans les plantules de 7 jours augmente suite à l'exposition à la lumière. En plus de ces effets, l'exposition des plantules de *Catharanthus* à la lumière initie l'apparition d'isoformes légèrement plus acides de la protéine D4H tel que démontré par l'analyse de type Western de gel bidimensionnel IEF-SDS-PAGE. Les effets de la lumière sur l'expression de la D4H sont dépendants du phytochrome puisque une exposition de 30 minutes de lumière rouge suivie de 24 h d'obscurité était suffisant pour produire la même réponse qu'une période de 24 h de lumière blanche en continu et puisque les effets de la lumière rouge sont abolis par une exposition subséquente à la lumière rouge sombre.

L'application exogène de jasmonate de méthyle, un inducteur chimique du métabolisme secondaire, produit un augmentation de 80% du niveau d'activité

enzymatique de la D4H dans les plantules cultivées à la lumière. Cependant, cet inducteur chimique n'a aucun effet sur la D4H dans les plantules cultivées à l'obscurité, ce qui confirme la dépendance à la lumière pour l'activation de la D4H et pour la biosynthèse de vindoline. De plus, l'application de l'inducteur n'a pas altéré la coordination de l'expression de la D4H au cours du développement avec la phase initiale de la biosynthèse de la vindoline, tel que la tryptophane décarboxylase (TDC).

L'analyse de la distribution des activités enzymatiques et des protéines TDC et d4H dans les différents tissus de plant mature suggère qu'à la fois la phase initiale et la phase terminale de la biosynthèse de la vindoline soit principalement exprimées dans les tissus juvéniles et en croissance active. En fait, même dans les feuilles immatures, la TDC et la D4H sont principalement détectées dans la base, la partie de la feuille qui présente le plus haut taux de division cellulaire.

L'immunolocalisation des protéines TDC et D4H dans les différents tissus de plant mature de *Catharanthus* a permis d'identifier des types cellulaires spécialisés responsable de la biosynthèse de la vindoline. La TDC est localisée exclusivement dans l'épiderme des feuilles et des tiges et distribuée dans la plupart des cellules de l'apex racinaire. En contrepartie, la D4H a été immunolocalisée dans les laticifères des feuilles et des tiges, et n'était pas présente dans les racines. La distribution cellulaire de TDC et D4H dans les cotylédons de jeunes plantules était similaire à celles des plants matures. Dans les cotylédons de plantules cultivées à l'obscurité ou à la lumière, aucune différence de distribution cellulaire de la D4H n'a été observée.

Les éliciteurs biotique et chimiques n'ont pas induit dans les cultures cellulaires de *Catharanthus* l'expression de niveau détectable de l'activité enzymatique D4H, pas plus que l'accumulation du transcrit de *d4h*, même si ces traitements ont stimulé l'activité enzymatique et l'accumulation de transcrits de la TDC.

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List of Tables.

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List of abrevations.

AVBL	α-3'4'-anhydrovinblastine.
bp	base pair
cDNA	complementary DNA
D4H	desacetoxyvindoline 4-hydroxylase
DAT	deacetylvindoline 4-*O*-acetyltransferase
G10H	geraniol 10-hydroxylase
IEF	isoelectrofocusing
kb	kilobase
kDa	kilodalton
LAMT	loganic acid methyltransferase
NMT	2,3-dihydro-3-hydroxytabersonine-*N*-methyltransferase
OMT	16-hydroxytabersonine-16-*O*-methyltransferase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming unit
SS	strictosidine synthase
T16H	tabersonine 16-hydroxylase
TDC	tryptophan decarboxylase
UTR	untranslated region
VBL	vinblastine
VCR	vincristine

Dedication:

A *Ojos Grises*, por su sonrisa llena de luz

A *Ojos Pardos*, por su compresión

A mis padres, por su apoyo

Verde que te quiero verde

(*Green since I want you green*)

Federico García Lorca

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It has been a long way since I first arrived to Montreal to initiate my doctoral studies. Now it is time to go back home; however, this journey would have been impossible without the collaboration of many people.

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Preface

The Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don produces the cytotoxic bisindole alkaloids vinblastine and vincristine (Fig. 1). These important anticancer drugs were discovered accidentally when searching for hypoglycemic agents in *Catharanthus* plants. According to Jamaican folk medicine, teas prepared from leaves of this plant were prescribed as a remedy against diabetes. However, when aqueous leaf extracts were orally administrated to mice, glucose levels in blood remained unchanged. In addition, injection of mice with the leaf extract caused death as a consequence of an opportunistic septicemia. Further studies showed that leaf extracts prevented white blood cell proliferation. Vinblastine and vincristine which are dimeric alkaloids composed of catharanthine and vindoline (Fig. 1), were identified as active components. After extensive studies, both drugs were marketed by Eli Lilly under the brand names of *Velban*® and *Oncovin*®, respectively (Johnson et al., 1963; Noble et al., 1958). Today, because of their powerful antimitotic effects, these drugs are still widely used in various cancer treatments, including Hodgkins' disease and certain types of leukemia (Johnson et al., 1963).

The economic value of these dimers, together with their low abundance in *Catharanthus* plants (Table 1), have prompted significant efforts to produce them through cell culture technology. However, despite many years of work by different research groups around the world, only a partial success has been achieved (see recent reviews by Moreno et al., 1995; and van der Heijden et al., 1989). Cell cultures are capable of producing high levels of catharanthine, as well as several other *Catharanthus* alkaloids, but they do not accumulate vindoline. The capacity of cell cultures to produce dimeric alkaloids *in vitro* is therefore hampered by this inability (but see O'Keefe et al., 1997; Parr et al., 1988). These results suggest that vindoline biosynthesis is regulated differently than the pathways governing the biosynthesis of other *Catharanthus* alkaloids.

Within the broader research context of the regulation of secondary metabolism in *Catharanthus roseus*, studies in this laboratory have focused on the purification, biochemical characterization and kinetic analysis of desacetoxyvindoline 4-hydroxylase

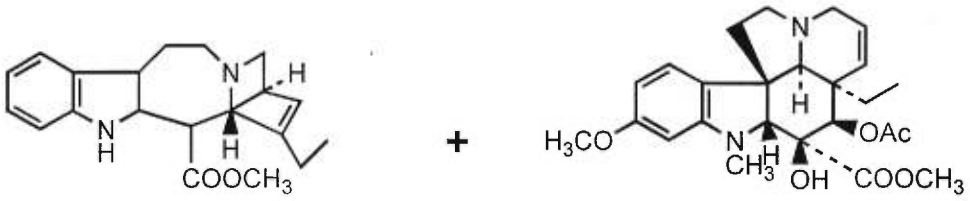
hydroxylase (D4H) (De Carolis, 1994), the enzyme which catalyzes the second to last step in vindoline biosynthesis. This thesis focuses on the molecular characterization of D4H and proposes to: **a)** further characterize the D4H *c*DNA clones previously isolated by De Carolis, (1994); **b)** perform molecular studies of the developmental and light regulation of D4H; **c)** determine the effects of chemical inducers of secondary metabolism on the developmental regulation of D4H and, **d)** identify the cell types where D4H is expressed.

This thesis has been written as a collection of 4 scientific articles which are preceeded by a general introduction (Chapter I) and are followed by a final discussion (Chapter VI).

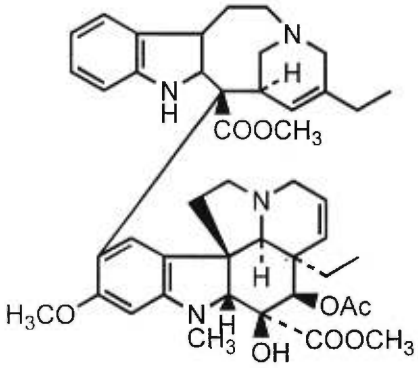
TABLE 1. Economic importance of the *Catharanthus* alkaloids.

Generic name	Trade name	Medical applications	Yield (% on dry basis)	Cost (USD/g)	Annual market (kg)
Vinblastine	Velban®	Hodgkin's disease	0.0005	$1,000	12.00
Vincristine	Oncovin®	Leukemias	0.0001	$3,500	1.00

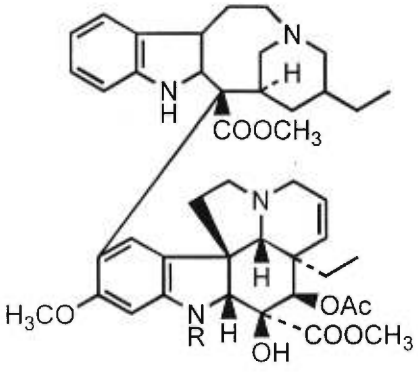
Modified from: **van der Heijden, R, Verpoorte, R, Ten Hoopen, JG** (1989) Cell and tissue culture of *Catharanthus roseus* (L.) G. Don: a literature survey. Plant Cell Tissue and Org Cult **18**: 231-280.



Catharanthine ***Vindoline***



α-3', 4'-anhydrovinblastine



R= CH_3 ***Vinblastine***

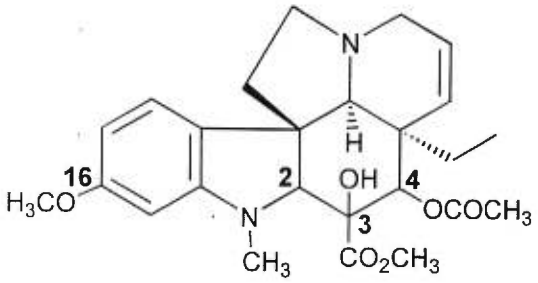
R=CHO ***Vincristine***

Figure 1. Structures of the dimeric alkaloids of *Catharanthus roseus* and the subunits which form them.

List of generic names.

desacetoxyvindoline	16-methoxy-2,3-dihydro-3-hydroxy-*N*(1)-methyltabersonine
desacetoxyvindorosine	2,3-dihydro-3-hydroxy-*N*(1)-methyltabersonine
deacetylvindoline	16-methoxy-2,3-dihydro-3,4-dihidroxy-N(1) -methyltabersonine
vindoline	16-methoxy-2,3-dihydro-3-hydroxy-4-acetoxy-tabersonine

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



vindoline

Chapter I. Review of Literature.

I.1. Enzymology of Monoterpenoid Indole Alkaloid Biosynthesis in *Catharanthus roseus* (L.) G. Don.

I.1.1. Introduction.

The genus *Catharanthus*, which is native to the island of Madagascar, belongs to the Apocynaceae family. This genus is composed of 7 species: *lanceous, longifolius, ovalis*, *pusillus*, *roseus, scitulus* and *trichophylus* (Stern, 1975), all of which produce different indole alkaloids. *Catharanthus roseus* has, however, received the most attention since it is the only commercial source for the valuable dimeric alkaloids, vinblastine and vincristine.

The biosynthesis of *Catharanthus* alkaloids involves the participation of two major metabolic routes which are the shikimate and the terpenoid pathways. Tryptophan-derived tryptamine is combined with geraniol-derived secologanin to form the central alkaloid intermediate, strictosidine. Intramolecular rearrangement of the terpene component of strictosidine results in the formation of the aspidosperma, the corynanthe, and the iboga families of alkaloids (Fig. 2). Extensive reviews on the enzymology of this pathway have been published in recent years (De Luca et al., 1992; De Luca, 1993; Meijer et al., 1993b). Furthermore, the *Introduction* of each manuscript in this thesis deals in detail with the enzymology of the late steps of vindoline biosynthesis. Only a brief review on the enzymology of the biosynthesis of the *Catharanthus* alkaloids will therefore be presented and special emphasis is given to those enzymes not discussed in the manuscripts.

I.1.2. Early stages of *Catharanthus* alkaloid biosynthesis.

Early steps in monoterpenoid indole alkaloids biosynthesis involve the conversion of tryptophan and geraniol into strictosidine (Fig. 3). These reactions are common to the synthesis of all *Catharanthus* alkaloids.

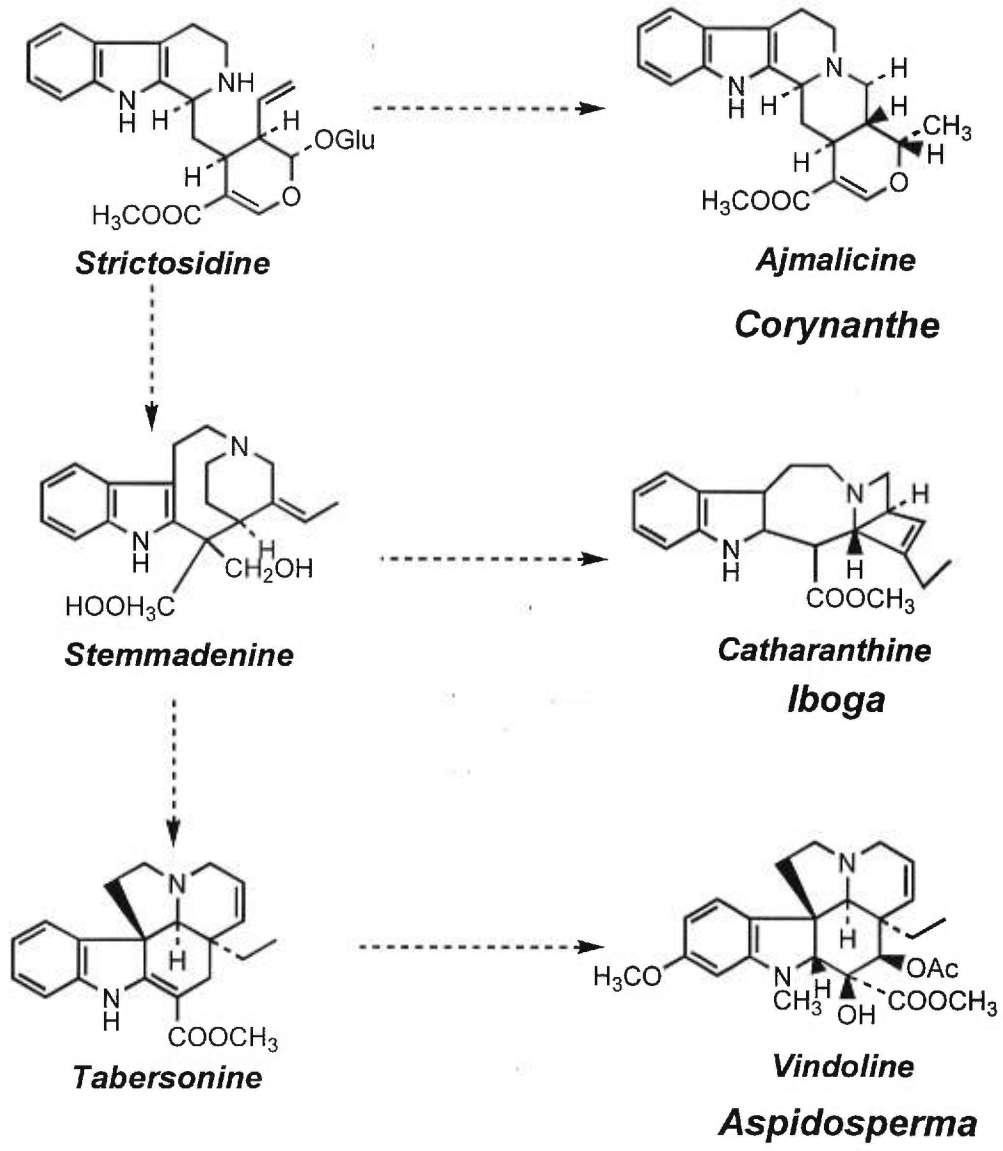


Figure 2. Biosynthetic relationships between Corynanthe, Iboga and Aspidosperma alkaloid families of *Catharanthus roseus*.

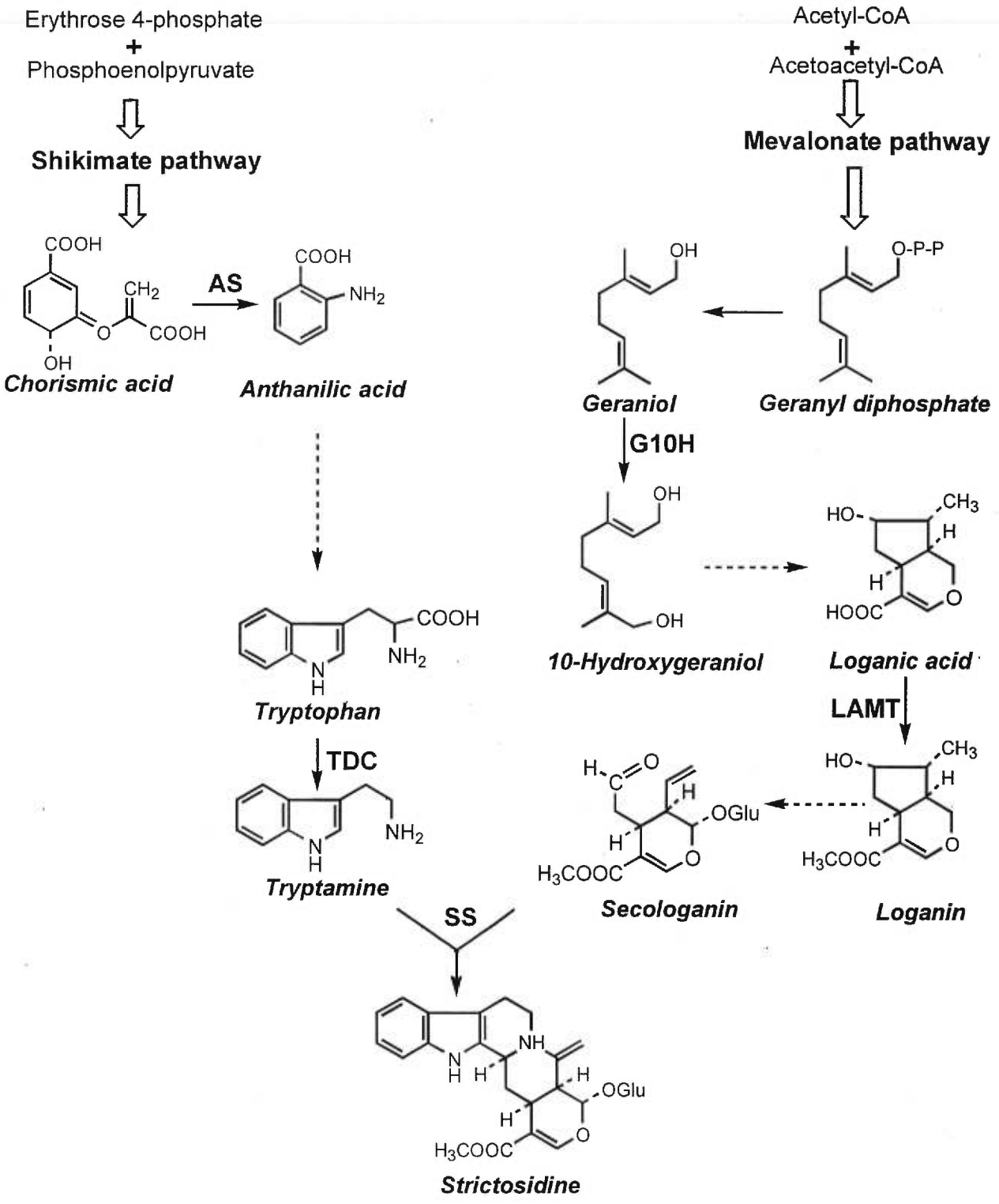


Figure 3. Early stages in alkaloid biosynthesis in *Catharanthus roseus*. (**AS**) Anthranilate synthase; (**G10H**) geraniol hydroxylase; (**LAMT**) S-adenosyl-L-methionine:loganic acid methyltransferase; (**TDC**) tryptophan decarboxylase; (**SS**) strictosidine synthase.

I.1.2.1. Formation of the indole component of *Catharanthus* alkaloids.

The indole nucleus of *Catharanthus* alkaloids is derived from tryptophan. This amino acid, as well as the other aromatic amino acids, are synthesized through the shikimate pathway (extensively reviewed by Bentley, 1990). Briefly, erythrose-4-phosphate and phosphoenolpyruvate are condensed to form 3-deoxy-D-arabino-heptulosonate (DAHP) by the enzyme DAHP synthase. Three further enzymatic reactions transform DAHP into shikimate which is subsequently converted into chorismate. This intermediate represents a critical branch point to the formation of prephenate or anthranilate which are early precursors of tyrosine and phenylalanine or of tryptophan, respectively (Fig. 3).

I.1.2.1.A. Anthranilate synthase. Anthranilate synthase (AS; EC. 4.1.3.27) is the only enzyme involved in tryptophan biosynthesis which has been studied in *Catharanthus roseus* (Poulsen and Verpoorte, 1992; Poulsen et al., 1993). In comparison with cell cultures from other plants, high levels of AS enzyme activity were detected in *Catharanthus* cell suspensions. A slight increase in this enzyme was noticed when the cultures were transferred to an alkaloid production medium, suggesting a possible involvement of the pre-tryptophan pathway in triggering the accumulation of alkaloids (Poulsen and Verpoorte, 1992). Initial experiments with crude extracts from cell cultures indicated that AS could be resolved as two peaks of activity by anion exchange chromatography, but further studies showed the enzyme to occur as a single isoform which was located in chloroplasts and which was strongly inhibited by tryptophan (Poulsen et al., 1993). The purified enzyme is a heterotetramer composed of two 67 and two 25 kDa subunits, respectively (Poulsen et al., 1993), which have yet to be cloned.

I.1.2.1.B. Tryptophan decarboxylase. The decarboxylation of tryptophan to produce tryptamine is catalyzed by the pyridoxal phosphate dependent tryptophan decarboxylase (TDC; EC 4.1.1.28). This cytosolic enzyme (De Luca and Cutler, 1997) is a dimeric protein composed of identical 54 kDa subunits (Alvarez-Fernandez et al., 1989; Noe et al., 1984). Complete TDC cDNA clones were isolated by antibody screening of a cDNA library produced from developing *Catharanthus* seedlings (De

Luca et al., 1989) and the identity of TDC was confirmed by expressing active enzyme in *E. coli* (De Luca et al., 1989) and in *Nicotiana tabacum* (Songstad et al., 1990). The sequence of a genomic clone together with Southern blot analysis suggested that *tdc* is present as a single, uninterrupted gene in the *Catharanthus roseus* genome (Goddijn et al., 1994). The regulation of this enzyme in relation to the biosynthesis of vindoline is discussed in section **I.3.3.1**.

I.1.2.2. Formation of the terpene moiety of *Catharanthus* alkaloids.

Mevalonate is the building block for the formation of a wide variety of plant products, including terpenoids. The biosynthesis of mevalonic acid involves the condensation of three units of acetyl-CoA, to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In plants, these reactions are catalyzed by either one or two enzymes (Bach et al., 1991). Two units of acetyl-CoA can be condensed into acetoacetyl-CoA by acetoacetyl-CoA thiolase (AACT; EC. 2.3.1.9) and the third acetyl-CoA unit is added by the action of HMG-CoA synthase (EC. 4.1.3.5) to form HMG-CoA. Bach et al (1991) also proposed that a single enzyme may catalyze the direct formation of HMG-CoA from three molecules of acetyl-CoA. Mevalonate is then formed irreversibly from HMG-CoA by the action of HMG-CoA reductase (EC. 1.1.1.34; Chappell, 1995).

I.1.2.2.A. Enzymes involved in mevalonate biosynthesis. Activities for both AACT and HMG-CoA synthase have been detected in *Catharanthus roseus* cell and root cultures, as well as in dark and light grown seedlings (van der Heijden et al., 1994). The roots, stems and flowers of mature plants compared to leaves contained the highest enzyme activities. However, it was suggested that the low activity observed in leaves was due to artefacts caused by chlorophyll interference in the enzyme assay (van der Heijden et al., 1994).

A putative HMG-CoA reductase cDNA clone has been isolated from a *C. roseus* cDNA library. The open reading frame of this clone encodes a 64 kDa membrane-associated protein that contains two predicted transmembrane spanning domains (Maldonado-Mendoza et al., 1992). HMG-CoA reductase transcripts occur at high levels in all tissues of mature plants (Maldonado-Mendoza et al., 1992) whereas it is

induced in cell cultures after methyl jasmonate treatment (Maldonado-Mendoza et al., 1994).

I.1.2.2.B. Synthesis of secologanin. The secoiridoid glycoside, secologanin, provides the C9-C10 unit for the biosynthesis of monoterpenoid indole alkaloids in *C. roseus* (Meehan and Coscia, 1973). This product is formed from mevalonate *via* geraniol which arises from the removal of two phosphate groups from geranyl diphosphate (Fig. 3). Nerol, the *cis* isomer of geraniol can also be used as a precursor in the synthesis of secologanin (Balsevich et al., 1982).

The first committed step in the formation of secologanin involves the conversion of either geraniol or nerol to the respective 10-hydroxy-derivatives. This reaction is catalyzed by geraniol-10-hydroxylase (G10H), a cytochrome P-450 dependent monooxygenase, which hydroxylates both geraniol and nerol at similar rates (Madyastha and Coscia, 1979a). This activity has been detected in 5 day old etiolated seedlings (Meehan and Coscia, 1973), as well as in cell cultures of *C. roseus* (Spitsberg et al., 1981). The enzyme is apparently located in a provacuolar membrane fraction (Madyastha et al., 1977) and it can be separated into cytochrome P-450 and NADPH cytochrome *c* reductase components by a combination of membrane solubilization and ion exchange chromatography (Madyastha et al., 1976). Enzyme activity can be restored by incubating the reductase component with a crude preparation of *Catharanthus* lipids containing the cytochrome P-450 component (Madyastha et al., 1976). NADPH cytochrome *c* reductase (EC. 1.6.2.4) was first purified to homogeneity from seedlings (Madyastha and Coscia, 1979a), and later from cell cultures (Meijer et al., 1993a). Polyclonal antibodies to the reductase were used to isolate a complete cDNA clone which was expressed in bacteria as an active protein with the expected *Mr* of 75 kDa (Meijer et al., 1993a). The reductase is present as a single copy gene (*Cpr*) in the *Catharanthus* genome, which suggests that all the cytochrome P-450 proteins in this plant are associated to this reductase for catalysis (Meijer et al., 1993a). The transcripts for this reductase were mainly detected in roots and flowers, but were also found in stems and leaves of mature plants. Cell cultures also contained *Cpr* transcripts, which were induced after treatment with fungal elicitors (Meijer et al., 1993a). Several *Catharanthus* cytochrome P-450 cDNA clones have been isolated by differential

screening, but none were shown to have G10H activity or any other alkaloid hydroxylase activity (Vetter et al., 1992; Mangold et al., 1994).

The formation of secologanin from 10-hydroxygeraniol involves the reduction of the latter to 10-oxogeranial, which is then transformed into iridodial. Some of the enzymes involved in these reactions have been characterized and partially purified in *Rauwolfia serpentina*, another member of the Apocynaceae family, but not in *Catharanthus roseus* (De Luca, 1993; Meijer et al., 1993b). Further uncharacterized enzyme reactions transform iridodial to loganic acid, which is then *O*-methylated at the carboxyl group to produce loganin. The enzyme which can also methylate secologanic acid, has been named *S*-adenosyl-L-methionine: loganic acid methyltransferase (LAMT; Fig. 3), and it has been partially purified from 6-8 day old etiolated seedlings, where it occurs at high levels (Madyastha and Coscia, 1979b).

I.1.2.3. The condensation of tryptamine with secologanin.

Strictosidine synthase (SS; EC. 4.3.3.2), the enzyme catalyzing the stereospecific condensation of tryptamine and secologanin, has been purified to homogeneity from cell (Treimer and Zenk, 1979) and root (De Waal et al., 1995) cultures. SS occurs as a single copy intronless gene in *Catharanthus* (Pasquali, 1994) and the numerous isoforms of this enzyme which have been isolated are based on a differential glycosylation pattern (De Waal et al., 1995; Pfitzner and Zenk, 1989). The predicted ORF of strictosidine synthase cDNA clone encodes a 39 kDa polypeptide (McKnight et al., 1990; Pasquali et al., 1992) which includes a putative signal peptide at the N terminus (McKnight et al., 1990). The presence of such a sequence is consistent with the vacuolar localization of this enzyme (McKnight et al., 1991). Transcripts for *ss* which were abundant in roots of mature plants, were also detected in lower amounts in leaves (Pasquali et al., 1992). The expression of *ss* transcripts could be transiently induced in cell suspension cultures with elicitor treatment, in coordination with the accumulation of *tdc* (Pasquali et al, 1992; Roewer et al, 1992) and *Cpr* transcripts (Pasquali et al, 1992). These results suggest that a common mechanism exists in *Catharanthus* for the activation of the early alkaloid biosynthetic pathway.

I.1.2.4. Post-strictosidine reactions.

The glucose moiety of strictosidine is removed by a specific glucosidase (Hemscheidt and Zenk, 1980) to produce a highly reactive aglycone precursor of the aspidosperma, corynanthe and iboga alkaloids (Fig. 2; Scott, 1970). The biosynthesis of the corynanthe alkaloids, ajmalicine and serpentine, has been studied at some length (reviewed by De Luca, 1993; Meijer et al., 1993b) and will not be discussed here. Stemmadenine apparently represents the branch point in the synthesis of both the iboga (catharanthine), and aspidosperma (vindoline) type alkaloids (Fig. 2). However, the enzymology of many of the reactions leading to the production of catharanthine and tabersonine, which is a late precursor of vindoline, remain to be studied (Scott, 1970).

I.1.2.5. The biosynthesis of vindoline from tabersonine.

Six enzymatic reactions are involved in the transformation of tabersonine to vindoline. These reactions include: 16-hydroxylation, 16-*O*-methylation, hydration of the 2,3 double bond, *N*-methylation, 4-hydroxylation and 4-*O*-acetylation (Fig. 4; Balsevich et al., 1986; De Luca et al., 1986). The enzymes involved in this part of the pathway are discussed in detail in the *Introduction* of each manuscript and only the recent molecular cloning and characterization of acetyl-CoA: deacetylacetylvindoline 4-*O*-acetyltransferase (DAT; EC 2.3.1-) is reviewed here (St-Pierre et al., 1998). DAT was cloned using a PCR approach based on the amplification of specific internal amino acid sequences of a peptide obtained from sequencing the purified protein (Alarco, 1995; Power et al., 1990). Degenerate oligonucleotides containing additional restriction cloning sites were produced corresponding to the N and C terminus of a 22 amino acid oligopeptide and were used to amplify reverse transcribed leaf RNA. The resulting 73 bp fragment was cloned and sequenced to reveal the corresponding DNA sequence of the peptide. This unique sequence was used as a probe to isolate cDNA and genomic clones (St-Pierre et al., 1998). The predicted amino acid sequence of a genomic clone confirmed the presence of several sequenced peptides obtained from the purified protein. These results suggest that DAT is a 45 kDa monomer and that the two subunit heterodimer previously isolated by Fahn et al (1985) and Power et al (1990) may have arisen as artefacts of protein purification (St. Pierre et al., 1998). The *dat* gene occurs in

a single copy in the *Catharanthus* genome and is uninterrupted by introns (St-Pierre et al., 1998). The tissue distribution and regulation of DAT is discussed in following sections.

I.1.2.6. Enzymes involved in the synthesis of dimeric alkaloids.

The oxidative coupling of catharanthine and vindoline results in the formation of α-3',4'-anhydrovinblastine (AVBL), which is the precursor of catharine, leurosine, vinblastine and vincristine (Endo et al., 1987). These coupling reactions can be performed in *in vitro* with cell-free extracts from *Catharanthus* plants and from cell cultures (Baxter et al., 1979; Kutney et al., 1982; 1988), as well as with commercial preparations of horseradish peroxidase (Goodbody et al., 1988).

It has been proposed that the coupling of vindoline with catharanthine may be catalyzed by a vacuolar peroxidase with specific AVBL synthase activity. However, attempts to purify this enzyme have yet to produce clear results (Sottomayor et al., 1996). The suspected location of this peroxidase activity in the tonoplast (Sottomayor et al., 1996) may have implications in the compartmentation of the dimeric alkaloids which, apparently, do not move freely across this membrane (McCaskill et al., 1988).

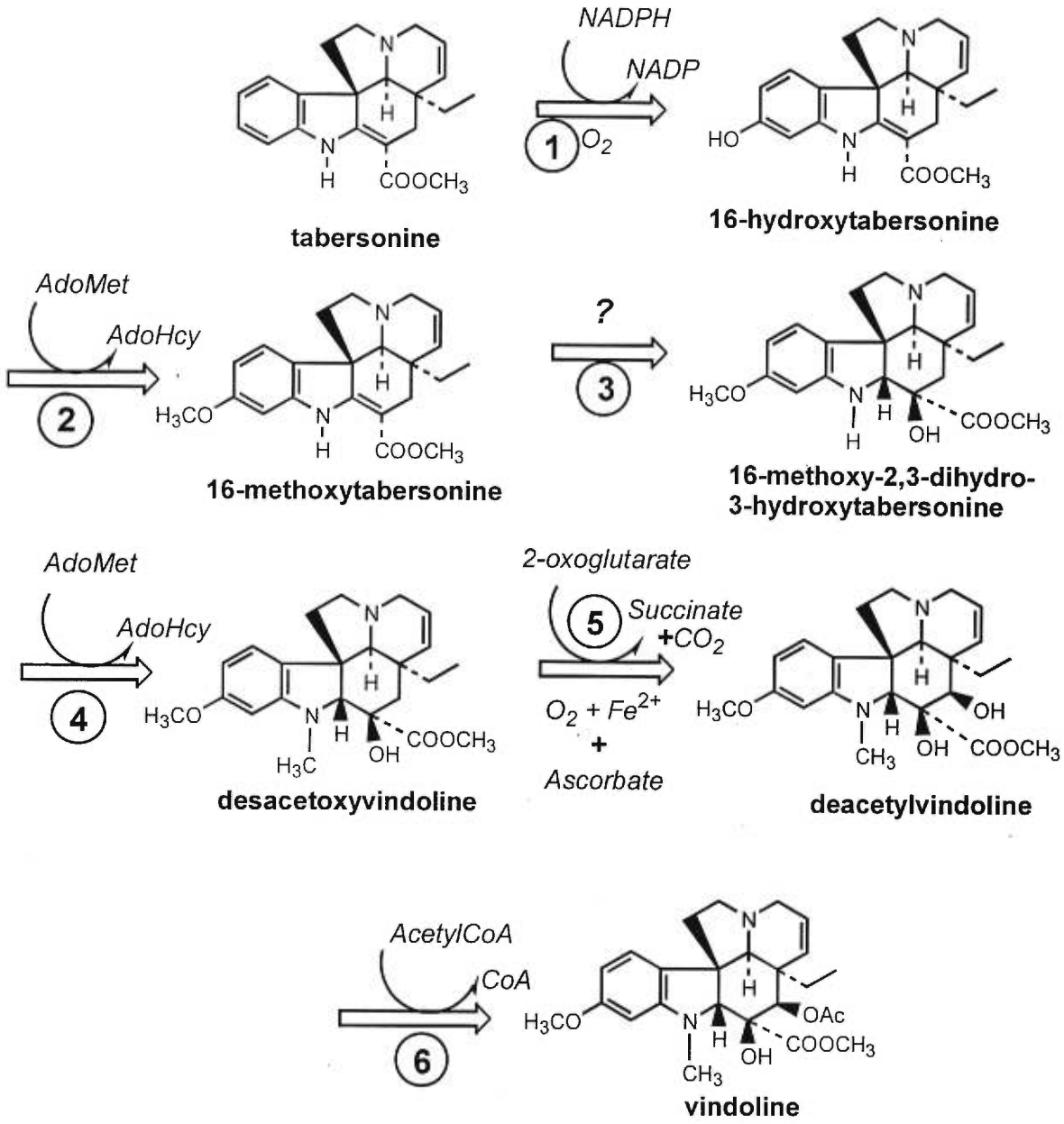


Figure 4. Conversion of tabersonine to vindoline. Tabersonine-16-hydroxylase (**1**); AdoMet:16-hydroxytabersonine-16-*O*-methyltransferase (**2**); an uncharacterized hydroxylase (**3**); AdoMet:2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase (**4**); desacetoxyvindoline 4-hydroxylase (**5**); and acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase (**6**).

I.2. Alkaloid Metabolism in *Catharanthus roseus.*

The biosynthesis of vindoline in *Catharanthus roseus* is regulated by development-, tissue- and environment-specific controls which are coordinated with the early phases of seedling germination through to the mature stages of vegetative growth. Even after normal cellular events are disrupted by production of *in vitro* cell cultures, some of these regulatory mechanisms tend to persist, leading to cell lines which produce a spectrum of monoterpenoid indole alkaloids.

The following section reviews the developmental aspects of alkaloid metabolism in germinating seedlings, mature plants and cell cultures. Certain data referring to the accumulation of catharanthine and dimers are also included.

I.2.1. Alkaloid metabolism in etiolated and light grown seedlings

Alkaloid metabolism appears to be a very active process in developing seedlings of *Catharanthus roseus*. More than 30% of the label in ^{14}[C]-tryptophan fed to 6 day old seedlings was incorporated into indole alkaloids over a 3 day period and 7% of the total radioactivity was recovered in vindoline (Scott et al, 1974). The accumulation of vindoline in relation to seedling development was first described by Mothes et al (1965), who observed that dry seeds were devoid of alkaloids. However, during germination and subsequent seedling unfolding, vindoline accumulation began after one week of growth and continued for a further two weeks of seedling development (Mothes et al., 1965). A more detailed seedling study was presented by Scott (1970) who described the developmentally regulated accumulation of vindoline precursors of increasing complexity. As early as 26 h after planting *Catharanthus* seeds in agar, low but significant amounts of strictosidine were recovered from germinating seedlings. More complex and elaborate alkaloids like tabersonine were detected after 2 days of growth while catharanthine became a major product after 4 days of seedling development. Despite the early accumulation of advanced vindoline precursors (tabersonine), vindoline was only detected after 8 days of growth (Scott, 1970). These data suggest that vindoline is only produced after seedlings had reached a more advanced

developmental growth stage than those required for catharanthine and tabersonine biosynthesis.

Besides this developmental control, the requirement for light to activate vindoline accumulation in developing seedlings, clearly differentiates the processes required for vindoline biosynthesis from those of the other *Catharanthus* alkaloids. When grown in the dark, seedlings accumulated catharanthine and tabersonine but not vindoline. Upon illumination, high levels of vindoline were detected while the levels of other alkaloids remained unaffected by the light regime (Mothes et al., 1965; Scott, 1970). However, light treatment only activated vindoline biosynthesis within a program already triggered by seedling development (Aerts et al., 1994; Balsevich et al., 1986; De Luca et al., 1986), since only older seedlings were capable of accumulating vindoline. Five- to seven-day old etiolated seedlings accumulated tabersonine as well as several post-tabersonine intermediates, whereas these alkaloids were quantitatively converted into vindoline when etiolated seedlings were exposed to light (Balsevich et al., 1986; De Luca et al., 1986). It should be noted that the tabersonine intermediates clearly reflect the biosynthetic pathway involved in the conversion of tabersonine to vindoline (Fig. 4).

I.2.2. Alkaloid metabolism during vegetative development.

As a rule, alkaloids are mainly accumulated in young and actively growing tissues. For this reason and due to their toxic nature, alkaloids have been associated with a protective or defensive function (Robinson, 1974; 1980; Frischknecht et al., 1987). In agreement with this rule, alkaloid biosynthesis in *Catharanthus* plants does not proceed at the same rate in young and mature tissues, suggesting that, as in seedlings, specific developmental stages for alkaloid biosynthesis also occur in the plant. This knowledge has been useful to commercial producers for estimating the best timing for the harvesting of *Catharanthus* plants (Morton, 1977).

Alkaloid metabolism in *Catharanthus* plants is particularly active during flowering (Balsevich and Bishop, 1989; Frischknecht et al., 1987; Reda, 1974) where important variations in alkaloid contents are observed in aerial and underground tissues. Leaf concentrations of catharanthine and vindoline peak right after full-flowering

(Frischknecht et al., 1987), whereas the amounts of the corynanthe alkaloids ajmalicine, serpentine (Levy et al., 1983) and perivine (Reda, 1974) reach their maximum levels during the period of fruit maturation. In contrast, no differences in the timing for maximum accumulation in corynanthe alkaloids were noticed between roots and leaves (Levy, 1983; Sevestre-Rigouzzo et al., 1993). These data may indicate different phenological requirements for the synthesis of structurally more complex aspidosperma and iboga alkaloids in comparison with the corynanthe type alkaloids.

I.2.2.1. Distribution of alkaloids in mature plants.

Except for vindoline and the dimeric alkaloids, which occur exclusively in aerial tissues, other alkaloids produced in *Catharanthus* have been detected in all plant tissues. Nevertheless, their distribution seems to follow a general pattern. The major corynanthe type alkaloids, ajmalicine and serpentine, occur predominantly in roots while catharanthine can be accumulated in roots and in leaves in a 6 to 4 ratio (Deuss Neumann et al., 1987). Although catharanthine was mainly detected in young tissues of both organs (Deus Neumann et al., 1987), its distribution profile in leaves seems more variable compared to that of vindoline. Some studies which determined the concentration of catharanthine in different leaves by chemical analytical methods (Balsevich and Bishop, 1989) or by radioimmunoassay (Deus Neumann et al., 1987) showed a 3 and 20-fold concentration difference, respectively, between the youngest and subsequent leaves. Other reports describe a more gradual decrease in the contents of catharanthine in leaves of different ages, following a similar pattern to that of vindoline (Frischknecht et al., 1987; Naaranlahti et al., 1991). Although variations in genotype, experimental procedures, or plant age may account for these described differences, the same factors did not seem to affect the pattern of vindoline accumulation. These data again suggest vindoline biosynthesis is more tightly regulated than those of other alkaloids.

Vindoline represents the dominant alkaloid in *Catharanthus* leaves (Westekemper et al., 1980), where the highest levels occur in young actively growing tissues, whereas older leaves contain progressively lower levels of vindoline (Balsevich and Bishop, 1989; Frischknecht et al., 1987; Naaranlahti et al., 1991; Westekemper et

al., 1980). In contrast, dimeric alkaloids are not primarily accumulated in young tissues (Balsevich and Bishop, 1989; Naaranlahti et al., 1991). In fact, as the levels of catharanthine and vindoline decrease from young to older leaves, there is an increase in dimer content, but not in an equivalent proportion, since the dimeric alkaloids only represent 1.0% of the combined catharanthine and vindoline levels in the plant (Hirata et al., 1993; Naaranlahti et al., 1991).

These data suggest that a certain degree of leaf maturity is necessary for the formation of the bisindole alkaloids. The capacity for dimeric alkaloid production is present in 11 day old seedlings (Aerts et al., 1996), although accumulation is not completely stable before plants have reached the 3 week stage of growth (Datta and Srivastava, 1997). The developmental control of dimer formation observed in these experiments further confirm the rigorous regulation which is apparent for alkaloid biosynthesis in *Catharanthus*. As stated earlier (section **I.1.2.6**), the enzymatic coupling reaction is catalysed *in vitro* by nonspecific commercial peroxidase preparations, and it has been suggested that endogenous peroxidases in tissues containing catharanthine and vindoline catalyse enzymatic coupling *in planta* (Kutney et al., 1982). These assertions do not, however, help to explain why plants only accumulate low levels of dimers.

I.2.2.2. Alkaloid metabolism in *in vitro* cultures.

The accumulation of secondary products in *in vitro* cultures is frequently associated with their level of cellular differentiation (Kutchan, 1983; Lindsey and Yeoman, 1983). In the case of *Catharanthus roseus*, most of the alkaloids found in the entire plant also occur in cell cultures. Vindoline however, only has been randomly reported in *in vitro* systems, despite the common accumulation of some of its advanced biosynthetic precursors (De Luca and Kurz, 1988; Moreno et al., 1995). Nevertheless, the biosynthetic capacity for vindoline reappears upon shoot regeneration (Constabel et al., 1982). These observations suggest that the last few steps of vindoline biosynthesis are indeed controlled differently than the rest of the pathway. The reader is referred to other extensive reviews, which illustrate the importance of this system in plant cell

biotechnology (Carew, 1975; De Luca and Kurz, 1988; Ganapathi and Kargi, 1990; Moreno et al., 1995; van der Heijden et al., 1989).

I.2.2.2.A. Alkaloid metabolism during callus formation. In this section, only those reports dealing with the association between vindoline production and cellular differentiation have been covered.

Callus formation from aerial tissues involves a shift from autotrophy to either total or partial heterotrophy (Constabel, 1984). As part of this dedifferentiation process in *Catharanthus roseus* leaf explants, alkaloid metabolism is also shifted towards a more root-like (but not identical) alkaloid profile (Endo et al., 1987a; Morris, 1986b). The capacity for vindoline biosynthesis decreased as leaf organization was lost in favor of dedifferentiated callus tissue and a pronounced increase in the ability of callus to synthesize catharanthine was noticed. The biosynthetic abilities of the callus continued to change with a gradual shift towards the accumulation of ajmalicine and serpentine which are the dominant alkaloids of roots, but this also resulted in a sharp decline in the levels of catharanthine (Morris, 1986b). Since catharanthine is also a predominant root alkaloid (Deus Neuman et al., 1987), these data cannot only be explained on the basis of a metabolic shift (Morris, 1986b). A careful analysis of the original data shows that the total molar concentration of alkaloids was constant during the 40 days of culture. These data may, therefore, be explained as a progressive loss in the capacity to synthesize structurally complex alkaloids, which may be coupled to the loss in tissue complexity. Since the capacity for vindoline biosynthesis was affected earlier, it seems that the leaf-based morphological structures required for vindoline biosynthesis are more complex than those required for ajmalicine, catharanthine, serpentine and tabersonine biosynthesis. In this context, specialized cell types are usually most sensitive to cell culture conditions (Constabel, 1984). It is interesting to note that the capacity for synthesis of catharanthine, but not vindoline, usually reappears when callus cultures have been well-established (Endo et al., 1987a; Morris, 1986a).

I.2.2.2.B. Recovery of vindoline production during shoot regeneration. Although the loss of photosynthetic capacity has often been associated with the inability

of cell cultures to produce vindoline, the induction of photoautotrophic undifferentiated cell cultures did not result in the accumulation of this alkaloid (Tyler et al., 1986). However, when calli of *Catharanthus roseus* were induced to form shoots, the capacity to synthesize vindoline and the dimeric alkaloids reappeared simultaneously (Constabel et al., 1982; Datta and Srivastava, 1997; Endo et al., 1987; Hirata et al., 1993; Miura et al., 1988). In fact, complete shoot formation is apparently not necessary as long some leafly structures have developed. Callus with such structures ("leafy callus") also recovered the ability to make vindoline (Krueger et al., 1982; O'Keefe et al., 1997). These data appear to suggest that root participation is not required for vindoline biosynthesis.

I.2.2.2.C. Vindoline production by cell and root cultures. The use of highly sensitive analytical techniques, such as mass spectroscopy (Naaranlahti et al., 1989; O'Keefe et al., 1997) or radioimmunoassays (Parr et al., 1988), have shown that low amounts of vindoline may accumulate in cell and root cultures. Tabersonine, 16-hydroxytabersonine and 16-methoxytabersonine (Fig. 4) have been reproduceably detected in cell (Kurz et al., 1980; Kutney et al., 1980) and root (Bhadra et al., 1993; Toivonen et al., 1989) cultures. A leaky vindoline pathway which accounts for the low levels of this alklaloid detected in etiolated seedlings (De Luca et al., 1986) could also account for the low levels of vindoline detected in cell cultures.

I.3. Factors Affecting Regulatory Control of Vindoline Biosynthesis.

I.3.1. Tissue-specific distribution of the early and late stages of vindoline biosynthesis in developing seedlings.

Vindoline is mainly accumulated in cotyledons of illuminated seedlings and in lower amounts in hypocotyls, but it is absent in roots (Balsevich et al., 1986; De Luca et al., 1986). The distribution of the enzymes responsible for the transformation of tabersonine into vindoline, such as NMT, DAT (De Luca et al., 1986; 1988) and D4H (Chapter V) corresponds exactly to the tissues where vindoline is accumulated. In contrast, both catharanthine and tabersonine, as well as the early pathway enzymes TDC and SS, are equally distributed throughout radicles, hypocotyls and cotyledons of both etiolated and light grown seedlings (Balsevich et al., 1986; De Luca et al., 1986).

I.3.2. Tissue-specific distribution of the early and late stages of vindoline biosynthesis in mature plants.

The profiles of enzyme activities involved in alkaloid biosynthesis followed closely the tissue-specific distribution of alkaloid products in the plant. Common indole alkaloid pathway enzyme activities, such as G10H, LAMT (Madyastha and Coscia, 1979b), TDC (Alvarez Fernandez et al., 1989) and SS (Pasquali, 1994) occurred at high levels in the youngest components of leaves, stems and roots. Among the enzymes involved in the late stages of vindoline biosynthesis, only NMT, D4H and DAT were restricted to leaves and stems (De Carolis et al., 1990; De Luca et al., 1985; 1987; St-Pierre et al., 1998), whereas 16-OHT, which was detected in roots, was also predominantly expressed in young leaves (St-Pierre et al., 1995). The distributions of D4H (De Carolis, 1994) and DAT (De Luca et al., 1985) were very similar, as their activities decreased continuously from terminal buds to more mature leaves. However, D4H enzyme activity could still be detected in the eighth pair of leaves, whereas DAT activity was absent after the fourth leaf pair (De Carolis, 1994; De Luca et al., 1985).

I.3.3. Coordinate regulation of the early and late stages of vindoline biosynthesis.

TDC and SS, which catalyse early steps of monoterpenoid indole alkaloid biosynthesis, peak about 24 to 36 hours before those of NMT, D4H and DAT (De Carolis et al., 1990; De Luca et al., 1986; 1987; 1988) which catalyze the terminal 3 steps of vindoline biosynthesis. The timing of TDC and SS expression which coincides with the timing of catharanthine and tabersonine accumulation, suggests that the remainder of the pathway leading to these products may be activated by a similar developmental program. In contrast the portion of the pathway leading to vindoline from tabersonine is regulated by different development-, tissue- and environment-specific controls than the rest of the pathway (Balsevich et al., 1986; De Luca, 1988).

I.3.3.1. Regulation of TDC during seedling development.

The pattern of TDC activation appears to be under complex transcriptional, post-transcriptional and post-translational control (Alvarez-Fernandez et al., 1989; Fernandez, 1989; Roewer et al., 1992). Northern blot analysis has revealed that *tdc* transcripts appear transiently during seedling development and that their levels increased simultaneously with enzyme activity over a narrow 36-48 h developmental window in 4- to 6-day old seedlings (Alvarez-Fernandez et al., 1989; De Luca et al., 1986, 1988; Roewer et al., 1992). However, later in seedling development, as TDC activity decreased, *tdc* transcript levels remained high and did not decrease as quickly (Roewer et al., 1992; Appendix II). In contrast, immunological studies showed a more direct correlation between TDC activity and accumulation of TDC protein. The transient appearance of TDC activity coincided with the immunological detection of a 55.0 and a related 54.8 kDa protein (Alvarez-Fernandez et al., 1989). TDC in its active form is a 110 kDa homodimer composed of two identical 55 kDa subunits (Noe et al., 1984; Noe and Berlin, 1985; Alvarez-Fernandez et al., 1989) and it has been suggested that regulation of TDC activity is controlled by an equilibrium between a stable dimer and an unstable monomer (Fernandez et al., 1989) which is subject to irreversible inactivation by an undetermined post-translational modification of TDC protein (Fernandez et al., 1989). Inactive protein is then subject to rapid degradation by a process requiring ATP

and which may involve the ubiquitin proteolytic pathway (Alvarez-Fernandez and De Luca, 1994).

I.3.3.2. Developmental regulation of secologanin biosynthesis.

Dry seeds of *Catharanthus* accumulate large pools of loganic acid which are practically depleted after 6 days of seedling growth (Guarnaccia et al., 1971). However throughout this growth period, loganin and secologanin levels remained low, suggesting a coordinate activation of the terminal reactions which convert loganic acid into secologanin with the alkaloid pathway enzymes that channel secologanin into indole alkaloids (Guarnaccia et al., 1971).

The first committed step in the formation of secologanin is catalysed by geraniol hydroxylase (G10H; section **I.1.2.2**). This cytochrome P450-dependent monooxygenase appears to regulate the entry of geraniol into secologanin biosynthesis and the supply of this precursor for the production of indole alkaloids (Meehan and Coscia, 1973). The appearance of G10H activity is detected in young developing seedlings with maximum activities occurring after 5 to 6 days of growth and decreasing thereafter (Madyastha and Coscia, 1974; Meehan and Coscia, 1973). This enzyme may also be subject to the same developmental control as for TDC (Meijer et al., 1993b). The enzymes involved in the early and late steps of secologanin formation also seem to be coordinately regulated during seedling development since loganic acid *O*-methyltransferase activity (LAMT; section I.1.2.2) peaked 36 to 48 h later than the G10H (Madyasta et al., 1973). It should be pointed out that after induction, both enzyme activities remained high for up to 11 days of seedling growth. Neither enzyme was induced by light treatment (Madyasta et al., 1973; 1976; Meehan and Coscia, 1973), and nor were those involved in the formation of mevalonate (van der Heijden et al., 1994).

I.3.3.3. Regulation of SS during seedling development.

The appearance of *ss* transcripts and enzyme activity during seedling development followed a similar transient induction pattern as those observed for TDC, suggesting a coordinate transcriptional regulation of these genes (Roewer et al., 1992;

Appendix II). The presence of SS enzyme activity, however, persisted for a longer developmental period than TDC (De Luca et al., 1988). These differences may in part be due to the fact that SS is a glycosylated vacuolar protein (De Waal et al., 1995; Pfitzner and Zenk, 1989) in contrast to the cytosolic TDC (De Luca and Cutler, 1987).

I.3.3.4. Light regulation of the late steps in vindoline biosynthesis.

The six step transformation of tabersonine into vindoline (section **I.1.2.5**) involves 3 hydroxylations, an *O*-methylation, a *N*-methylation and an *O*-acetylation which occur in a specific order depicted in Figure 4. Five enzymes involved in this pathway (16-OH, 16-OMT, NMT, D4H and DAT) have been characterized. Expression of most of these activities appears to be confined to leaves, stems, cotyledons and hypocotyls (De Carolis et al., 1990; De Luca et al., 1987; 1988; St-Pierre and De Luca, 1995). The levels of T16H, D4H and DAT activities are very low or absent in dark grown seedlings and light treatment induces a 6-, 8- and 9-fold increase of the respective enzyme activities (De Carolis et al., 1990; De Luca et al., 1985; 1986; 1988; St-Pierre et al., 1998), which coincides with the accumulation of vindoline (Aerts et al., 1994; 1996; De Luca et al., 1986).

The phytochrome photoreceptor may be involved in the light activation of D4H and DAT, since treatment of etiolated seedlings with short pulses of red light could replace the white light required to induce these enzyme activities (Aerts and De Luca, 1992; De Carolis, 1994). Red light activation of D4H and DAT could be reversed with a subsequent exposure to far red light and re-induction was achieved by re-exposure to red light (Aerts and De Luca, 1992; De Carolis, 1994). Although the light-mediated control of D4H and DAT differentiates them from the majority of enzymes in the rest of this pathway, each enzyme also had different minimal light requirements for full activation. A short 5 min red light pulse (Aerts and De Luca, 1992) was sufficient to fully activate DAT, whereas a 30 min red light treatment was required to completely activate D4H (De Carolis, 1994).

I.3.4. Modulation of vindoline biosynthesis by chemical effectors.

The external application of auxin and ABA growth regulators (Aerts et al., 1992; 1996), or of chemical inducers, such as methyl jasmonate and salicylic acid (Aerts et al.,1994; 1996), can alter the pattern of enzyme activity and alkaloid accumulation in developing seedlings.

I.3.4.1. Jasmonate enhancement of alkaloid biosynthesis.

The exposure of 3 day old light grown seedlings to jasmonate vapors for 96 h caused a more than 200% increase in the accumulation of catharanthine, tabersonine and vindoline, together with an increase in TDC, SS, D4H and DAT enzyme activities (Aerts et al., 1994). However, the developmental profile for alkaloid accumulation and the timing of appearance of each enzyme activity remained unaltered by this treatment (Aerts et al., 1996). Furthermore, the stoichiometry between the concentrations of catharanthine, tabersonine and vindoline remained the same as in untreated seedlings (Aerts et al., 1994; 1996). These data indicate that the enhancing effects of jasmonate on alkaloid metabolism occurs within the framework of the developmental program which regulates this pathway (Aerts et al., 1994). The modulatory effects of jasmonate on alkaloid biosynthesis appeared to be specific, since other chemical inducers of secondary metabolism, including salicylic acid, did not have this effect (Aerts et al., 1996). However, the application of a jasmonate metabolic precursor or of an inhibitor of jasmonate synthesis did not affect the alkaloid profile in seedlings. These results suggest that jasmonate may not be part of a signaling cascade which triggers alkaloid biosynthesis during seedling development (Aerts et al., 1996). Nevertheless, jasmonate may be involved in the additional alkaloid accumulation occurring as a response to injury or to other types of stresses (Farmer and Ryan, 1992; Frischknecht et al., 1987).

Even when jasmonate treatment caused an important accumulation of catharanthine and vindoline, the levels of vinblastine remained unaffected in comparison with seedlings of untreated controls (Aerts et al., 1996). The coupling of catharanthine and vindoline into dimers will, therefore, not necessarily increase simultaneously when the levels of these 2 alkaloids increase. A possible explanation for this may be due to

undetermined differences in the mechanisms controlling the formation of the monomeric subunits and their subsequent coupling into AVBL.

I.3.4.2. Auxin effects on vindoline biosynthesis.

Auxins supplied externally to 3 day old seedlings produced a noteworthy increase in immunologically detectable TDC proteins and in TDC activity in radicles. In cotyledons and hypocotyls, 2,4-dichlorophenoxyacetic acid (2,4-D), but not indolbutyric acid (IBA), extended the developmental period when high TDC antigen and enzyme activity could be detected (Aerts et al., 1992). In contrast to the positive effects observed with expression of TDC, both auxins seemed to delay and reduce the light-mediated induction of DAT (Aerts et al., 1992). Perhaps these effects were associated with the morphological changes produced by auxin treatment, which included hypocotyl shortening and thickening. The morphological changes described for auxin treated seedlings are similar to those occurring in the early stages of callus formation (Carew, 1975), which also have drastic effects on alkaloid metabolism (Morris et al., 1986; see section **I.2.2.2.A**).

1.3.5. Cellular and subcellular location of the enzymes involved in alkaloid biosynthesis.

I.3.5.1. Cell types involved in alkaloid metabolism.

Cytochemical and immunolocalization studies of vindoline have suggested that it is accumulated in vacuoles (Brisson et al., 1992), which coincides with the previously reported localization of the dimeric indole alkaloids (McCaskill et al., 1988). However these studies failed to reveal if particular leaf cells were involved in vindoline biosynthesis. *Catharanthus roseus* possesses unbranched laticifer cells which are distributed throughout leaves, stems and fruits (Mersey and Cutler, 1986; Eilert et al., 1985; Yoder and Mahlberg, 1976). Laticifers are specialized cell types containing latex and other excretory substances (Fahn, 1988). These cells were shown to react specifically with histochemical alkaloid dyes (Yoder and Mahlberg, 1976) and the reaction products present a characteristic emission spectra when cells are observed by

epifluorescence microscopy (Mersey and Cutler, 1986). In addition to these laticifers, *Catharanthus* leaves and fruits also have idioblasts (Constabel, 1982; Mersey and Cutler, 1984). Idioblasts are specialized cells occurring in several plant families which are probably morphologically related to laticifers (Fahn, 1988). Such cell types are usually associated with the biosynthesis and accumulation secondary products (Eilert et al., 1986; Platt and Thomson, 1992; Postek and Tucker, 1983). Neither laticifers nor idioblasts appear to be produced in cell cultures (Eilert et al., 1985) and in roots (Yoder and Mahlberg, 1976) and it has been speculated that the lack of these structures may account for the inability of cell and root cultures to produce vindoline.

I.3.5.2. Subcellular localization of enzymes involved in vindoline biosynthesis.

The enzymes involved in vindoline biosynthesis occur in different subcellular compartments. Biochemical and molecular studies suggest that TDC, strictosidine glucosidase, OMT, D4H and DAT are cytosolic enzymes (De Luca and Cutler, 1987; De Carolis and De Luca, 1993; Stevens et al., 1993; St-Pierre and de Luca, 1995). In contrast, the predicted amino acid sequence of SS includes a putative transit peptide characteristic for proteins localized in plant vacuoles (McKnight et al., 1990). Immunological studies in *Catharanthus roseus* and in SS expressing transgenic tobacco confirmed the vacuole localization of this enzyme (McKnight et al., 1991). The cytochrome P-450 dependent monooxygenases, G10H (Madyastha and Coscia, 1979) and 16-OHT (St-Pierre and De Luca 1995) were localized in microsomal fractions, in agreement with the well-known association of this family of mono-oxygenases with the external face of the endoplasmic reticulum (Donaldson and Luster, 1991). The NMT appears to be tightly associate with thylakoid membranes of mature chloroplasts (De Luca and Cutler, 1987; De Luca et al., 1987; Dethier and De Luca, 1994). However, since high levels of NMT can be detected in etiolated seedlings (De Luca et al., 1988; section **II.1.1**), complete chloroplast development may not be absolutely required for expression of this activity (De Luca et al., 1988). In fact, the NMT activity of etiolated seedlings was associated with both endoplasmic reticulum and plastid markers (De Luca and Cutler, 1987; De Luca et al., 1988). Although the significance of the latter results remain to be explained, a complex traffic of alkaloid pathway intermediates within the cell is suggested by the participation of the cytosol, endoplasmic reticulum,

plastid and vacuole compartments in vindoline biosynthesis. The mobilization of precursors from one compartment to another may, therefore, be an additional component in the regulation of this pathway (De Luca et al., 1992).

I.4. Concluding Remarks.

This short review illustrates the close relationship which appears to exist between plant development and induction of monoterpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. This association is particularly evident in the case of vindoline biosynthesis, which apparently requires a certain degree of tissue organization, as suggested by the inability of cell cultures to synthesize this alkaloid , or by the precise developmental stages where it can be detected during seedling germination and development. The participation of light is also critical for the process of vindoline biosynthesis. The close association between light, development and the onset of vindoline biosynthesis observed in *Catharanthus*, suggests that these components may operate in a coordinate manner. Since the developmental regulation of vindoline biosynthesis appears to be exerted mainly through the controlled expression of the terminal hydroxylase and *O*-acetyltransferase, these enzymes could be used as biochemical markers for vindoline biosynthesis.

The developmental and light induction of D4H activity has previously been studied at the level of enzyme activity (De Carolis et al., 1990) and the present study focuses on the molecular regulation of D4H. In order to gain a better understanding of the regulatory levels which control D4H expression, the present thesis proposes to perform complementary molecular, immunological and enzymatic studies of D4H. The identification of the components involved in the activation of this enzyme by light would help to understand the nature of the relationship between vindoline biosynthesis and light-induced development in *Catharanthus* seedlings. The results obtained indicate that a complex ontogenic program may be involved in controlling the level of this enzyme through a multilevel regulatory process.

Chapter II. Molecular Cloning and Characterization of Desacetoxyvindoline 4-Hydroxylase. A 2-Oxoglutarate Dependent-Dioxygenase Involved in the Biosynthesis of Vindoline in *Catharanthus roseus* (L.) G. Don.

Objectives.

General objective.

1) To isolate and characterize D4H cDNA and genomic clones.

Specific objectives.

1) To complete the characterization of D4H cDNA clones previously isolated by De Carolis (1994).
2) To isolate and characterize the *d4h* gene.
3) To compare the D4H protein sequence and *d4h* gene to other 2-oxoglutarate dependent dioxygenases.
4) To express active D4H protein in in *Escherichia coli* cells.
5) To estimate *d4h* gene copy number.
6) To analyse the tissue and cellular distribution of D4H protein.
7) To analyse the effect of light and development on expression of D4H.

Publication of Chapter II: *Plant Molecular Biology* **34:** 935-948 (1997). Emidio De Carolis isolated and partially characterized 3 D4H cDNA clones. Anne-Marie Alarco made the *Catharanthus roseus* genomic library and helped to sequence one cDNA clone (*cD4H-3*).

MOLECULAR CLONING AND CHARACTERIZATION OF DESACETOXYVINDOLINE 4-HYDROXYLASE. A 2-OXOGLUTARATE DEPENDENT-DIOXYGENASE INVOLVED IN THE BIOSYNTHESIS OF VINDOLINE IN *CATHARANTHUS ROSEUS* (L.) G. DON.

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ABSTRACT.

A 2-oxoglutarate-dependent dioxygenase (EC 1.14.11.11) which catalyzes the 4-hydroxylation of desacetoxyvindoline was purified to homogeneity (according to DeCarolis and De Luca, *J. Biol. Chem.* **268**, 5504-5511). Three oligopeptides isolated from a tryptic digest of the purified protein were microsequenced and one oligopeptide showed significant homology to hyoscyamine 6ß-hydroxylase from *Hyoscyamus niger*. A 36-mer degenerate oligonucleotide based on this peptide sequence was used to screen a *C. roseus* cDNA library and three clones, *cD4H-1* to *3* were isolated. Although none of the three clones were full length, the open reading frame of each clone encoded a putative protein containing the sequence of all three peptides. Primer extension analysis suggested that *cD4H-3*, the longest cDNA clone, was missing 163 bp at the 5' end of the clone and sequencing of the genomic clone, *gD4H-8,* confirmed these results. Southern blot analysis suggested that *d4h* is present as a single copy gene in *C. roseus* which is a diploid plant, and the significant differences in the sequence of the 3'UTR between *cD4H-1* and *-3* suggested that they represent dimorphic alleles of the same hydroxylase. The identity of the clone was further confirmed when extracts of transformed *Escherichia coli* expressed D4H enzyme activity. The D4H clone encoded a putative protein of 401 amino acids with a calculated molecular mass of 45.5 kDa and the amino acid sequence showed a high degree of similarity with those of a growing family of 2-oxoglutarate-dependent dioxygenases of plant and fungal origin. The similarity was not restricted to the dioxygenase protein sequences but was also extended to the gene structure and organization since the 205 and 1627 bp introns of *d4h* were inserted around the same highly conserved amino acid consensus sequences as those for *e8* protein, hyoscyamine 6ß-hydroxylase and ethylene forming enzyme. These results provide further support that a common ancestral gene is responsible for the appearance of this family of dioxygenases.

Hydroxylase assays and RNA blot hybridization studies showed that enzyme activity followed closely the levels of *d4h* transcripts, occurring predominantly in young leaves and in much lower levels in stems and fruits. In contrast, etiolated seedlings which contained considerable levels of *d4h* transcripts had almost undetectable hydroxylase activity, whereas exposure of seedlings to light resulted in a rapid increase

of enzyme activity without a significant further increase in *d4h* transcripts over those detected in dark grown seedlings. These results suggest that the activating effect of light may occur at a point downstream of transcription which remains to be elucidated.

Key Words: *Catharanthus roseus*, dioxygenases, indole alkaloids, molecular regulation, secondary metabolism.

INTRODUCTION.

The cytotoxic dimeric indole alkaloids vinblastine and vincristine, which accumulate in the leaves of *Catharanthus roseus*, are composed of catharanthine and vindoline monomers (Svoboda and Blake, 1975). Vinblastine is employed in the treatment of Hodgkin's disease while vincristine is used to treat certain types of leukemia (Jonhson et al., 1960). The pharmaceutical importance of these dimers, together with the high cost of extraction and purification of these products from the plant, have prompted many efforts to produce them in *Catharanthus* cell culture systems. These efforts which resulted in the successful production of high catharanthine-accumulating cell lines, failed to produce lines which also accumulated vindoline monomers (Moreno et al., 1995; van der Heijden et al., 1989). Further studies have shown that the genetic potential for the biosynthesis of vindoline is not lost, but only repressed in cell cultures, since vindoline accumulation reappears concomitantly with shoot regeneration (Constabel et al., 1982).

The early stages of vindoline biosynthesis involve the formation of tryptamine from tryptophan followed by the condensation of tryptamine with secologanin to produce the central intermediate, strictosidine. The enzymes catalyzing these two reactions are tryptophan decarboxylase (TDC) and strictosidine synthase (SS), respectively. Studies with developing *Catharanthus* seedlings have suggested that the appearance of TDC and SS activities do not coincide with vindoline accumulation (Alvarez-Fernandez et al., 1989; De Luca et al., 1986) and that the post-tabersonine pathway is under additional tissue-specific and developmental control compared to earlier stages of vindoline biosynthesis. Transformation of strictosidine into tabersonine requires the participation of several enzymes and hydroxylation at C-16, 16-*O*-methylation, hydration of the 2,3-double bond, *N*(1)-methylation, hydroxylation at C-4, and 4-*O*-acetylation (Fig. 4; De Luca et al., 1986) is required to convert tabersonine into vindoline. The first of these reactions is catalyzed by tabersonine 16-hydroxylase (T16H), a cytochrome P-450 dependent monooxygenase associated with the microsomal fraction while the following *O*-methylation apparently occurs in the cytosol and it is catalyzed by AdoMet:16hydroxytabersonine *O*-methyltransferase (16-OMT; St-Pierre and De Luca, 1995). The third to last reaction in the pathway is catalyzed by an AdoMet:2,3-dihydro-

3-hydroxytabersonine-*N*-methyltransferase (NMT) associated with the thylakoid membrane (De Luca et al., 1987; Dethier and De Luca, 1993), whereas 4-hydroxylation is catalyzed by a soluble 2-oxoglutarate dependent dioxygenase named desacetoxyvindoline 4-hydroxylase (D4H; De Carolis et al., 1990; De Carolis and De Luca, 1993) and the final step in vindoline biosynthesis is catalyzed by a cytosolic Acetyl-CoA: deacetylvindoline-*O*-acetyltransferase (DAT; De Luca et al., 1985; Power et al., 1990).

Catharanthus cell suspension cultures which contain T16H and 16-OMT enzyme actitivities (St-Pierre and De Luca, 1995), lack the enzymes responsible for the last three steps in vindoline biosynthesis (De Carolis et al., 1990; De Luca et al., 1987; Appendix III) and this may explain why cell cultures fail to accumulate vindoline and its immediate precursors, desacetoxyvindoline and deacetylvindoline (Kurz et al., 1980; Kutney et al., 1980). Similarly, dark grown *C. roseus* seedlings accumulate high levels of tabersonine together with traces of other post-tabersonine intermediates as well as low levels of vindoline (Balsevich et al., 1986; De Luca et al., 1986). Exposure of etiolated seedlings to light induces D4H and DAT enzyme activities in cotyledons (De Carolis et al., 1990; De Luca et al., 1986; De Luca et al., 1989) and the appearance of these enzymes results in the quantitative conversion of tabersonine and intermediates into vindoline (Balsevich et al., 1986; De Luca et al., 1986). The induction of D4H (De Carolis, 1994) enzyme activities and the quantitative conversion of tabersonine and intermediates into vindoline also occured when etiolated seedlings were treated for short periods with red light. The likely involvement of the phytochrome photoreceptor in this process was shown when far red light treatment reversed the effect of red light. In addition, treatment of developing seedlings with methyl jasmonate (Aerts et al, 1994) and auxin growth regulators (Aerts et al, 1992) also modulated the levels of several enzymes in the pathway for vindoline biosynthesis and affected the accumulation of pathway intermediates as well as vindoline. The studies performed with *Catharanthus* cell cultures and with developing seedlings illustrate the additional development-, tissue- and environment-specific controls which appear to regulate the late steps in vindoline biosynthesis.

Recently, D4H (EC.1.14.11.11) was purified to homogeneity and characterized in our laboratory (De Carolis et al., 1990; De Carolis and De Luca, 1993). This 2-oxoglutarate dioxygenase typically requires alkaloid substrate, 2-oxoglutarate, ascorbate, ferrous ions and molecular oxygen for activity (De Carolis et al., 1990; De Carolis and De Luca, 1993). This member of a growing number of dioxygenases belongs to a subclass of genes which include anthocyanidin synthase (Davis, 1993), hyoscyamine 6ß-hydroxylase (H6H; Matsuda et al., 1991), flavonol synthase (Holton et al., 1993), and flavanone 3ß-hydroxylase (Bristsch et al., 1993). A second subclass of dioxygenases which include ethylene forming enzyme (EFE; Holdsworth et al., 1987a), gibberellin 20-oxidase ($G_{20}O$; Phillips et al., 1995) and a fungal isopenicillin *N*-synthase (IPNS; Ramon et al., 1987), do not require 2-oxoglutarate as a cosubstrate. Amino acid sequence comparisons from dioxygenases of both subclasses reveal a minimum 30% amino acid identity and 45% amino acid similarity which is generally shared throughout most of the protein sequence (De Carolis and De Luca, 1994). In addition, the initial event in the reaction mechanism of both subclasses of dioxygenase, involves the formation of a ferryl oxidant by the binding of dioxygen to the non-heme iron in the active site of the enzyme (Ming et al., 1991).

Using internal peptide sequences from the purified D4H, degenerate oligonucleotide probes were synthesized in order to isolate a cDNA clone for D4H. The identity of this clone was confirmed after identifying all three peptides in the predicted amino acid sequence of the cDNA clone and by the heterologous expression of active D4H in *E. coli*. This paper describes the molecular cloning and characterization of D4H and its position in a growing family of plant dioxygenases. The availability of this clone represents a unique complementary tool to previously isolated vindoline pathway genes (Kutchan et al., 1988; McKnight et al., 1990; De Luca et al., 1989) for the study of vindoline biosynthesis in this interesting medicinal plant.

MATERIALS AND METHODS.

Plant material. *Catharanthus roseus* (L.) G. Don cv. Little Delicata plants (W.H. Perron, Laval, Québec) were grown under greenhouse conditions. For the time course of D4H expression, seedlings were germinated in the dark at 25 °C and 80% relative humidity (De Luca et al., 1986). For the light treatments, seedlings were exposed for specified times (Fig. 7) to a 18 h photoperiod (10 µmol m^{-2} s^{-1}) after 7 days of dark growth. Seedlings were collected, frozen in liquid nitrogen and kept at -80 °C until analysis.

Screening of cDNA and genomic libraries. The purified D4H (De Carolis and De Luca, 1993) was submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically from the gel onto nitrocellulose and the protein was submitted to *in situ* enzymatic cleavage (Aebersol et al., 1987). Trypsin digestion was performed in 0.1 M ammonium bicarbonate (pH 8.2) containing 0.1 M calcium chloride for 16 h at room temperature (enzyme/substrate ratio 1:20). Tryptic peptides were fractionated by reverse-phase high pressure liquid chromatography (HPLC) on a Vydac C-18 column (2.1 x 150 mm; Alltech Inc, Deerfield IL) and eluted with a linear 0-60% acetonitrile-isopropanol 1:1 (v/v) gradient in 0.1% trifluoroacetic acid. Three peptides (# 48, 61, and 92) were selected for sequencing by automated Edman degradation. Enzyme digestion, chromatography and sequencing services were performed by Harvard Microchemistry, Harvard University, Cambridge, Mass. Since peptide # 48 (ELISEENPPIYK) showed significant homology to amino acids 304-316 of H6H from *Hyoscyamus niger* (Matsuda et al., 1991), a degenerate oligonucleotide based on this peptide was used to screen the cDNA library. Inosine was incorporated when convenient to reduce the final degeneracy of the probe, which was 144. The sequence of the 36 mer oligonucleotide probe, named 48c, was:

5'GAGTTIAT(H)(W)(S)(I/C)GAGGAGAATCCICC(I/C)AT(H)TATAAG**3'**

The oligonucleotide was end labeled with [^{32}P]ATP and was used to screen a λ *ZAP* cDNA library made from light-induced *C. roseus* seedlings (De Luca et al., 1989). Approximately 2 x 10^5 pfu were screened using standard protocols (Sambrook et al.,

1989) and three clones, *cD4H-1* to *3* were isolated according to the manufacturer's instructions (Stratagene, San Diego, CA). An EMBL3 (Clonetech, Palo Alto, CA) genomic library was constructed from DNA isolated from *C. roseus* which had been partially digested with *Mbo*I (Sambrook et al., 1989). A primary library of 1.0 x 10^7 plaques was obtained and 2 x 10^5 pfu of the amplified library were screened with a 1.2 kb *EcoR*I fragment isolated from *cD4H-3* (Fig. 5). Genomic fragments were cloned into the *Sal*I site of pBluescript II (SK^+) (Stratagene) for further characterization (Sambrook et al., 1989).

DNA sequencing. Putative D4H genomic and cDNA clones were sequenced on both strands by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a commercial kit (USBiochemicals, Cleveland, OH) and synthetic oligonucleotide primers (16-18 mers).

Sequence alignment. Sequences of other dioxygenases were obtained from the literature and from the GenBank data base (home page: http://www.ncbi.nlm.nih.gov). Alignment of D4H with the other dioxygenases was made using the MultiAlin software package (Corpet, 1985), which is based in the FASTP algorithm (Lipman and Pearson, 1985).

Isolation and analysis of nucleic acids. Genomic DNA was isolated from *C. roseus* leaves (Murray and Thompson, 1980) digested with different restriction enzymes and separated on 0.8% agarose gels. After electrophoresis, the digested DNA was depurinated with 0.2 N HCl and then denatured and transfered by capillarity to nylon membranes (Hybond-N+, Amersham, Airlington Heights, IL) in the presence of 0.4 M NaOH (Sambrook et al., 1989). The blots were hybridized under high stringency conditions (65°C in 0.25M sodium phosphate buffer, pH 8.0, 7% SDS, 1% BSA, 1mM EDTA) with the 1.2 kb *EcoR*I fragment from *cD4H-3* (Fig. 5) labeled by random priming with [^{32}P]dCTP (Feinberg and Vogelstein, 1984). After hybridization, the blots were washed at 55°C, once with 4X SSC, 0.1% SDS, then with 2X SSC, 0.1% SDS and finally twice with 0.2X SSC, 0.1% SDS (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) (Sambrook et al., 1989). Blots were autoradiographed with an intensifying screen on Fuji RX 100 X-ray film at -80°C.

Total RNA was prepared from different tissues of mature 6 month old plants or from developing seedlings by phenol/chloroform extraction followed by lithium chloride precipitation (Jones et al., 1985). Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography (Gubler and Hoffman, 1983). For RNA blots, 4 mg of poly(A)$^+$ RNA or 10 mg of total RNA were fractionated on 1% agarose gels containing formaldehyde and transferred by capillarity to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) with 10X SSC. Hybridization probes, hybridization conditions, washes and autoradiography methods used for the RNA blots were identical to those used for DNA blots described above.

Heterologous expression of D4H activity in *E. coli*. Since clone *cD4H-3* had the longest open reading frame (ORF), it was selected for heterologous expression. However, the D4H ORF in this clone was in a -1 reading frame in respect to the ß-galactosidase promotor of the vector (pBluscript). This clone also contained additional unrelated cDNAs attached to the 3' end which were eliminated by digestion of the clone with *BstX*I. A 1.2 kb (Fig. 5) fragment which included an additional 44 bp segment between the *BstX*I and *EcoR*I sites of the pBluescriptII polylinker (Appendix I), was isolated after purification by PAGE (Sambrook et al., 1989). After filling the ends with free nucleotides in the presence of T4 DNA polymerase, the DNA fragment was digested with *Xba*I and subsequently inserted back into pBluescriptII (SK$^+$) between *Xba*I and *EcoR*V in order to to regain the multiple cloning site. This new construct, *cD4H-3A* (Appendix I) which contained the complete ORF of D4H plus 53 nucleotides of the 3' untranslated region (3'UTR), was digested with *BamH*I and *Sal*I, and the resulting restriction fragment was directionally cloned into the expression vector pQE30 (QIAgene, Chatsworth, CA). Plasmids carrying the *D4H* insert (*pQD4H-19*) also contained the *BamH*I-*EcoR*I fragment of the pBluescriptII (SK$^+$) polylinker which encode eight amino acid belonging to the N terminus of the b-galactosidase polypeptide (Appendix I). *E. coli* cells BB4 (Stratagene) harboring *pQD4H-19* were grown at 37 °C in 60 ml of Luria-Bertani medium to OD_{600}=0.5, and expression of the protein was induced by addition of isopropyl-1-thio-ß-D-galactopyranoside to a final concentration of 3 mM. Cells were collected by centrifugation after 3 hours of incubation with the inducer at 30°C. The cell pellet was washed once and then resuspended with 2 ml of the

extraction buffer (Tris/HCl 200 mM, pH 7.5, EDTA 5 mM, DDT 10 mM). Extractions were performed by sonicating (Bronson Ultrasonic Corporation, Danbury, CT) freeze-thawed bacterial suspensions. Sonicated extracts were desalted in PD-10 columns (Pharmacia, Uppsala Sweden) and protein was eluted with Tris-HCl 50 mM, pH 7.5 containing 28 mM 2-mercaptoethanol.

D4H enzyme assay. D4H was assayed by the direct method as described by De Carolis et al (1990). The labeled alkaloid substrate ([^{14}C]H_3-*N*-desacetoxyvindorosine) was prepared enzymatically (De Luca et al., 1987). The reaction mixture contained 0.56 nmol of the labeled alkaloid substrate (containing 44,600 dpm), 10 mM 2-oxoglutarate, 7.5 mM ascorbate, 0.1 mM Tris-Cl, pH 7.5 and protein extract in a final volume of 150 ml. After incubation at 30°C for 30 min, the reaction was stopped with 100 ml of 1 M NaOH and the products were extracted twice with ethyl acetate. The unreacted substrate (R_f 0.52) was separated from product (R_f 0.22) by thin layer chromatography (silica plates 0.2 mm, Merk, Darmstadt, Germany) using 10% methanol in ethyl acetate as solvent. The chromatograms were visualized under UV light (366 nm) and the spots corresponding to the product were scraped from the plates and counted in an aqueous-based scintillation fluid (Optiphase Hisafe II, LKB/Pharmacia).

RESULTS.

Characterization of the D4H clones. Three cDNA clones named *cD4H-1*, *cD4H-2* and *cD4H-3*, were isolated by screening the λ *ZAP* cDNA library (2 x 10^5 pfu) with oligonucleotide probe 48c end-labeled with [^{32}P]ATP. The size of each clone was unusually large for the size of D4H protein (De Carolis et al., 1990), approximadely 3 kb in size and this was due to the presence of additional unrelated cDNA sequences (Appendix I), which is a characteristic of this cDNA library (De Luca et al., 1989). Sequencing each clone revealed that *cD4H-3* contained the longest insert corresponding to the cDNA which we were interested in (Fig. 5). The cDNA insert corresponding to the *d4h* transcript in the *cD4H-3* clone was 1626 bp in length where 1160 bp encoded a putative ORF of 386 amino acids. The 3'-untranslated region (3'UTR) was 463 bp in length and included a 24 base poly(A)$^+$ tail. The corresponding clones, *cD4H-2* and *cD4H-1*, were shorter than *cD4H-3* at the 5'-end by 11 and 29 bp, respectively. The sequence of *cD4H-2* was otherwise identical to *cD4H-3* whereas *cD4H-1* differed significantly in the sequence of the 3'UTR which was 306 bp long and included an 8 base poly(A)$^+$ tail (Appendix I). Sequence analysis or the 3'UTR revealed that *cD4H-1* was missing 163 nucleotides and contained two extra bases before the start of the poly(A) tail compared to *cD4H-3* (Appendix I). The putative polyadenylation signals (Joshi, 1987a) were identical for both sequences (Fig. 5; Appendix I) and comparisons of the 3'UTRs of the 2 sequences revealed that unlike the upstream regions which were absolutely conserved, only 64% of the bases were identical. The predicted ORFs of *cD4H-1* and *-3* were absolutely identical with both clones encoding a predicted amino acid sequence containing the three tryptic peptides obtained from amino acid sequencing (Peptides # 91, DLNFHAATLSEEESLR; # 62, YGFFQIVNHGIPQDVMAK and # 48, ELISEENPPIYKE) beginning at amino acids 28, 120 and 361, respectively (Figure 5).

Primer extension analysis which was performed to identify if *cD4H-3* represented a complete *d4h* transcript, revealed that the clone was missing 163 bp at the 5' end of the clone (Appendix I). In order to obtain the complete *d4h* transcript sequence, a genomic library was screened using *cD4H-3* as a probe and seven clones (11 to 30 kb in size) out of 2 x 10^5 pfu analyzed were obtained. Three putative genomic clones of

d4h were sub-cloned into the *Sal*I site of pBluescriptII (SK^+) and characterized. A single genomic clone called *gD4H-8* (11 kb in size), contained the complete gene including 4 kb at the 5' end (Fig. 6). DNA sequencing of this clone confirmed that *cD4H-3* was missing the first 15 amino acids and that the putative transcription start site appears to be at -113 bp. The sequence around the first methionine initiation codon (AAAAATGCC) matched in five out of eight bases normally occurring in typical plant consensus sequences (Joshi, 1987b). The clone possesses no identifiable signal peptide which confirms D4H to be a cytosolic protein (De Carolis and De Luca, 1993). Considering the additional sequence obtained from analysis of the *gD4H-8*, the estimated size of the *d4h* transcript was determined to be 1782 bp while the predicted *Mr* of 45.5 kDa matched closely the *Mr* of 45 kDa obtained for the purified protein as determined by high performance gel filtration and by SDS-PAGE (De Carolis et al., 1990; De Carolis and De Luca, 1993).

The genomic clone also contained a 205 bp and an 1627 bp intron which are located at the equivalent positions of 729 and 1052, respectively of the *d4h* transcript (Figs. 5 and 6). The two introns occurred within corresponding stretches of amino acids which are highly conserved in three other available plant dioxygenases genes (Kanegae et al., 1994; Appendix I). The sequence of *gD4H-8* corresponding to the 3'UTR of *cD4H-2* and *-3* was identical and confirmed this to be the parent gene of these cDNA clones. The genomic clone of *cD4H-1* remains to be isolated.

Similarity of D4H with other dioxygenases. The amino acid sequence of D4H was aligned with those of five representative plant dioxygenases and one fungal dioxygenase using the MultiAlin program (Corpet, 1985). Although the areas of amino acid similarity to the other dioxygenases extended throughout the protein, areas of greater homology were observed in the C-terminus domain of D4H (Fig. 7). The overall D4H amino acid identity with the other dioxygenases ranked between 27% for IPNS from *Aspegillus nidulans* and 33% for $G_{20}O$ from *Arabidopsis thaliana* and anthocyanidin synthase from apple whereas the amino acid similarity varied between 48% for IPNS to 54% for $G_{20}O$. Highly conserved residues include Ala_{106}, Gly_{111}, Gly_{117}, His_{116}, His_{268} His_{324}, Pro_{257}, Leu_{285}, Gln_{286} and Arg_{334}. Identical residues at equivalent positions have been identified in other 23 dioxygenases from plants, fungi and bacteria

(Bristsch et al., 1993). The high conservation of key amino acid residues for proteins involved in such a diverse set of oxidative reactions which may or may not require 2-oxoglutarate, indicates that these types of enzymes may have diverged from a common ancestral gene. The triad His_{268}-X-Asp_{270}, together with His_{324} deserve special attention since in IPNS, they represent the binding site for ferrous ions (Chen et al., 1989; Ming et al., 1991, Roach et al., 1995). Hydropathy plots of the enzymes aligned in figure 7 indicate that those residues are located within highly conserved hydrophilic stretches which would make them accessible to water and ions. Nevertheless, crystallographic analysis of recombinant IPNS from *A. nidulans*, has revealed that the iron-binding site is located within a hydrophobic pocket (Roach et al., 1995). The strict conservation of the same amino acids for binding ferrous ions between members of this dioxygenase family and the similar mechanism proposed for dioxygen activation (Chen et al., 1989; Ming et al., 1991) suggests that this hydrophobic pocket is likely to be a ubiquitous feature of this type of enzyme. This is also suggested by biochemical studies on the effect of diethyl pyrocarbonate on flavanone 3ß-hydroxylase (Bristsch et al., 1993) and on D4H (De Carolis, 1994) which imply that histidine residues are involved in the catalytic activity of these enzymes. This hypothesis was recently confirmed when site-specific mutagenesis of histidine residues of IPNS from *Cephalosporium acremonium* resulted in the elimination of enzyme activity (Tan and Sim, 1996).

***In vitro* expression of D4H.** The *cD4H-3* clone containing the longest ORF was chosen for heterologous expression by subcloning it into the expression vector pQE-30 to produce *pQD4H-19*, and expressing this vector in BB4 *E. coli* cells. In spite of missing the first 15 amino acid residues, the recombinant D4H protein was active and indicated that these residues are not essential to produce a functional enzyme. The functionality of the heterologously expressed protein was confirmed in enzyme assays showing the conversion of alkaloid substrate into product in the presence of cosubstrates and cofactors. The removal of O_2, 2-oxoglutarate or ascorbate from the reaction mixture abolished the activity of the recombinant protein, as well as D4H extracted from *C. roseus* leaves (Table 2). The addition of ferrous ions to desalted D4H preparations isolated from either bacteria and or leaves (De Carolis and De Luca, 1993) did not increase enzyme activity, whereas the addition of ferrous ions to leaf preparations was

absolutely necessary for enzyme activity following a dye-ligand affinity chromatographic step (De Carolis and De Luca, 1993).

Gene copy number of *D4H*. Southern blots of genomic DNA isolated from *C. roseus* plants were probed with a 1.2 kb *EcoR*I fragment containing 800 bp encoding part of the ORF and the complete 3'UTR of *cD4H-3* (Fig. 5; Appendix I). Single hybridization bands of 10, 4.6, 2.8 and 4 kb sizes were observed for *C. roseus* DNA digested with *Xba*I, *Hind*III, *EcoR*V and *EcoR*I, respectively (Fig. 8). The size of the fragment detected for the *EcoR*V digest corresponds to the *EcoR*V restriction fragment size obtained from sequencing *gD4H-8*. When the blot was probed with the 1.2 kb *BstX*I fragment from *cD4H-3* (Appendix III) additional hybridization bands were present for the *Hind*III and *EcoR*I restriction pattern (discussed in Appendix III). The pattern obtained was that expected from the restriction profile deduced from the sequence of the genomic clone which suggests that *d4h* is present as single copy gene in the diploid genome of *C. roseus*. In this way, the differences observed between the sequences of clone *cD4H-1* and clones *cD4H-2* and *-3* may be a cloning artifact or may be resulted from the isolation of dimorphic alleles. To rule out the possibility of such an artifact, the region accounting for these different nucleotide sequences was amplified by polymerase chain reaction using reverse transcribed RNA isolated from seedlings (RT-PCR; Frohman, 1990). Two products of the expected sizes (461 and 529 bp for the transcripts represented by *cD4H-1* and *cD4H-3,* respectively) were obtained. Futhermore, an oligonucleotide complementary to common sequences in both clones hybridized to the two PCR products (Appendix I). These results indicated that *cD4H-1* and *-3* represent actual transcripts occurring in *C. roseus* seedlings.

The genes involved in the early stages of vindoline biosynthesis, such as *tdc* (Goddijn et al., 1994), *ss* (Pasquali et al., 1992) as well as in the later stages, such as *dat* (St-Pierre et al, in preparation) were also shown to occur as single copy genes.

Tissue specific expression of D4H. Vindoline, deacetylvindoline and desacetoxyvindoline have only been shown to occur in the aerial parts of *C. roseus* plants (De Luca et al., 1986, Westerkemper et al., 1980). Enzyme assay of D4H in different tissues showed that by far the highest activity occurred in leaves, whereas

stems and fruits had only about 8 and 5% of the activity found in leaves, respectively (Fig. 9). When flowers and roots were assayed for D4H activity, none was detected. The *d4h* transcripts, which followed closely the pattern obtained with enzyme assays, were mainly detected in leaves, with significantly lower levels occurring in fruits as well as in stems and none were detected in flowers or roots (Fig. 9).

Catharanthus roseus cell cultures can be induced to express high levels of *tdc* and *ss* transcripts as well as enzyme activity and to accumulate indole alkaloids under suitable conditions (Eilert et al., 1986; Roewer et al., 1992). Submission of these cultures to growth on maintenance medium ($1B_5$ plus 1 mg/L 2,4-dichlophenoxyacetic acid; Gamborg et al., 1968) or to biotic stress with a *Phythium aphidermatum* elicitor (Roewer et al., 1992) or to methyl-jasmonate vapours (1 ppm) did not, however, induce *d4h* transcript accumulation nor was D4H enzyme activity detected under any of the conditions tested (Appendix III) .

Developmental and light regulation of D4H. Although vindoline and deacetylvindoline may be detected in dark grown seedlings, they only occur in trace amounts at later stages of seedling development (De Luca et al., 1986). Since light plays a critical role in the regulation of the late stages of vindoline biosynthesis (De Carolis et al., 1990; De Luca et al., 1986) and its accumulation (Balsevich et al., 1986), the role of light in the activation of D4H during seedling development was investigated. *C. roseus* seedlings were germinated and were either grown under continuous darkness or they were transferred to a 18 h. photoperiod after seven days of growth. Low levels of D4H enzyme activity were found in etiolated seedlings (Fig. 10), whereas light treatment resulted in a rapid increase in enzyme activity which was easily detected in the first twelve hours of growth. The enzyme activity reached a maxima after 24-36 hours of growth under this photoperiod and this level of activity remained up to the end of the experiment (Fig. 10). *d4h* transcripts were clearly observed in 6 day old dark grown seedlings, as well as at earlier developmental stages (Chapter III), even though they contained barely detectable levels of enzyme activity (Fig. 10), and in fact transcript levels from earlier developmental stages were as high if not higher than those detected in 6 day old etiolated seedlings (Chapter III). The levels of *d4h* mRNA decreased sharply with continued growth of seedlings in the dark whereas exposure of seven day

old dark grown seedlings to light resulted in maintenance and a subsequent increase of *D4H* transcripts for the balance of the time course (Fig. 10). These results indicate that, at least during some developmental stages, the simple presence of *d4h* transcripts is not sufficient to ensure the expression of D4H enzyme activity and that light is required to trigger expression of enzyme activity by an undetermined and potentially complex mechanism.

DISCUSSION.

The development of a protocol to purify desacetoxyvindoline 4-hydroxylase to near homogeneity (De Carolis and De Luca, 1993) was used to obtain and sequence peptides # 48, #61 and # 92 which were of 12, 16 and 17 amino acids in length, respectively. A *Catharanthus roseus* cDNA library (De Luca et al., 1989) was screened with a degenerate oligonucleotide probe whose sequence was based on peptide # 48 and three cDNA clones were isolated. Although none of the clones were full length, two (*cD4H-2* and *-3*) were identical apart from a 11 bp difference in their size and the third (*cD4H-1*) was identical to the other 2 except for significant sequence differences occurring in the 3'UTR of these clones. In addition, since the ORF encoded by all 3 clones is absolutely conserved, it suggests that they encode the same protein and that the screening procedure may have resulted in the isolation of dimorphic alleles of the same hydroxylase from *Catharanthus roseus* which is a diploid plant species (Stearn, 1975). This hypothesis is in agreement with the Southern blot analysis which only revealed the presence of a single copy gene for *d4h* (Fig. 8), and with the results obtained from the RT-PCR of seedlings which confirmed the ocurrence of two different transcripts (Appendix I). The accumulation of mutations through evolution in non-coding regions has been widely reported for eukaryotes (French et al., 1989). The strict conservation of the *d4h* sequence within the dimorphic alleles encoding the ORF may indicate that *Catharanthus roseus* is under evolutionary pressure to maintain a functional *d4h*. Other genes involved in vindoline biosynthesis (*tdc*, *ss* and *dat*) also have been detected as single copy genes (Goddijn et al., 1994; Pasquali et al., 1992; St-Pierre et al, 1998) and although more evidence is required to prove this, it may indicate that the complete pathway for vindoline biosynthesis may have evolved as single copy genes. The absence of genetic redundancy for this pathway may have implications in its regulation.

The three clones were confirmed to be authentic *d4h* clones when all three peptides which were obtained by sequencing of the purified enzyme were clearly identified in the deduced ORF (Fig. 5) and by heterologous expression of the recombinant protein to demonstrate desacetoxyvindoline 4-hydroxylase enzyme activity

(Table 2). These results also show that the first 15 amino acid residues which were absent in the expression vector were not required for enzyme activity.

The deduced amino acid sequence of *cD4H-3* showed clear homology to plant and fungal dioxygenases which was more extensive in the C-terminal domain (Fig. 7). The conservation was not restricted to the dioxygenase protein sequences but it also extended to gene structure and organization. In a recent review (Prescot and John, 1996) on this subject, the positions for four introns found on plant dioxygenases were identified. Dioxygenases genes such as the *efe* homolog, *e8* from tomato (Deikman and Fischer, 1988), *efe* from tomato (Holdsworth et al., 1987b), and *h6h* from *H. niger* (Matsuda et al., 1991), contain two (*e8*) or three (*efe*, *h6h*) introns at four possible positions and these are inserted around highly conserved amino acid consensus sequences (Kanegae et al., 1994; Appendix I). The *d4h* gene (Fig. 6) contains two introns which are also inserted at two of the same highly conserved sites (Appendix I). These results increase the probability that this *d4h* has diverged from a common ancestral gene which has been suggested to contain three intron positions (Prescot and John, 1996). This common ancestry has also been suggested for other plant and fungal dioxygenases (Matsuda et al., 1991; Bristsch et al., 1993). However, other dioxygenases such as the bacterial 2,3-dihydroxybiphenyl, 1,2-dioxygenase (Han et al., 1995) and catechol 1,2-dioxygenase (Nakai et al., 1995) or the bovine aspartyl ß-hydroxylase (Jia et al., 1992) may have a separate evolutionary origin since they did not show any significant levels of homology to any of the enzymes in Figure 7.

The degree of sequence similarity reported here and in other work between dioxygenases that oxidize different substrates suggests a similar reaction mechanism common to this family of enzymes (Ming et al., 1991). The strict conservation of the His_{268}-X-Asp_{270} motif as well as His_{324}, which have been shown in structure solving studies of IPNS (Roach et al., 1995) to participate in the binding of iron, in the sequence of D4H provides evidence of a similar mechanism for dioxygen activation in this enzyme. The poorly conserved N-terminal domain may represent the binding sites for specific alkaloid substrates (De Carolis and De Luca, 1994) although no homology was observed between this domain and DAT (St-Pierre et al, unpublished results) which catalyzes the last step in vindoline biosynthesis (Fig. 4).

It has been proposed that the biosynthesis of vindoline comprises a complex cellular compartmentation of biosynthetic enzymes which require related transport mechanisms to permit movement of pathway intermediates from one compartment to the other. The four cellular compartments which appear to be involved in the biosynthesis of vindoline are the endoplasmic reticulum, the vacuole, the chloroplast and the cytosol (St-Pierre and De Luca, 1995). Three early steps in indole alkaloid biosynthesis catalyzed by TDC, geraniol 10-hydroxylase and SS occur in cytosol (De Luca et al., 1987; De Luca and Cutler, 1987), endoplasmic reticulum (Madyastha et al., 1976) and in vacuoles (McKnight et al., 1991), respectively. Four late steps in vindoline biosynthesis (Fig. 4) T16H, 16-OMT (St-Pierre and De Luca, 1995), NMT (De Luca et al., 1987) and DAT (De Luca and Cutler, 1987) occur in endoplasmic reticulum, cytosol, chloroplast thylakoids and in cytosol, respectively. Since D4H displays no identifiable signal peptide at the N-terminal of the deduced amino acid sequence of this clone (Fig. 5), the data corroborate biochemical localization studies which indicated that D4H is also a cytosolic protein (De Carolis et al., 1990; Table 2) and this increases our understanding of the complex cellular compartmentation of vindoline biosynthesis.

The leaf-specific distribution of enzymes involved in the late stages of vindoline biosynthesis has been well-documented (De Luca et al., 1989; St-Pierre and De Luca, 1995). The contrasting whole plant distribution of most of the rest of the pathway has been used to explain why *C. roseus* cell cultures accumulate catharanthine but not vindoline (De Luca et al., 1989; Alvarez-Fernandez et al., 1989; Pasquali et al., 1992; data not shown). The molecular studies reported here suggest that genes involved in the late stages of vindoline biosynthesis are indeed expressed in a tissue-specific manner. There was an apparent direct correlation between the level of *d4h* transcripts and the level of enzyme activity observed in specific tissues (Fig. 9) where D4H enzyme activity and mRNA appeared mainly in leaves, with much lower levels occurring in fruits and stems whereas neither enzyme activity nor *d4h* transcripts could be found in roots. In this respect, young leaves represent the only tissue where early and late stages of vindoline biosynthesis are strongly expressed and where they appear simultaneously (Alvarez-Fernandez et al., 1989).

The light regulation of the late stages of vindoline biosynthesis has been reported previously (Aerts and De Luca, 1992; Balsevich et al., 1986; De Luca et al., 1986; De Luca et al., 1989). Phytochrome appears to participate in the light-mediated activation of developing seedlings which results in the appearance of D4H and DAT enzyme activities (Aerts and De Luca, 1992; De Carolis, 1994). The detection of *d4h* transcripts in dark grown seedlings (Fig. 10) without the corresponding appearance of D4H enzyme activity (Fig. 10), suggests that the activating effect of light may not solely be at the transcriptional level, but instead also affects a point downstream of transcription which remains to be elucidated.

The early (TDC and SS) and late (NMT, D4H and DAT) stages of vindoline biosynthesis are developmentally regulated during seedling growth, with the late stages appearing 24 to 36 h after the rest of the pathway (De Luca et al., 1986; De Luca et al., 1989). The appearance of TDC is subjected to very strict transcriptional, post-transcriptional and post-translational controls (De Luca et al., 1989; Alvarez-Fernandez et al., 1989; Alvarez-Fernandez and De Luca, 1994). The data shown in Figures 9 and 10 suggest that a similar multilevel regulatory mechanism may also operate to regulate the late stages of vindoline biosynthesis. What are the elements involved in these mechanisms and what are the signals that activate it are fascinating questions that remain to be solved. The answers to these questions have both biotechnological and basic biology implications since, they will help us to understand why cell cultures fail to produce vindoline and, more importantly, to understand the biological role of these secondary metabolites in *Catharanthus roseus*. The avalability of molecular tools, such as cDNA clones and antibodies (Chapter III) against D4H will help us to solve some of these questions.

TABLE 2. HETEROLOGOUS EXPRESSION OF *pQD4H-19* IN *E. COLI* CELLS.

	Reaction Mixture			
	Enzyme Activity (pkat/mg protein)			
Protein source[(b)]	Complete	(-)2-OG	(-)Asc	(-)O_2[(c)]
C. roseus leaves	1.40	0.06	0.05	0.01
pQD4H-19[(d)]	17.70	0.70	0.70	0.10
pQE30[(d)]	0.00	----	----	----

[(a)]The complete reaction mixture contains O_2, 10 mM 2-oxoglutarate (**2-OG**), 7.5 mM ascorbate (**Asc**) and 0.56 nmol of labeled substrate (44,600 dpm) in a total volume of 150 µl 50 mM Tris-HCl pH 7.5 plus 28 mM ß-mercaptoethanol. Ferrous ions however, are only necessary when certain purification protocols are used (*De Carolis and De Luca, 1993*).

[(b)]Crude, PD-10 (Pharmacia) desalted extracts were used in the assays.

[(c)]Oxygen was removed from the reaction preincubating the enzyme extract and all reaction components, except the alkaloid substrate, with 10 U of glucose oxidase and 1 mM glucose for 5 min at 30 °C in a sealed vial.

[(d)]*E. coli* BB4 cells containing either *pQD4H-19* or pQE30 plasmids were grown for three hours in the presence of 3 mM isopropyl-1-thio-ß-D-galactopyranoside prior to extraction for enzyme assay.

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   1  GGA AAA AAT AAA TTA TAA AAA AAA TAT ATT ATA GAA GGA AAA TTT AAA CGA TAA TAC GGT    60

   1                                                                   M   P          2
  61  TAT TAT ATT TGA CTG GCA GAT TTA GAC TGT CCG GCA GTC TGT AAT CCT ATA AAA ATG CCT   120

   3   K   S   W   P   I   V   I   S   S   H   S   F   C   F   L   P   N   S   E   Q   22
 121  AAG TCT TGG CCA ATT GTG ATA TCA TCT CAT AGT TTC TGT TTT CTT CCA AAT TCT GAG CAA   180

  23   E   R   K   M   K   D   L   N   F   H   A   A   T   L   S   E   E   E   S   L   42
 181  GAA AGA AAA ATG AAG GAC TTG AAC TTT CAT GCT GCT ACA CTC TCA GAA GAA GAA TCT TTA   240

  43   R   E   L   K   A   F   D   E   T   K   A   G   V   K   G   I   V   D   T   G   62
 241  AGG GAA TTG AAG GCT TTT GAT GAG ACA AAG GCT GGT GTA AAA GGG ATT GTA GAT ACT GGG   300

  63   I   T   K   I   P   R   I   F   I   D   Q   P   K   N   L   D   R   I   S   V   82
 301  ATA ACC AAA ATT CCA CGT ATC TTT ATC GAT CAA CCA AAA AAT CTT GAC CGG ATT TCA GTG   360

  83   C   R   G   K   S   D   I   K   I   P   V   I   N   L   N   G   L   S   S   N   102
 361  TGT AGA GGA AAA TCC GAT ATC AAG ATT CCA GTT ATA AAC TTG AAT GGC CTC AGT AGC AAT   420

 103   S   E   I   R   R   E   I   V   E   K   I   G   E   A   S   E   K   Y   G   F   122
 421  TCA GAA ATA CGG CGT GAG ATT GTG GAG AAA ATT GGA GAA GCG AGT GAG AAA TAT GGA TTC   480

 123   F   Q   I   V   N   H   G   I   P   Q   D   V   M   D   K   M   V   D   G   V   142
 481  TTC CAG ATA GTT AAT CAT GGG ATT CCA CAA GAT GTT ATG GAT AAA ATG GTA GAT GGA GTT   540

 143   R   K   F   H   E   Q   D   D   Q   I   K   R   Q   Y   Y   S   R   D   R   F   162
 541  CGT AAG TTT CAT GAA CAA GAT GAT CAA ATC AAG AGA CAA TAT TAC TCC CGT GAC CGC TTC   600

 163   N   K   N   F   L   Y   S   S   N   Y   V   L   I   P   G   I   A   C   N   W   182
 601  AAC AAA AAT TTT CTA TAT AGC AGT AAT TAC GTT TTG ATT CCA GGA ATT GCT TGC AAT TGG   660

 183   R   D   T   M   E   C   I   M   N   S   N   Q   P   D   P   Q   E   F   P   D   202
 661  AGG GAT ACT ATG GAA TGC ATT ATG AAT TCT AAT CAA CCT GAT CCT CAG GAA TTC CCA GAT   720

 203   V   C   R   D   I   L   M   K   Y   S   N   Y   V   R   N   L   G   L   I   L   222
 721  GTA TGC AGa GAC ATA TTG ATG AAG TAC TCA AAT TAT GTA AGA AAT TTG GGG CTT ATT CTC   780

 223   F   E   L   L   S   E   A   L   G   L   K   P   N   H   L   E   E   M   D   C   242
 781  TTT GAA TTA CTA TCA GAA GCT TTG GGG CTC AAA CCA AAT CAT CTT GAA GAA ATG GAT TGT   840

 243   A   E   G   L   I   L   L   G   H   Y   Y   P   A   C   P   Q   P   E   L   T   262
 841  GCT GAA GGA CTT ATA CTT CTT GGT CAT TAC TAC CCT GCA TGC CCT CAA CCA GAG TTG ACA   900

 263   F   G   T   S   K   H   S   D   S   G   F   L   T   I   L   M   Q   D   Q   I   282
 901  TTT GGG ACA AGC AAG CAC TCA GAC AGT GGT TTC TTA ACC ATA CTT ATG CAG GAT CAA ATT   960

 283   G   G   L   Q   I   L   L   E   N   Q   W   I   D   V   P   F   I   P   G   A   302
 961  GGC GGC CTT CAA ATT CTT CTT GAG AAT CAA TGG ATT GAT GTC CCC TTC ATT CCT GGA GCT  1020

 303   L   V   I   N   I   A   D   L   L   Q   L   I   T   N   D   K   F   K   S   V   322
1021  CTA GTT ATT AAC ATT GCA GAT CTT TTA CAG cTG ATC ACG AAC GAC AAG TTC AAG AGT GTC  1080

 323   E   H   R   V   L   A   N   K   V   G   P   R   I   S   V   A   V   A   F   G   342
1081  GAA CAT AGA GTA CTG GCA AAC AAA GTT GGT CCG AGG ATT TCT GTT GCT GTT GCT TTT GGT  1140

 343   I   K   T   Q   T   Q   E   G   V   S   P   R   L   Y   G   P   I   K   E   L   362
1141  ATA AAA ACA CAA ACT CAA GAA GGG GTT TCG CCA AGA TTG TAC GGA CCA ATT AAA GAG TTA  1200

 363   I   S   E   E   N   P   P   I   Y   K   E   V   T   V   K   D   F   I   T   I   382
1201  ATA TCA GAG GAA AAT CCA CCC ATT TAC AAG GAG GTC ACT GTT AAA GAT TTT ATA ACA ATT  1260

 383   R   F   A   K   R   F   D   D   S   S   S   L   S   P   F   R   L   N   N       401
1261  CGA TTT GCC AAA CGC TTT GAC GAT AGC TCT TCC TTA TCT CCT TTC AGG TTA AAC AAT TAG  1320

1321  ATC GGG AGA AGC TAA TAC TAG CTT AAG TGT TTC TAT ATA AAT CTC CAC CTA TTT GGC GCT  1380
1381  GAA ACT GAT AAT TTT TGC ATG TTG GAC TAA ATA AGC GTC GGC GAA TAT TTG TTT CGT CTC  1440
1441  ATT TTC AAT AAT CAA ATA AAG CAA ATG GTC TGC TAG AGA GTT TGG CCA AAC TAT ACT TGC  1500
1501  GAT TCG AGT AGC CTT TCT ATT TTC TAC AAT TTG AGA GAT CTT CCG GTG AAC ATT AGA GGT  1560
1561  AAA TAT TTC GTG GAT ATC TCA ATT ACC CCT TAT CAT CAG TGT GTT TTG ACC CCT TAT CAT  1620
1621  CAG TGT GTT TTG ACA GGG TCT TAA TTT TCC TTT ATT TTT CTA CCT CGT ACC TTG TTT TCT  1680
1681  TTA TGG ATT AAG GTA AGT TTT ATG CAC TGC CTT TTG TAA TTT ATT TCA CTT GAT TAT TAA  1740
1741  TAA AAC CGA GCA TCG CCC GAA AAA AAA AAA AAA AAA AAA A                            1783
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Figure 5. Nucleotide sequence of the *d4h* transcript and its predicted amino acid sequence. The sequence obtained from the genomic clone (*gD4H-8*) is underlined whereas the rest of the sequence is from *cD4H-3*. Recognition sequences for *EcoR*I, located at position 684 and 708; *Hind*III, at 797; and *BstX*I, at 1380; are in bold italics. The two intron insertion positions are underlined and are represented as bold lower case letters in the ORF. The putative polyadenylation signal is represented in underlined italics in the 3' UTR. Boxed areas represent the tryptic peptides obtained from sequencing D4H. The shaded box shows the amino acid sequence from which probe **48c** was designed.

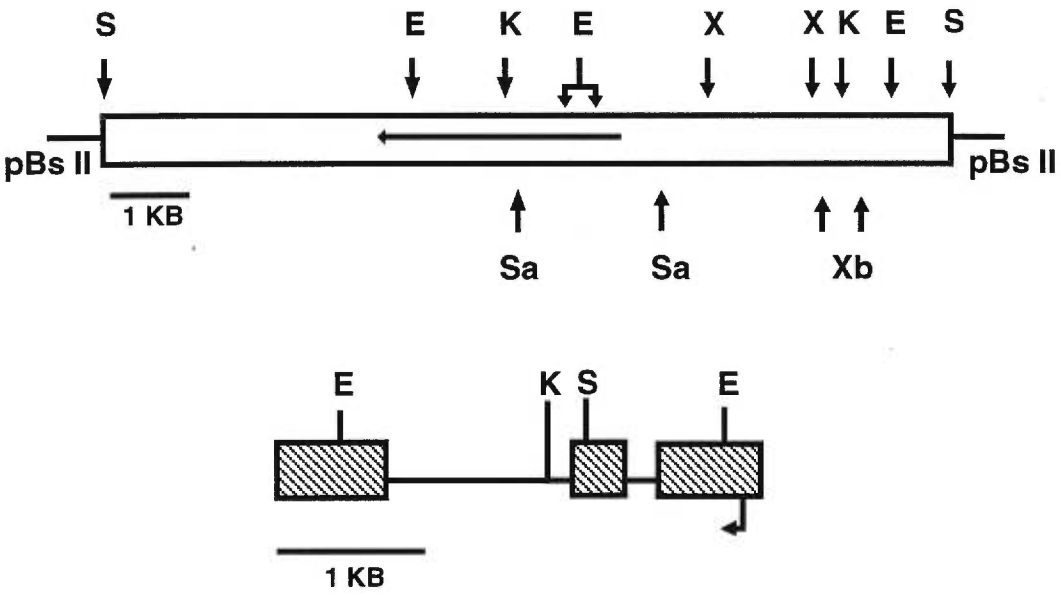

Figure 6. Organization of the genomic clone, *gD4H-8* which was 11kb in size. The *d4h* (arrow orients the 5' to 3' end) gene sequence illustrated in Fig. 5 occurs near the center of *gD4H-8.* The magnified diagram illustrates the three exons (hatched boxes) and two introns composing the *d4h* gene. Vertical arrows show restriction sites for (**EI**) *EcoR*I, (**E**) *EcoR*V, (**S**) *Sal*I, (**X**) *Xho*I, and (**Xb**) *Xba*I.

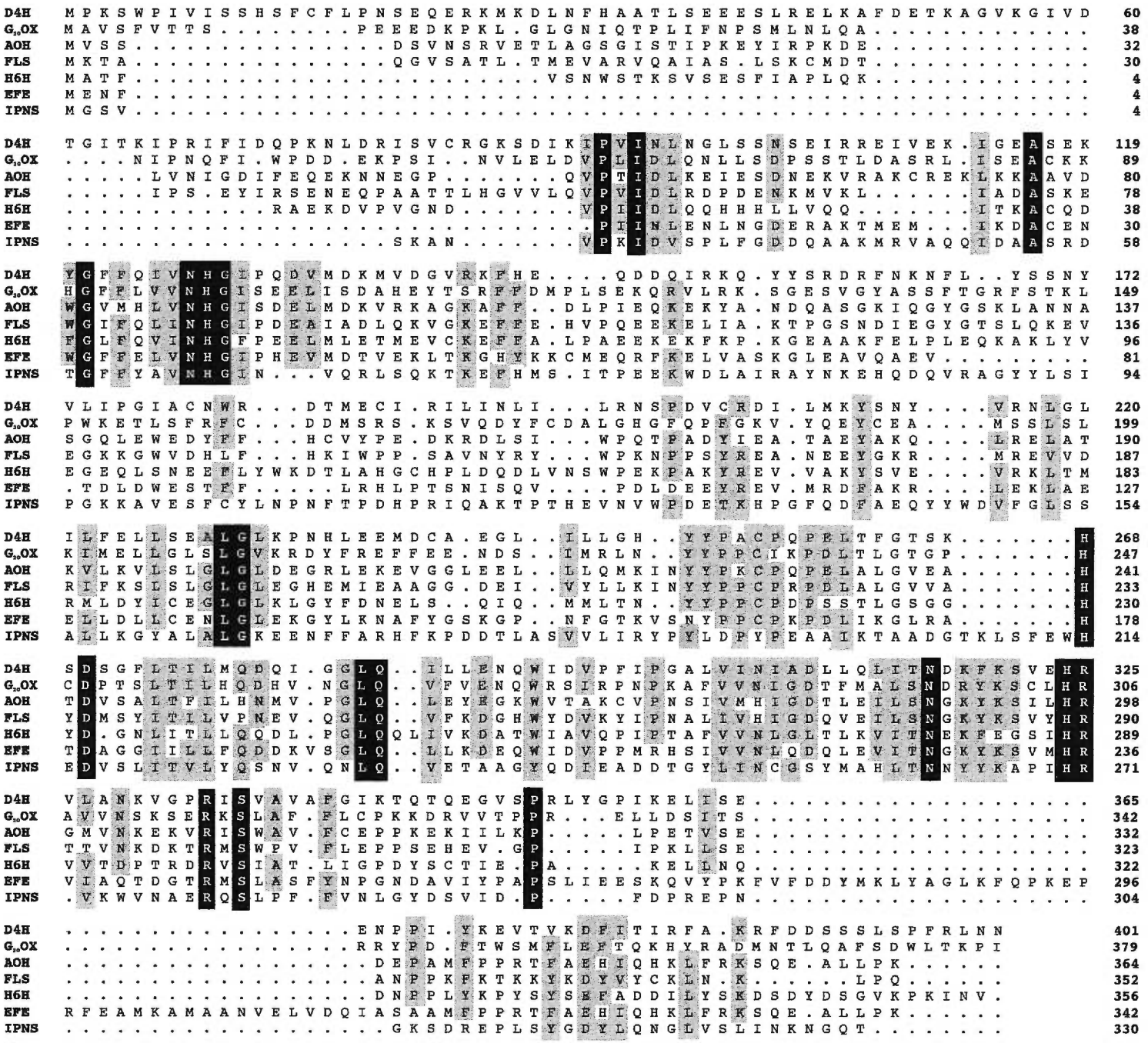
D4H
G3OX
AOH
FLS
H6H
EFE
IPNS

Figure 7. Alignment of **D4H** with other dioxygenases: gibberellin 20-oxidase from *A. thaliana* (**$G_{20}O$**; Phillips et al., 1995; GenBank acc. X83379); anthocyanidin synthase from apple tree (**AOH**; Davis, 1993; GenBank acc. X71360); flavonol synthase from *Petunia hybrida* (**FS**; Holton et al., 1993; GenBank acc. Z22545); hyoscyamine 6ß-hydroxylase from *H. niger* (**H6H**; Matsuda et al., 1991; GenBank acc. M62719); ethylene forming enzyme from tomato (**EFE**; Holdsworth et al., 1987a; GenBank acc. X58885); isopenicillin *N*-synthase from *A. nidulans* (**IPNS**; Ramon et al., 1987; GenBank acc. M18111). White characters in black boxes represent identical amino acids in all the sequences aligned; shaded boxes represent conserved residues.

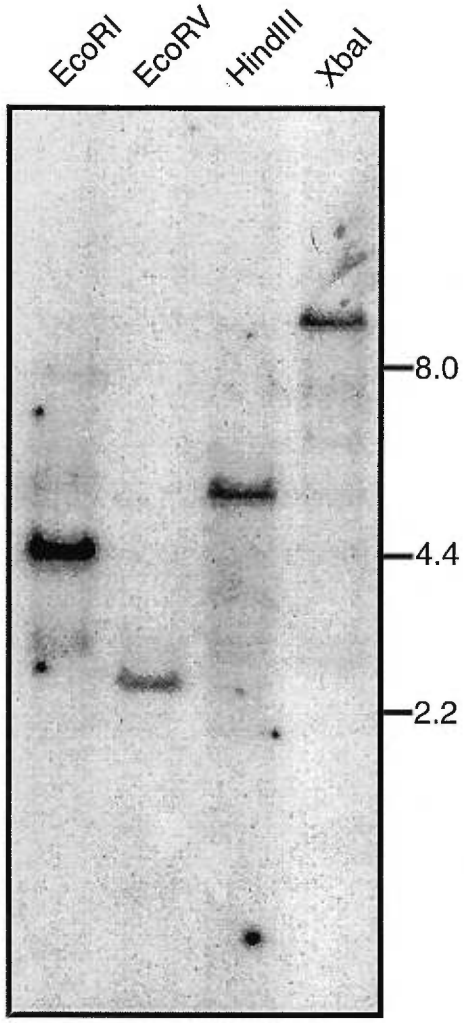

Figure 8. Southern Blot analysis of the *d4h gene*. Genomic DNA isolated from *C. roseus* leaves was digested with *EcoR*I; *EcoR*V; *Hind*III and *Xba*I. The membrane was probed with a 1.2 kb *EcoR*I fragment isolated from *cD4H-3* (see Fig. 5) end labelled by random-priming with [^{32}P]dCTP. The molecular weight markers (kb) are shown on the right of the figure.

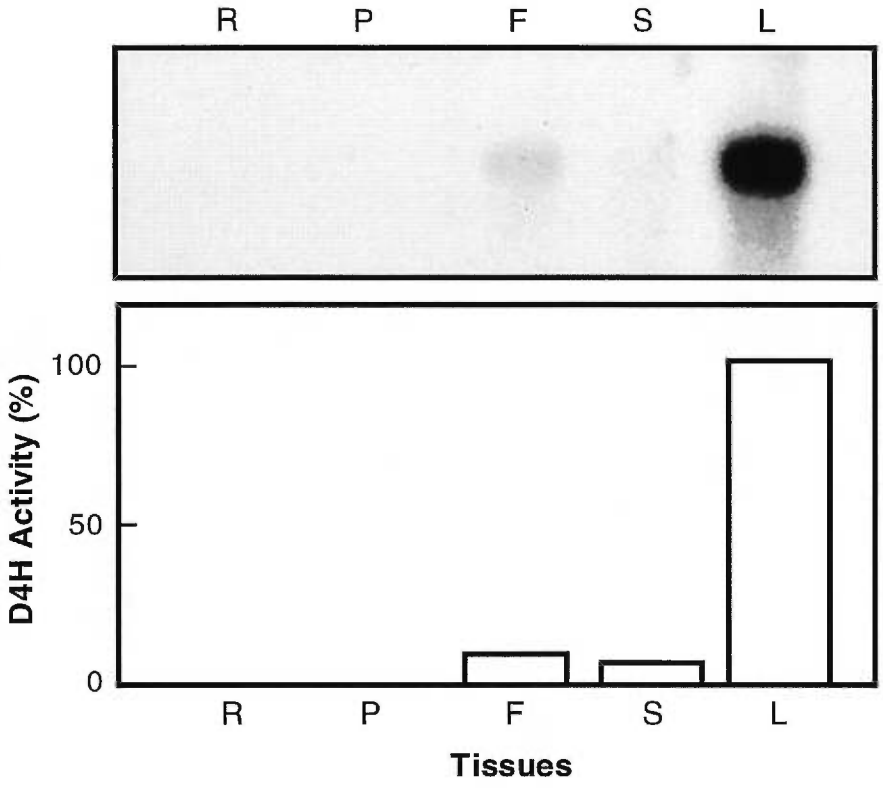

Figure 9. Tissue specific distribution of *d4h* transcripts (top panel) and D4H enzyme activity (bottom panel). (**R**) roots; (**P**) petals; (**F**) fruits; (**S**) stems; (**L**) leaves. Enzyme activity is expressed as (%) of the activity observed in leaves where 100% represents 0.81 pkat/mg protein.

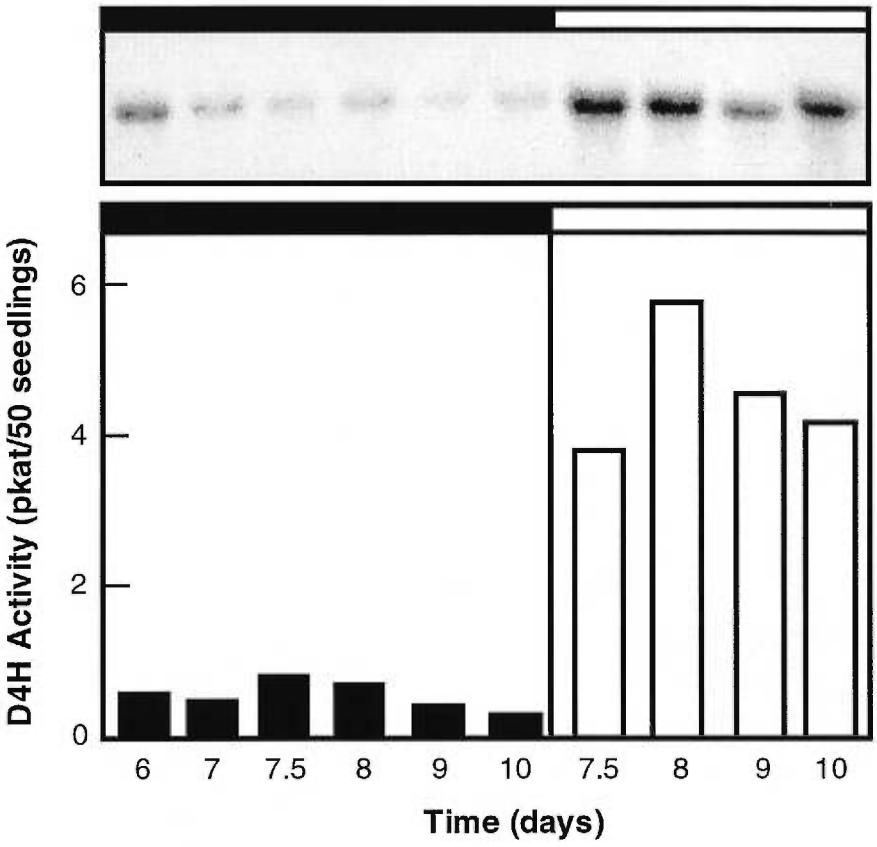

Figure 10. Effect of light on the induction of *d4h* transcripts (top panel) and D4H enzyme activity (bottom panel) during seedling development. Seeds were germinated and grown in the dark for ten days (black horizontal box, black bars) or were exposed to a 18 h photoperiod after 7 days of dark growth (white horizontal box). Northern blots of *d4h* transcript levels in dark and light grown seedlings are shown in the upper panel whereas D4H enzyme activities are shown in the lower panel.

Chapter III. Developmental and Light Regulation of Desacetoxyvindoline 4-Hydroxylase in *Catharanthus roseus* (L.) G. Don. Evidence of a Multilevel Regulatory Mechanism.

Objectives.

General objectives

1) To investigate the regulation of D4H expression.

2) To analyse the possible relationship between light and development in this regulation.

Specific objectives

1) To raise polyclonal anti-D4H antibodies in rabbits.

2) To monitor the steady state levels of *d4h* transcript, D4H antigen and enzyme activity during the course of development of *Catharanthus* seedlings grown in the dark and in the light.

3) To compare the light activation of D4H at different developmental stages.

4) To investigate the role of phytochrome in the light activation of D4H.

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DEVELOPMENTAL AND LIGHT REGULATION OF DESACETOXYVINDOLINE 4-HYDROXYLASE IN *CATHARANTHUS ROSEUS* (L.) G. DON. EVIDENCE OF A MULTILEVEL REGULATORY MECHANISM.

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ABSTRACT.

The expression of desacetoxyvindoline 4-hydroxylase (D4H) which catalyzes the second to last reaction in vindoline biosynthesis in *Catharanthus roseus* appeared to be under complex multilevel developmental and light regulation. Developmental studies with etiolated- and light-treated seedlings suggested that while light had variable effects on the levels of *d4h* transcripts, those of D4H protein and enzyme activity could be increased, depending on seedling development, up to 9- and 8-fold, respectively, compared to etiolated seedlings. However, light treatment of etiolated seedlings could stop and reverse the decline of *d4h* transcripts at later stages of seedling development. Repeated exposure of seedlings to light was also required to maintain the full spectrum of enzyme activity observed throughout seedling development. Further studies showed that a photoreversible phytochrome appeared to be involved in the activation of D4H since red-light treatment of etiolated seedlings increased the detectable levels of *d4h* transcripts, D4H protein and enzyme activity, whereas far red-light treatment completely reversed this process. Additional studies also confirmed that different major isoforms of D4H protein exist in etiolated- (pI 4.7) and light-grown (pI 4.6) seedlings, suggesting that a component of the light-mediated activation of D4H may involve an undetermined post-translational modification. The biological reasons for this complex control of vindoline biosynthesis may be related to the need to produce structures which could sequester away from cellular activities the cytotoxic vinblastine and vincristine dimers which are derived partially from vindoline.

INTRODUCTION

Alkaloids are physiologically active secondary metabolites containing heterocyclic nitrogen in their structures (Pelletier, 1970). These complex molecules are widely spread in the plant kingdom and it is estimated that about 30% of all plants contain alkaloids (Robinson, 1981). Most theories propose a role for alkaloids in the interaction of plants with their environment, either by providing a chemical defense against pathogens or by participating in different plant-insect interactions (Bennet and Wallsgrove, 1994; Grayer and Harbone, 1994; Rhodes, 1994). These possible contributions of alkaloids to plant fitness to their surroundings may be modulated by the rate and type of alkaloids produced in response to biotic and abiotic factors (Robinson, 1981; Bennet and Wallsgrove, 1994). Some aspects of the molecular basis for pathogen-induced alkaloid synthesis have been studied in *Papaver somniferum* (Facchini et al., 1996), in *Eschscholtzia californica* (Dittrich and Kutchan, 1991; Kutchan, 1993) and in *Catharanthus roseus* (Eilert et al., 1987; Pasquali et al., 1992; Roewer et al., 1992). Cell suspensions from these species responded to the addition of fungal elicitors by activating the transcription of key alkaloid pathway genes, which was followed by the appearance of corresponding enzyme activities and the accumulation of indole alkaloids.

The molecular mechanisms mediating the effects of other environmental factors on alkaloid biosynthesis are less documented. Light which plays a critical role in plant growth and development may also affect alkaloid biosynthesis. For example, during the early stages of tobacco seedling development, the rate of nicotine biosynthesis was associated with radicle elongation. A brief pulse of light interfered with radicle growth and also reduced nicotine accumulation (Weeks and Bush, 1974). However, after cotyledons were open, a 10 h photoperiod triggered a 70% increase in nicotine content over untreated etiolated seedlings (Weeks and Bush, 1974). Light-dependent enhancement of nicotine biosynthesis was also observed in 6-week old plants where a correlation between photoperiod length and nicotine accumulation was found (Tso et al.,

1970). Phytochrome seems to be involved in this process since a red light pulse, given at the end of the day, promoted a further nicotine accumulation while a similar far-red light treatment reversed these effects (Tso et al., 1970).

The effects of light on alkaloid accumulation have also been studied in *Catharanthus roseus*. This plant, which belongs to the Apocynaceae family, produces more than 100 monoterpenoid indole alkaloids, including the powerful cytotoxic drugs vinblastine (VBL) and vincristine (VCR). These alkaloids are dimers formed from the condensation of catharanthine and vindoline (Svodoba and Blake, 1975). Early studies have shown that the pattern of alkaloids extracted from *Catharanthus* seedlings was greatly affected by development and light (Mothes et al., 1965; Scott, 1970). Etiolated seedlings contained high levels of the late vindoline precursor, tabersonine, which upon illumination was transformed stoichiometrically into vindoline (Balsevich et al., 1986; De Luca et al., 1986). In contrast, catharanthine which accumulated to high levels in etiolated seedlings, was hardly affected by the light regime (Scott, 1970; Balsevich et al., 1986). These studies suggested that light was a major limiting factor in the conversion of tabersonine into vindoline and in the formation of dimeric indole alkaloids (Balsevich et al., 1986; De Luca et al., 1986).

The transformation of tabersonine into vindoline involves six strictly ordered enzyme reactions (Fig. 4): aromatic hydroxylation, *O*-methylation, hydration of the 2,3-double bond, *N*(1)-methylation, hydroxylation at position 4 and 4-*O*-acetylation (Balsevich et al., 1986; De Luca et al., 1986). The first of these reactions is catalyzed by tabersonine 16-hydroxylase (16-OHT), a cytochrome P_{450} dependent monooxygenase associated with microsomal cell fractions, while the next reaction is catalyzed by a cytosolic *S*-Adenosyl-*L*-methionine: 16 hydroxytabersonine *O*-methyltransferase (16-OMT) (St-Pierre and De Luca, 1995). The enzyme involved in the hydration of the double bond of the 16-methoxy compound has yet to be characterized, but the product from this hydroxylase is *N*-methylated by a thylakoid associated S-adenosyl-L-methionine: 16-methoxy-2,3-dihydro-3-hydroxytabersonine-*N*-methyltransferase (NMT)

which forms desacetoxyvindoline (De Luca et al., 1985; Dethier and De Luca, 1993). The second to last reaction involves the 4 hydroxylation of desacetoxyvindoline and is catalyzed by a cytosolic 2-oxoglutarate-dependent dioxygenase, known as desacetoxyvindoline 4-hydroxylase (D4H; De Carolis et al., 1990; De Carolis and De Luca, 1993). Final *O*-acetylation of deacetylvindoline to yield vindoline is catalyzed by a cytosolic acetyl CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase (DAT; De Luca et al., 1986; Powers et al., 1990; St-Pierre et al., submitted). In addition, these studies revealed that expression of 16-OHT, D4H, and DAT in developing *Catharanthus* seedlings is light-regulated. However, while D4H and DAT activities are detected exclusively under conditions resulting in vindoline biosynthesis, expression of 16-OHT occurs at low levels in *Catharanthus* cell cultures which don't accumulate vindoline (St-Pierre and De Luca, 1995).

We have recently isolated cDNA and genomic clones of D4H which display a high degree of similarity with a well-characterized family of plant and fungal dioxygenases (Chapter II). Expression of D4H appears to be regulated by cell-, tissue-, development- and environment-specific controls. Enzyme assays and RNA blot hybridization studies showed that hydroxylase activity followed closely the levels of *d4h* transcripts, occurring predominantly in young leaves and in much lower levels in stems and fruits. In contrast, etiolated seedlings which contained considerable levels of *d4h* transcripts had almost undetectable hydroxylase activity. Exposure of seedlings to light resulted in a rapid increase of enzyme activity without any further increase in transcript levels and continued exposure to light was necessary to maintain transcript levels later in seedling development (Chapter II).

The present study describes in greater detail the relationship between seedling development and the role played by light in the activation of D4H. The results indicate that expression of D4H may be regulated by transcriptional, translational and post-translational controls.

MATERIALS AND METHODS.

Plant materials. Batches of seeds (1.0 g, *ca.* 800 seeds) of *Catharanthus roseus* (cv. Little Delicata, Sakata Seed America Inc., Salinas CA) were sterilized in 70% ethanol for 30 s and were thoroughly washed with sterile water. Seeds were imbibed in sterile water for 12 h and then plated on Petri dishes containing 3 layers of filter paper (Whatman 3 MM, Whatman International Ltd., Maidstone England) wetted with 3.5 mL of sterile water. Approximately 100 seeds were applied per plate and care was taken to avoid any contact between seeds. Plates were sealed with laboratory film (Parafilm M, American Ntl. Can., Neenah WI) and kept in the dark under controlled conditions (25 °C, 70% RH) in growth chambers (Conviron CMP 3023, Pembina ND). Day zero was taken as the time when seeds were placed on the Petri dish.

Seedling treatments. *Light treatments.* Etiolated seedlings were exposed to white light (60 W cool white fluorescent tubes from General Electric Sylvania, Drummondville, Quebec and 60 W incandescent bulbs from Phillips Royale, Scarborough, Ontario) at the times indicated in the figures. Photon fluence rate was calibrated at 20 μmol m^{-2} s^{-1} with a Li-Cor photometer Li-189 (Lincoln, NB). Unless otherwise specified, light-exposed seedlings received an 18 h photoperiod. For red light treatments, white light was provided as described above and filtered through a Roscolux 19 filter (Port Chester, NY). This filter transmitted only wavelengths longer than 575 nm and transmitted greater than 90% of the irradiance at wavelengths longer than 650 nm. These characteristics produced red light with approximately the same photon fluence rate as the red component of the original white light source (Aerts and De Luca, 1992). Far-red light irradiation was obtained by filtering white light through Roscolux 19, 83, 89 filters. Such a filter combination transmitted only wavelengths longer than 710 nm (Aerts and De Luca, 1992). The spectral quality of light transmitted through the filters was verified using a Beckman DU-65 spectrophotometer (Beckman Instruments, Palo Alto CA).

External carbon source application. Etiolated seedlings received 1 mL of filter sterilized (Millex-GP Millipore, Bedford MA) sucrose stocks to give a final concentration of 100 or 300 mM per petri and were treated as described in Fig. 13.

Enzyme analysis. Batches of 100 seedlings which were submitted to various treatments, were collected under a dim green safe light (25 W; DecoColor, General Electric Lighting Canada, Mississauga Ontario), were frozen in liquid nitrogen and were kept at -80 °C until analysis. D4H was extracted and assayed by the direct method described in De Carolis et al., 1990, and enzyme activity was expressed on a per seedling basis.

Immunological studies. *Protein purification and antibody production.* Anti-D4H antibodies were raised against recombinant D4H protein in New Zealand female rabbits. The expression construct, *pQD4H-19* was engineered from the cDNA clone *cD4H-3* into the expression vector pQE30 (QiaGene, Chatsworth CA) as described previously (Chapter II). This vector provides an affinity tag containing 6 histidine residues at the N-terminus of the recombinant protein which allowed the specific purification of D4H (Appendix II). *E. coli BB4* cells (Stratagene, San Diego CA) containing the expression construct *pQD4H-19* were grown at 37 °C and 300 rpm in 250 ml of Luria Bertani media (Sambrook et al., 1989) until an OD_{600} = 0.6, and bacterial cultures were induced and extracted as described previously (Chapter II). The sonicated bacterial extract (Brandon, Danube CT) was loaded onto a 5 cm Ni-NTA agarose affinity resin (QiaGene) packed in a 1 X 10 cm chromatographic column (C-10 Pharmacia, Uppsala Sweden). The buffers and procedures used for purification of recombinant D4H were those recommended by the manufacturer (Appendix II). Fractions which eluted from the Ni-NTA column were tested for enzyme activity and were checked for purity by SDS-PAGE. Antibodies were produced by Cocalico Biologicals, Reamstown, PA. Aliquots (50 µg) of pure recombinant protein were mixed with complete Freund's adjuvant and were used to immunize rabbits by subcutaneous injection. Rabbits received four extra biweekly boosts with 50 µg of protein mixed in Freund's incomplete adjuvant before the final

bleeding. The titer of D4H antiserum was tested one week after each boost by quantitative immunoblot with known amounts of the pure recombinant protein. A 1:10,000 dilution of the final antiserum easily recognized 100 picograms of pure recombinant protein and a single band of the expected *Mr* was recognized in crude extracts of *C. roseus* seedlings (10 mg protein) submitted to SDS-PAGE and Western immunoblotting.

Immunological analysis. Desalted seedling extracts (Pharmacia PD-10 columns) were diluted with Laemmli buffer (Laemmli, 1970) and equivalents of a half- seedling (protein content 8-12 µg; Bradford, 1976) were submitted to 12% SDS-PAGE. After electrophoretic transfer (Towbin et al., 1979) to nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH), they were blocked with 9% skim milk powder diluted in TBST (TBST is 10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween; Sambrook et al., 1989), washed twice with TBST, and then incubated for 1 h at room temperature with the primary antibody diluted 10,000-fold in 3% skim milk powder suspended in TBST (total antiserum protein approx. 70 µg). Membranes were washed twice with TBST and probed with horseradish peroxidase conjugated to donkey anti-rabbit IgG diluted 1:4000 in TBST (Amersham, Arlington Heights, IL). The immunoblots were revealed with a mixture of oxidizing and luminescence reagents (Renaissance Chemiluminescence Reagent, NEN Life Science Products, Boston MA) following the instructions of the manufacturer. D4H antigens were detected by autoradiography on Kodak X-OMAT AR X-ray film (Eastman Kodak Co., Rochester, NY). In order to distinguish variations in the amounts of immunoreacted protein, films were exposed to probed membranes from 30 to 120 s.

Two dimension IEF-SDS PAGE and immunoblotting. Seedlings were grown for 8.5 days in continuous darkness or 7-day old etiolated seedlings were exposed for a further 36 h to white light before harvesting. Seedlings were extracted and fractionated with 30 to 70 % ammonium sulphate as described previously (DeCarolis et. al, 1990) and were desalted on PD-10 columns. Protein was mixed to yield a final concentration

of 9 M urea, 1% Triton-X-100, 5% 2-mercapthoethanol and 2% ampholytes (1.6% pH range 5-7; 0.4% pH range 3-10; BioLytes, BioRad Hercules CA). Forty µg of total protein from dark- or light-induced seedlings, respectively, were submitted to isolectrofocusing (IEF; O'Farrel, 1975), followed by 12% SDS-PAGE in the second dimension (Laemmli, 1970). An identical IEF-SDS PAGE gel was also run with a mixture of 25 µg protein each from extracts of dark- and light-induced seedlings. The pH gradient (range 3-10) was calibrated with IEF standards (Bio-Rad) which had been applied simultaneously and which were detected with Ponceau red reversible staining (Sambrook et al., 1989). The IEF-SDS PAGE gels were then processed for immunoblotting as described in the previous section for SDS PAGE gels.

Nucleic acid extraction and analysis. Total seedling RNA was extracted, submitted to electrophoresis on agarose gels and transferred onto nitrocellulose membranes as described previously (Chapter II). Transcripts were detected by hybridization to the radioactive cDNA clone *cD4H-3* under conditions reported previously (Chapter II). Equal loading of RNA was ensured by inspection of gels stained with ethidium bromide.

RESULTS.

Developmental and light regulation of D4H. The appearance of D4H enzyme activity in developing etiolated seedlings was detected at low levels with the highest activities observed between days 5 to 7 (Fig. 11A). Despite the low levels of enzyme activity observed, D4H protein was easily detected during early stages of growth, and it appeared to decrease continuously after 7.5 days until it could no longer be detected by day 11 (Fig. 11A). The levels of *d4h* transcript also increased from the beginning of the experiment (day 4), reached a maximum 24 to 48 h later and decreased thereafter (Fig. 11A; Chapter II).

Etiolated seedlings were exposed to light at 5- and 7-days of seedling development. The cotyledons, hypocotyls and radicles were easily distinguished in 5-day old seedlings (average length 8 mm), but the seed coats were firmly attached to cotyledons. In contrast, the seeds coats were loosely attached to cotyledons of 7-day old seedlings (average length 12 mm) and were removed prior to extraction. Exposure of 5-day old seedlings to light resulted in an 8-fold increase in D4H activity after 24-48 h, and although it decreased with further seedling development, enzyme activity remained several-times higher than in untreated control seedlings (Fig. 11B). Light-induced D4H activity was accompanied by a corresponding 9-fold (as determined by densitometry) rise in the level of immunoreactive D4H protein (Fig. 11B) over the typical maximal levels appearing during etiolated growth (Fig. 11B). The relative levels of *d4h* transcripts in 6-day old seedlings treated with light for 24 h (Fig. 11B, top panel, 5/24) were identical to dark controls of the same developmental age (Fig. 11A and C, top panels, 6-day) as determined by densitometry scanning. Exposure of 7-day old etiolated seedlings to light (Fig. 11C, bottom panel) also resulted in an increase of D4H enzyme activity which peaked by day 8. However, the maximal enzyme activities obtained with light-treatment (Fig. 11C, bottom panel, 8-day) were identical to light-treated 5-day old etiolated seedlings of the same developmental age (Fig. 11B, bottom panel, 8-day). Light-induced D4H activity was not accompanied by a significant rise in the level of

immunoreactive D4H protein over the typical maximal levels appearing during etiolated growth (Fig. 11B, middle panel). The decline of *d4h* transcripts occurring by 7-days of etiolated growth (Fig. 11C, top panel, 6- and 7-days) could be reversed by light treatment (Fig 11C, top panel, 7.5- to 8-days), where *d4h* transcript levels increased again to those of 6-day old etiolated seedlings (Fig. 11A). The results clearly suggest that the ability of light to modulate D4H activity is controlled by seedling development and that regulation of this hydroxylase is under complex control.

Appearance of different isoforms of D4H protein in etiolated and light grown seedlings. The expression of *d4h* transcripts and the presence of significant amounts of D4H protein in etiolated seedlings (Fig. 11) which contain little or no measurable enzyme activity suggested that some modification of D4H protein was required for enzyme activity. It was previously shown that D4H exists as three charged isoforms (De Carolis and De Luca, 1993), and it was speculated that light-treatment might also trigger a posttranslational modification leading to the generation of one of these isoforms and to the activation of D4H activity. When desalted protein extracts from etiolated and light grown seedlings were submitted to 2-dimensional protein electrophoresis and to immunoblot with anti-D4H antibody, different isoforms of D4H protein could be observed (Fig. 12). Blots of extracts from 7-day old etiolated seedlings contained one main isoform with a pI of 4.7 and a second minor isoform with a slightly higher pI of 4.8. A 36 h light treatment resulted in the disappearance of the 4.7 pI isoform and the appearance of a new slightly more acidic isoform with a pI of 4.6. Blots of combined protein extracts from etiolated and light treated seedlings contained all three pI isoforms, confirming the existence of different isoforms in etiolated- and light-treated seedlings. Another low molecular weight antigen (around 31 kDa) was detected in all cases in the immunoblots below the pI 4.7 isoform (Fig. 12). This protein may represent a degradation product since IEF-SDS-PAGE of D4H purified from leaves also showed the presence of a peptide with a similar *Mr* (De Carolis and De Luca, 1993).

The light stimulation of D4H is not caused by an increase in carbon availability. To investigate if light activated D4H is a result of increased carbon availability due to the activation of photosynthesis, 7 day old etiolated seedlings were fed an external carbon source. Neither etiolated seedlings nor light-treated etiolated seedlings grown in the presence of sucrose (Fig. 13) or glucose (data not shown) displayed enhanced levels of *d4h* transcripts, D4H protein or enzyme activity compared to the light treatment alone (Fig. 13). These initial experiments suggested that light may exert a more direct effect on D4H and that induction may not be due to the activation of photosynthesis. These results were not unexpected since etiolated seedlings do accumulate equivalent concentrations of vindoline pathway intermediates which are quantitatively converted into vindoline by light treatment (Balsevich et al., 1986; De Luca et al., 1986).

D4H requires light to remain fully active. Studies were also conducted to determine if the continuous presence of light is necessary to maintain high levels of enzyme activity. Seven-day old etiolated seedlings which were exposed to light for a 24 h period were returned to dark conditions for 24 or 48 h and each dark treatment was subsequently re-exposed to a 24 h light period. The results indicate that *d4h* transcripts, D4H protein and D4H activity decreased as a result of the light/dark transition (Fig. 14). The maximum decreases in these three parameters were observed after 48 h in the dark and the levels were comparable with those detected at the beginning of the experiment (Fig. 14). Re-exposure of 24 h and 48 h dark-treated seedlings to light caused an increase in *d4h* transcripts, D4H protein and D4H activity (Fig. 14) indicating that regular exposure to light is necessary to maintain the levels of D4H activity during seedling development.

Phytochrome is involved in D4H light-activation. Previous studies have suggested that phytochrome may be involved in the light activation of D4H (De Carolis, 1994) and DAT (Aerts and De Luca, 1992). Five- and 7-day old etiolated seedlings were treated with red light for various lengths of time at a continuous photon fluence of 20 μM

m^{-2} sec^{-1} and were kept in the dark for a further 24 h before processing. As noticed in earlier figures, 5-day old seedlings showed a more pronounced response to red light treatment than the older developmental stage (Fig. 15A and B). When 5-day old seedlings were exposed to red light for a minimum of 15 min, both *d4h* transcripts and D4H protein levels increased, respectively, but D4H activity remained at background levels. A minimum 30 min red light treatment of 5-day old etiolated seedlings was required in order to obtain maximum D4H activity (Fig. 15A), whereas longer exposures to red-light did not result in any further increase in enzyme activity. Red-light treatment of 7-day old etiolated seedlings resulted in a slight but continuous increase in enzyme activity with 15 to 120 min of exposure (Fig. 15B). In general, the accumulation of D4H protein increased coordinately with the length of red light treatment and correlated with the timing of appearance of enzyme activity in both stages of seedling development (Fig. 15A and B). In contrast to this, the level of *d4h* transcripts which seemed to peak at 30 min of red-light exposure, either remained at this level (Fig. 15A) or decreased with prolonged red light treatment. These data strongly support an involvement of phytochrome in the pathway leading to *d4h* gene expression and/or enzyme activity.

Further studies on the kinetics of D4H activation by red-light and its reversal by far red-light were conducted. Five- and 7-day old etiolated seedlings which were exposed to 30 min of red-light were harvested after 8, 16 and 24 h of dark growth (Fig. 16). While there was little modulation of *d4h* transcript levels by red-light treatment (Fig. 16A, top panel), both D4H protein levels and enzyme activities increased 5- and 3-fold, respectively, in 5-day old red-light treated seedlings. In contrast, red-light treatment of older seedlings significantly increased the levels of *d4h* transcripts and enzyme activities after 16 h of dark growth, but not D4H protein levels, compared to those found in 7-day old dark controls (Fig. 16B). In both developmental stages enzyme activity increased up to 16 h after the red-light pulse (Fig. 16A), and it decreased significantly after 24 h (data not shown).

Typical phytochrome responses involve reversibility of red-light activation by far red light. Some of these processes include the regulation of hypocotyl shortening in etiolated seedlings, the control of flowering (McNellis and Deng, 1995) and the control of terpenoid biosynthesis (Tanaka et al., 1989; Yamamura et al., 1991). In *Catharanthus* seedlings, red light activation of D4H (Fig. 15A; De Carolis, 1994) and DAT (Aerts and De Luca, 1992) appear to be under this type of photoreversible control. The inducing effects of a 30 min red-light pulse on the accumulation of *d4h* transcripts, D4H protein and enzyme activity were reversed by a 30 min far red-light treatment and the reversion was prevented by a subsequent 30 min red-light pulse (Fig. 16A and B).

DISCUSSION.

The availability of a rapid and sensitive assay for D4H enzyme activity (De Carolis et al., 1990; De Carolis and De Luca, 1993), D4H cDNA clones (Chapter II) and a highly specific anti-D4H antibody has made it possible to study the expression of D4H at multiple levels.

Light participates in processes controlled by seedling development. Developmental studies with etiolated seedlings (Fig. 11A) confirmed that the *d4h* gene is expressed in the dark (Chapter II). The appearance of D4H protein followed closely the levels of hydroxylase transcripts in etiolated seedlings but these produced only low D4H enzyme activities throughout the time course. Treatment of etiolated seedlings with light did activate D4H enzyme activity, but this depended on the age at which seedlings were exposed (Fig.11). Five-day old seedlings appeared to be optimally primed to respond to light treatment, producing the highest D4H activities (Fig. 11B) which also correlated with the most appropriate developmental stage for vindoline accumulation (De Luca et al., 1986; Aerts et al., 1994). In contrast, younger (data not shown) seedlings did not respond well to light treatment and older seedlings (Fig. 11C) were only capable of a more limited response producing maximal D4H activities directly related with their developmental stage of growth (Compare Fig. 11B and C, 8 day time point). The importance of seedling development in the light response was also corroborated when 9- and 11-day old etiolated seedlings were treated with light and the D4H activities only reached those of later stages of development of continuously illuminated seedlings (Fig. 11C, bottom panel, data not shown). The light treatment, therefore, appears to activate processes already triggered and controlled by seedling development.

Appearance of D4H enzyme activity is under complex regulatory control. The differential effect of light on the expression of D4H transcripts, protein, and enzyme activity (Fig. 2) at various stages of seedling development suggest that multiple levels of

control may be involved in the regulation of D4H. The results shown in Figure 11A and B show that, while D4H transcripts and protein appear in dark grown seedlings, light is required for the appearance of significant hydroxylase activity. The modulation by light of these three parameters appears to vary with seedling development and decreases progressively with the age of etiolated seedlings. A possible explanation of these results may involve several levels of control in which light modulates development-related transcription, translation and undetermined post-translational modifications (Fig. 12) which would activate or inactivate the enzyme.

The occurrence of post-translational modifications in D4H protein have been suggested by previous studies involving the purification of this protein to homogeneity from *Catharanthus* leaves (De Carolis and De Luca, 1993) and by the fact that D4H exists as a single copy gene (Chapter II). The purified protein could be resolved by isoelectric focusing and SDS-PAGE into 3 isoforms with isoelectric points of 4.6, 4.7 and 4.8 of a 45 kD protein. The results presented in this report suggest that the 4.7 pI isoform which also occurs in dark grown seedlings (Fig. 12A) may be inactive and that light treatment may convert this isoform into an active more acidic isoform by an undetermined post-translational modification (Fig. 12B). In this context, it is interesting to note that DAT which is involved in the last step of vindoline biosynthesis also appears to exist as isoforms with various specific activities (Fahn et al., 1985; Powers et al., 1990).

A photoreversible phytochrome is involved in the activation of D4H. The red light activation of D4H could be reversed by a subsequent far red light treatment and strongly suggested the involvement of phytochrome in the light regulation of D4H (Fig. 15 and 7). A minimum 30 min red light pulse was necessary and sufficient to saturate the D4H response (Fig. 16A) resulting in increased production or accumulation of *d4h* transcripts and of D4H protein, whereas far red light treatment completely reversed this process (Fig. 16). The significant increase of D4H protein appearing within 8 h of red light treatment of 5-day old etiolated seedlings (Fig. 16A) suggests that the signal

transduction pathway between photoreception of the light stimulus and activation of D4H may be shorter than previously anticipated (Aerts et al., 1992; De Carolis, 1994). It is interesting to note that the kinetics of D4H activation by red light was time dependent (Fig. 16A and B). The minimal dose of red light required for the induction of D4H does not allow its classification as a low fluence response. However, the relatively short time span between reception of the light stimulus on the induction of D4H activity and its photoreversibility, strongly suggest that vindoline biosynthesis is closely associated with the photomorphogenetic process in *Catharanthus roseus*. These results, together with those showing that regular intervals of light treatment were required to maintain enzyme activity (Fig. 14), suggest the involvement light-requiring factors in the transduction of the light stimulus resulting in the activation of D4H.

Our results suggest that the phytochrome receptor may be involved in the transcriptional, translational and post-translational regulation of D4H. Other light regulated plant proteins displaying this behavior are usually enzymes involved in basic metabolic activities, such as nitrate reductase (Naussaume et al., 1995), the small subunit of rubisco (Keller et al., 1991) and starch phosphorylase (St-Pierre et al., 1996). This report suggests that these mechanisms may regulate alkaloid biosynthesis for an undetermined, but important reason. Developmental studies have shown that the complete pathway leading to catharanthine biosynthesis occurs in etiolated seedlings, whereas several of the terminal steps in vindoline biosynthesis appear only upon light stimulation. Chemical inducers of vindoline biosynthesis such as methyl jasmonate (Aerts et al., 1994) appear only to be effective if light is applied and only within a specific developmental time frame (Vazquez-Flota and De Luca, submitted), suggesting an intimate association between the light-activation of vindoline biosynthesis and light-dependent developmental processes. *In vitro* experiments have shown that enzymatic coupling of vindoline and catharanthine can be carried out by rather nonspecific peroxidase preparations (Endo et al., 1988). It is reasonable, therefore, to suggest that the combined presence of catharanthine and vindoline in the cell would lead to the production of the antimitotic dimers, VBL and VCR. In this way light activation of the

terminal steps in vindoline biosynthesis may be coupled with essential and undetermined ontogenetic processes required to sequester cytotoxic vinblastine and vincristine dimers, which would otherwise kill the plant. Specialized laticifers and idioblasts have been shown to exist in *Catharanthus roseus* (Yoder and Mahlberg, 1976; Eilert et al., 1985; Mersey and Cutler, 1986), but their potential roles in alkaloid biosynthesis/accumulation remain to be shown.

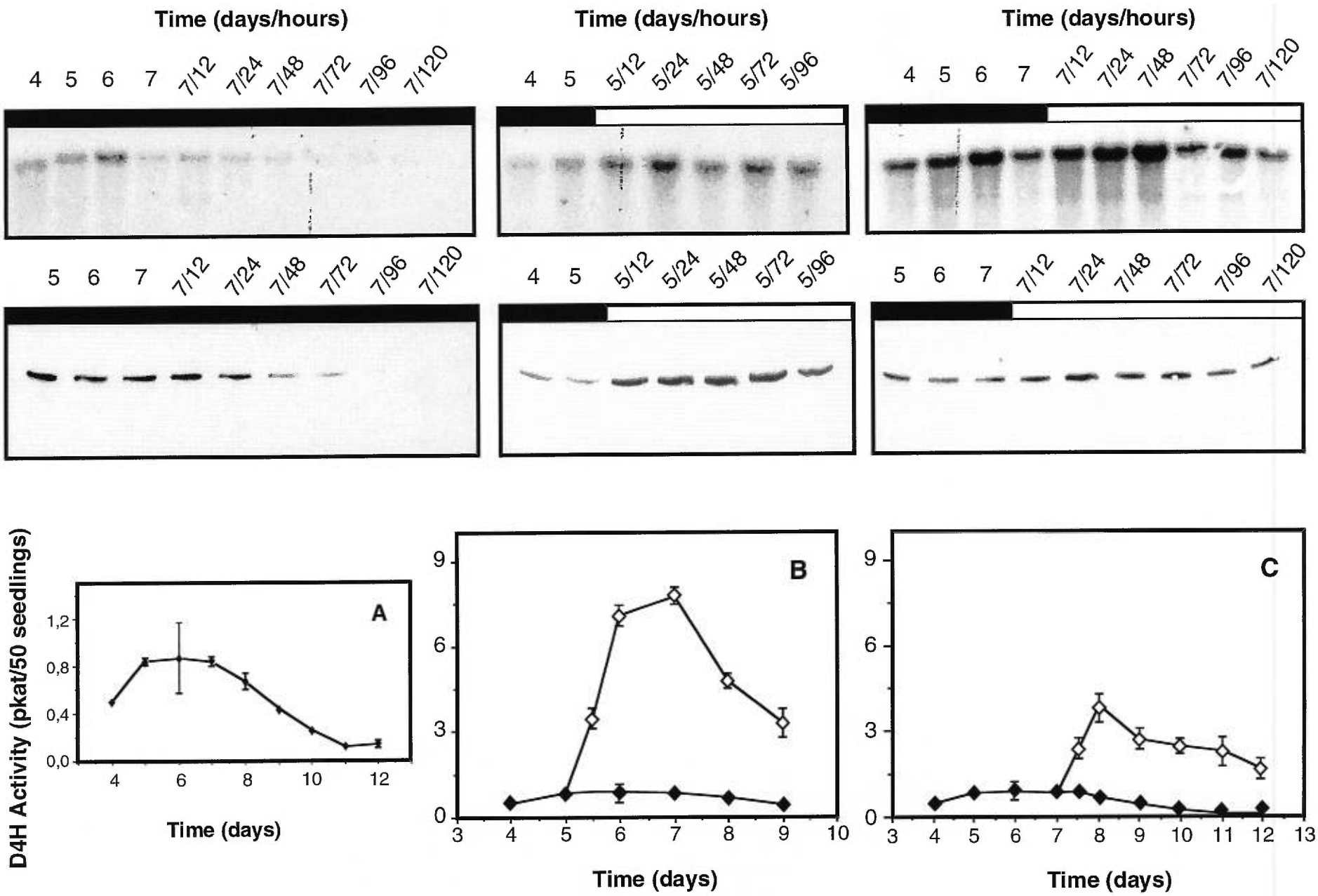
Time (days/hours)
4 5 6 7 7/12 7/24 7/48 7/72 7/96 7/120
Time (days/hours)
4 5 5/12 5/24 5/48 5/72 5/96
Time (days/hours)
4 5 6 7 7/12 7/24 7/48 7/72 7/96 7/120
5 6 7 7/12 7/24 7/48 7/72 7/96 7/120
4 5 5/12 5/24 5/48 5/72 5/96
5 6 7 7/12 7/24 7/48 7/72 7/96 7/120
D4H Activity (pkat/50 seedlings)
A
1,2
0,8
0,4
0,0
4 6 8 10 12
Time (days)
B
9
6
3
0
3 4 5 6 7 8 9 10
Time (days)
C
9
6
3
0
3 4 5 6 7 8 9 10 11 12 13
Time (days)

Figure 11. Profiles of *d4h* transcripts (top panels), D4H protein (middle panels) and D4H enzyme activities (bottom panels) during seedling development. Seedlings were grown in the dark (**Fig. 11A**) or exposed to light following 5- or 7-days (**Fig. 11B** or **C**) of etiolated growth. Black boxes in top and middle panels represent when seedlings were kept in darkness and white boxes represent when seedlings were exposed to light. The black symbols in the bottom panels represent D4H enzyme activities during etiolated seedling growth whereas white symbols represent D4H enzyme activities in light grown seedlings. The data in the bottom panel is represented as an average of three separate experiments and standard deviations are shown. The equality of RNA and protein loading were ensured by visual inspection of ethidium bromide stained gels and by Bradford assays (*Bradford, 1976*), respectively. These controls were performed for all subsequent Figures as well.

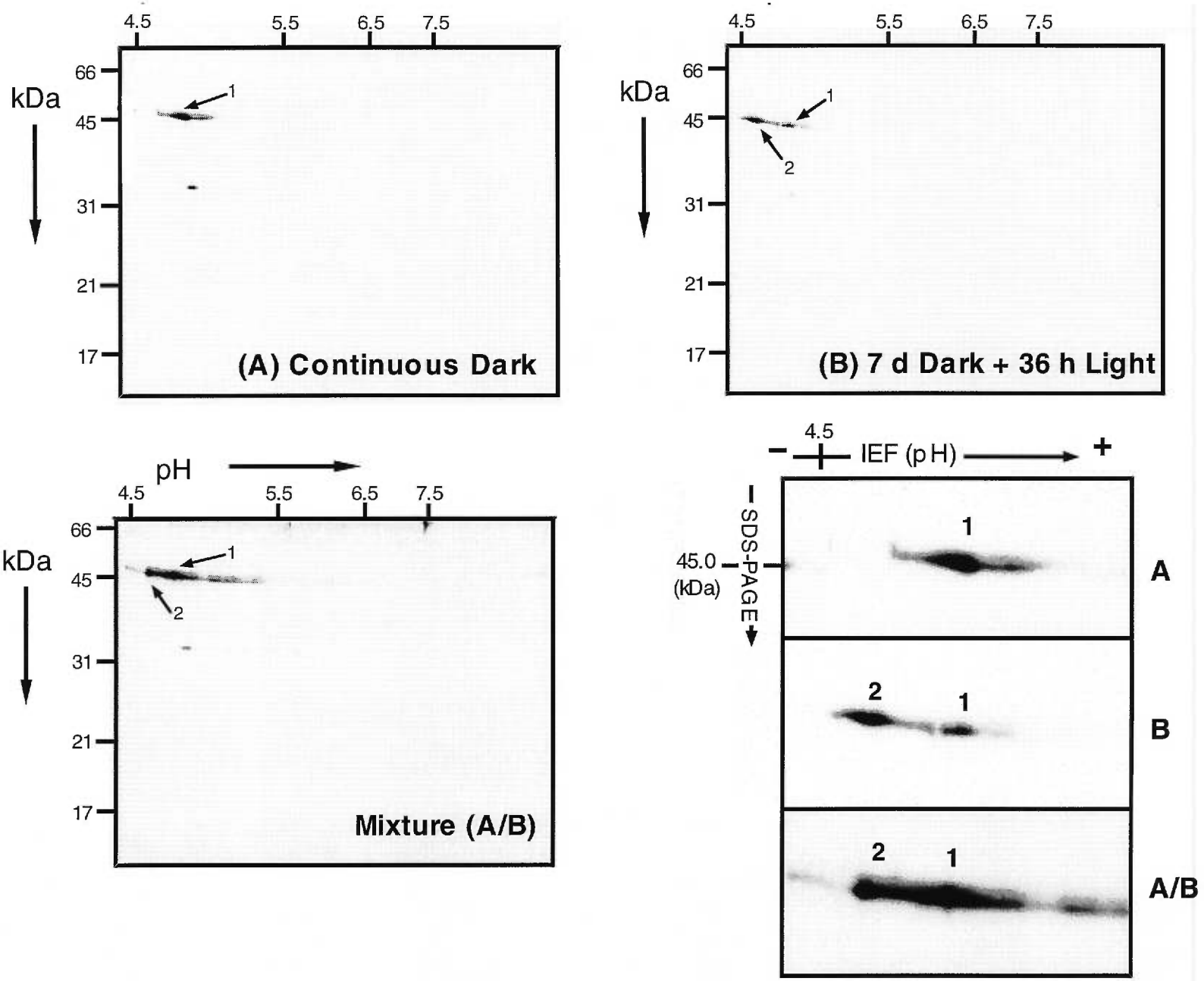
4.5
5.5
6.5
7.5
kDa
66
45
31
21
17
1
(A) Continuous Dark
2
(B) 7 d Dark + 36 h Light
pH
Mixture (A/B)
IEF (pH)
SDS-PAGE
45.0
(kDa)
A
B
A/B

Figure 12. IEF-SDS PAGE immunoblots of crude protein extracts. Seven-day old etiolated seedlings were grown for a further 36 h in the dark (**A**) or were treated with light for 36 h (**B**) before processing. Equal amounts of dark and light induced extracts were pooled and were also submitted to IEF-SDS PAGE immunoblot (**A/B**). The lower right panels shows a magnification of the immunoblot area where the D4H antigens were resolved. (**1**) and (**2**) represent isoforms with pI's of 4.7 and 4.6, respectively. The pH gradient (range 3-10, right pointing arrow) and the molecular weight (in kDa) range (down pointing arrow) were calibrated with commercial standards (2D-SDS-PAGE standards and SDS-PAGE low range molecular weight standards, respectively, from Bio-Rad).

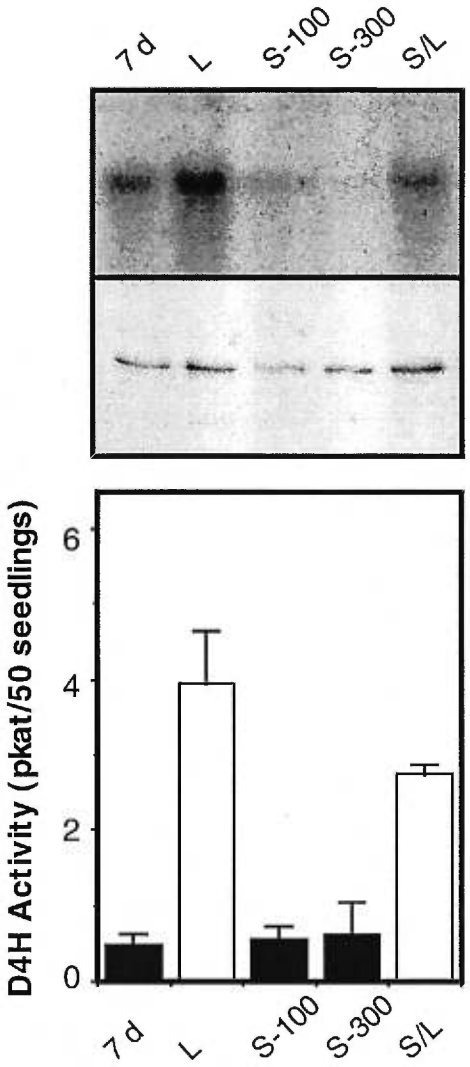

Figure 13. Effects of addition of sucrose to etiolated seedlings on the levels of *d4h* transcripts (top panel), D4H antigen (middle panel) and D4H enzyme activity (bottom panel). Seven-day old etiolated seedlings (**7 d**) were either exposed to: 24 h of light (**L**), 100 µMoles of sucrose in the dark (**S100**), 300 µMoles of sucrose in the dark (**S300**), or to a combination of 100 µMoles of sucrose and 24 h of light (**S/L**). The data in the bottom panel is represented as an average of three separate experiments and standard deviations are shown.

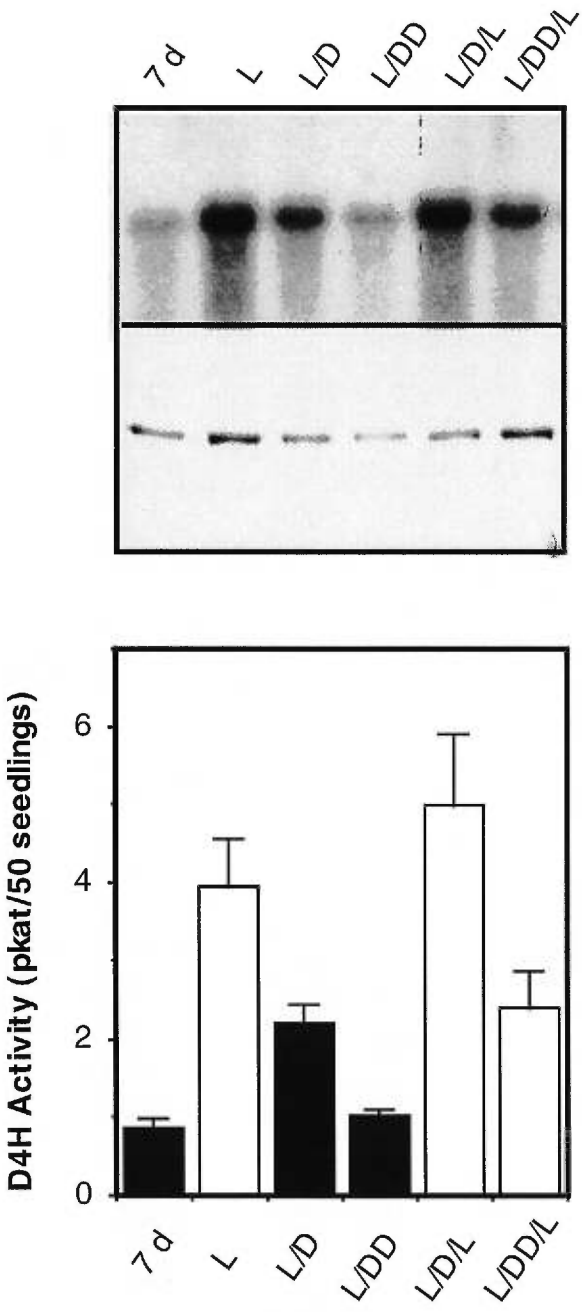

Figure 14. Effects of light/dark transitions on the levels of *d4h* transcripts (top panel), D4H antigen (middle panel) and D4H enzyme activity. Seven-day old etiolated seedlings (**7 d**) were exposed to 24 h of light (**L**), followed by a 24 (**L/D**) or 48 h (**L/DD**) dark period. After the 24 or 48 dark periods, seedlings were re-exposed to light for 24 h (**L/D/L** or **L/DD/L**). The data in the bottom panel is represented as an average of three separate experiments and standard deviations are shown.

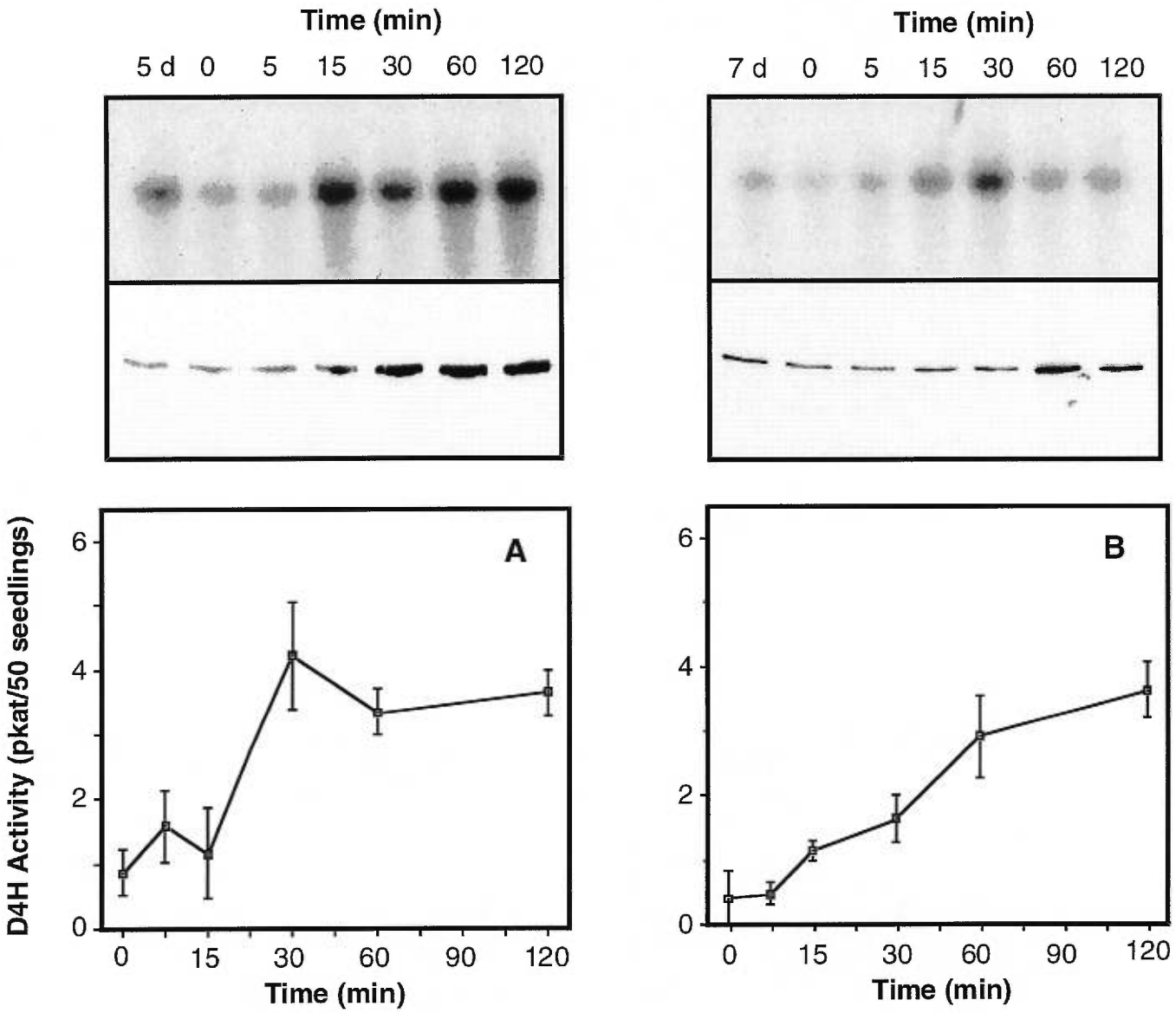
Time (min)
5 d 0 5 15 30 60 120
Time (min)
7 d 0 5 15 30 60 120
D4H Activity (pkat/50 seedlings)
A
B
0
2
4
6
0 15 30 60 90 120
Time (min)

Figure 15. Time course of red-light treatment on the levels of *d4h* transcripts (top panels), D4H antigens (middle panels) and D4H enzyme activities in 5- (**A**) and 7-day (**B**) old etiolated seedlings. After each red light treatment (denoted in minutes), seedlings were returned to dark conditions for 24 h before prior to processing for analysis. The data in the bottom panels are represented as an average of three separate experiments and standard deviations are shown.

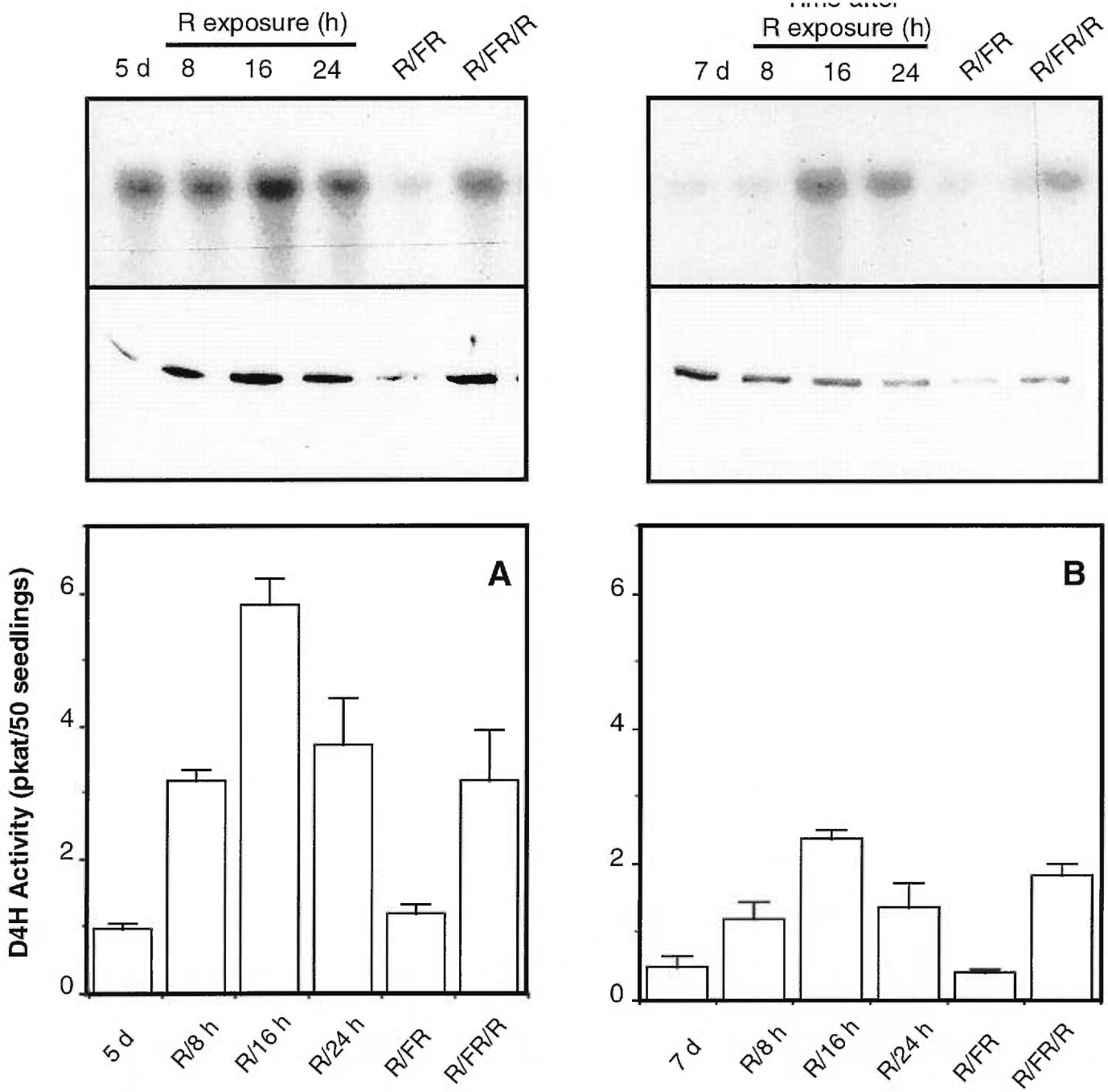
R exposure (h)
5 d
8
16
24
R/FR
R/FR/R
R exposure (h)
7 d
8
16
24
R/FR
R/FR/R
A
B
D4H Activity (pkat/50 seedlings)
0
2
4
6
5 d
R/8 h
R/16 h
R/24 h
R/FR
R/FR/R
7 d
R/8 h
R/16 h
R/24 h
R/FR
R/FR/R

Figure 16. Red light kinetics of D4H activation and its photoreversibililty by far red light in 5- (**A**) and 7-day old etiolated seedlings (**B**). For the kinetic analysis, 5- and 7-day old etiolated seedlings were either harvested immediately **(5 d** and **7 d)** or were exposed to red light **(R)** for 30 min and were harvested following **8**, **16** and **24** h of further dark growth. Five- and 7-day old etiolated seedlings were also exposed to repetitive 30 min red and 30 min far red light treatments (**R/FR** and **R/FR/R**) and samples were harvested after a further 24 h of dark growth. The top, middle and bottom panels display the levels of *d4h* transcripts, D4H antigens and D4H enzyme activities, respectively. The data in the bottom panels are represented as an average of three separate experiments and standard deviations are shown.

Chapter IV. Jasmonate Modulates Developmental and Light Regulated Alkaloid Biosynthesis in *Catharanthus roseus*.

Objectives.

General objective.

1) To analyze if the developmentally coordinated regulation of the early (TDC) and late (D4H) stages of vindoline biosynthesis persist under conditions which alter indole alkaloid biosynthesis in *Catharanthus roseus* seedlings.

Specific objective.

1) To study the effects of methyl jasmonate and salicylic acid on the levels of TDC and D4H transcripts, proteins and enzyme activities during the course of seedling development.

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JASMONATE MODULATES DEVELOPMENT- AND LIGHT-REGULATED ALKALOID BIOSYNTHESIS IN *CATHARANTHUS ROSEUS*.

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ABSTRACT

Methyl jasmonate, which is a chemical inducer of secondary metabolism, has been shown to promote the biosynthesis of vindoline in developing seedlings as a result of induction of tryptophan decarboxylase (TDC) and desacetylvindoline 4-hydroxylase (D4H). The present studies suggest that jasmonate-based induction of TDC and D4H activities involves modulation of transcriptional, post-transcriptional and post-translational controls. The effects of jasmonate on both enzymes were transient with maximum TDC activity appearing 12 h earlier than the D4H peak. Jasmonate treatment of etiolated seedlings could not replace the light requirement for induction of D4H. Jasmonate, therefore, appears to modulate events which are already triggered by development- and environment-specific controls. Salicylic acid, another chemical inducer of secondary metabolism, was ineffective in activating either TDC or D4H under the experimental conditions used.

Key words: *Catharanthus roseus*; desacetoxyvindoline 4-hydroxylase; methyl jasmonate; salicylic acid; tryptophan decarboxylase; vindoline.

INTRODUCTION

Numerous monoterpenoid indole alkaloids, including catharanthine and vindoline, are produced by the Madagascar periwinkle (*Catharanthus roseus*). As independent agents, both compounds have a limited ecological and therapeutic value (Frischknecht et al., 1987; Prakash et al., 1987). However, the oxidative coupling of these alkaloids produces the highly cytotoxic bisindole alkaloids vinblastine and vincristine. These dimeric alkaloids are currently used in cancer chemotherapy and they may represent important defensive/anti-feeding chemicals (Frischknecht et al., 1987). Unfortunately, both valuable anticancer agents are accumulated at very low levels in the aerial parts of *Catharanthus roseus* plants and intensive research has shown that this may be related to the strict controls which regulate the biosynthesis of the vindoline component of these dimers (De Luca et al., 1986; St-Pierre and De Luca, 1995). Developing seedlings accumulate vindoline mostly in cotyledons, where its biosynthesis is activated by a specific developmental program and by light (De Luca et al., 1986; 1988), whereas catharanthine accumulates throughout the seedling without light stimulation. Cell and organ cultures which accumulate catharanthine are unable to produce vindoline since they may lack certain cotyledon-specific enzyme activities involved in the late stages of vindoline biosynthesis (De Carolis, 1994; De Luca et al., 1986; 1988). However, a recent study has shown that transformation of cell cultures with different strains of *Agrobacterium tumefasciens* and *Agrobacterium rhizogenes* produced cell lines which express deacetylvindoline 4-*O*-acetyltransferase and accumulate low levels of vindoline (O'Keefe et al., 1997).

Catharanthine and vindoline are derived from tryptamine and secologanin (reviewed by De Luca, 1993). Tryptophan decarboxylase (TDC; EC, 4.2.1.28) converts tryptophan into tryptamine and the iridoid glucoside secologanin is produced from geraniol by a series of enzyme reactions. The condensation of tryptamine and secologanin is catalyzed by the vacuolar enzyme strictosidine synthase (SS) to produce the key central intermediate strictosidine and several enzymatic reactions transform

strictosidine by a series of different molecular rearrangements to form iboga alkaloids, such as catharanthine, or aspidosperma-type alkaloids, such as tabersonine and vindoline.

The position of TDC at the interphase between primary and secondary metabolism suggests that it may play a critical role in controlling carbon flux towards alkaloid biosynthesis in *Catharanthus roseus* (Alvarez-Fernandez et al., 1989; De Luca et al., 1986; 1988). However, the appearance of maximum TDC activity in developing seedlings does not correspond with the time of vindoline accumulation which occurs 24 to 36 h after the peak of TDC activity. Accumulation of vindoline does, however, coincide with the appearance of maximal enzyme activities which catalyze the terminal steps of vindoline biosynthesis (De Carolis et al., 1990; De Luca et al., 1986; Fig. 4). The second to last step in vindoline biosynthesis is catalyzed by a 2-oxoglutarate dependent dioxygenase known as desacetoxyvindoline 4-hydroxylase (D4H; EC. 1.14.11.11; De Carolis and De Luca, 1993) which hydroxylates desacetoxyvindoline at position 4 to produce deacetylvindoline. The last reaction in this pathway involves the *O*-acetylation of deacetylvindoline which is catalyzed by acetyl CoA:deacetylvindoline 4-*O*-acetyltransferase (DAT; De Luca et al., 1986, 1988). The enzyme activities for D4H and DAT are almost exclusively detected in the cotyledons of light-exposed seedlings and in leaves of mature plants (De Carolis et al., 1990; De Luca et al., 1986), which coincides with the sites of vindoline accumulation. Recent studies reported that the methyl ester of jasmonic acid, methyl jasmonate, can augment the accumulation of vindoline in developing seedlings in part by increasing TDC, SS, D4H and DAT enzyme activities (Aerts et al., 1994; 1996). Jasmonates act as chemical messengers in the transduction of environmental signals which may activate defense mechanisms including secondary metabolism (Gundlach et al., 1992). Salicylic acid has also been found to participate in this defense-related increase of secondary metabolism (Sharma et al., 1996).

We are interested in the regulation of vindoline biosynthesis and in the external factors that affect it. In this article we report the effects of jasmonate and salicylic acid,

two chemical inducers of secondary metabolism, on two key enzymes, TDC and D4H, involved in vindoline biosynthesis.

RESULTS AND DISCUSSION

Catharanthus roseus seedlings were germinated in darkness for 7 days and were then exposed to methyl jasmonate (1.25 ppm) or salicylic acid (70 pMoles/seedling) (Aerts et al., 1996), under either a dark or a light regime. Light treatment was necessary to induce D4H activity (Aerts et al., 1994; De Carolis et al., 1990, Chapter II) and vindoline accumulation, whereas the TDC activity profile was not altered significantly by light treatment (De Luca et al., 1986; 1988).

Effects of MeJa and SA on the development- and environment-specific regulation of TDC. The appearance of TDC activity has been shown to be under developmental and hormonal control (Alvarez-Fernandez et al., 1989; Fernandez et al., 1989; Goddijn et al., 1992; Noe and Berlin, 1985; Pasquai et al., 1992; Roewer et al., 1992) and the enzyme peaks transiently for a brief 48 h period between day 4 to day 6 of seedling development (Alvarez-Fernandez et al., 1989; Roewer et al., 1992). Jasmonate treatment of seven-day old seedlings which only contain 25 % of the maximal activity found in 5-day old seedlings, resulted in a transient reactivation of TDC enzyme activity. A 12 h exposure of 7-day old seedlings to jasmonate vapors produced a transient 400 % increase in TDC enzyme activity in both dark- (Fig. 17A) and light-grown (Fig. 17B) seedlings compared to untreated control seedlings, whereas TDC activity almost decreased to control levels after a further 36 hours of exposure to jasmonate. Previous studies showed that polyclonal anti-TDC antibodies reacted positively against several discrete proteins in crude seedling extracts which were resolved by SDS-PAGE and Western immunoblotting (Alvarez-Fernandez et al., 1989). The slowest migrating proteins (72, 68 and 65 kDa) were thought to represent ubiquitinated forms of TDC, whereas more rapidly migrating proteins were proposed to be degradation products (Alvarez-Fernandez et al., 1994). The appearance of TDC enzyme activity in developing seedlings coincided with the detection of a 54.8 and a 55 kD immunoreactive protein (Alvarez-Fernandez et al., 1989). SDS-PAGE and immunoblotting of protein extracts from jasmonate-treated *Catharanthus roseus*

seedlings, did not reveal any increase in the 55 kD immunoreactive protein over initial levels after a 12 h MeJa exposure (Fig. 18), but the levels of this immunoreactive protein decreased more slowly in jasmonate treated dark- or light-exposed seedlings than in the corresponding untreated light-control (Fig. 18). As TDC activity decreased after 24 and 48 h of jasmonate treatment, a corresponding reduction in both the 54.8 and 55 kD proteins was observed (Fig. 18) and these ultimately returned to the TDC antigen levels detected in untreated seedlings (data not shown).

In its active form TDC appears to be a 110 kD homodimer (Fernandez et al., 1989, Noe and Berlin, 1985) composed of two 55 kD subunits. The equilibrium between the stable active dimer and an unstable monomer may involve post-translational modifications of the monomer which lead to inactivation and degradation of the enzyme (Fernandez et al., 1989). In view of this proposed regulatory mechanism, a plausible interpretation of our results could be that jasmonate favors the maintenance of the stable active dimeric form of the enzyme (Fig. 18, 12 h treatment) compared to the situation in untreated control seedlings. Although jasmonate may stabilize active TDC protein, the transient induction of enzyme activity observed in Figure 17 may also be due to increased transcription of TDC mRNA. In fact MeJa treatment for 24 h also caused a 3- to 4- fold increase of *tdc* transcript levels in dark- or light- grown seedlings (Fig. 19). These results corroborate previous findings which suggested that TDC is regulated by complex transcriptional, post-transcriptional and post-translational controls (Alvarez-Fernandez et al., 1989; Alvarez-Fernandez and De Luca, 1994; Fernandez et al., 1989; Goddijn et al., 1992; Roewer et al., 1992). In contrast, salicylic acid did not promote increases in either TDC activity (Fig. 17), antigen accumulation (data not shown) or TDC transcript levels (Fig. 19).

Effects of MeJa and SA on the development- and environment-specific regulation of D4H. The effects of jasmonate and salicylic acid on the expression of D4H during seedling development were also studied. Very low levels of enzyme activity were observed in 7-day old etiolated seedlings (Fig. 20A), and neither jasmonate nor

salicylic acid were effective in inducing D4H activity in the dark. Exposure of etiolated seedlings to light increased D4H enzyme activity (Chapter II; Fig. 20B, Ctl) and MeJa treatment could further transiently activate this enzyme. The increase of D4H enzyme activity was about 85% higher in seedlings exposed to jasmonate (Fig 20B, MeJa at 24h) than in controls (Fig. 20B, Ctl. at 24h). Longer exposures to MeJa resulted in a decrease of enzyme activity back to the corresponding illuminated control level (Fig. 20B).

It was noted previously (Chapter III) that depending on seedling development and the light regime, similar levels of D4H antigens could account for variations of up to 300% in enzyme activity. Under the conditions used in this experiment, significant levels of D4H protein (Fig. 21) were detected in the etiolated seedlings which have low levels of enzyme activity (Fig. 20A). Light-treatment for 48 h increased D4H activity up to 7-fold compared to dark controls (Fig. 20B). In contrast, the level of D4H protein did not correlate with the enzyme activity as it decreased slightly over the dark control and subsequently surpassed slightly the dark control after 48 hours of light treatment. Recent studies in our laboratory which indicate that different isoforms of antigenic D4H protein exist in dark- and light-grown seedlings (Chapter III) may explain the lack of correlation observed in Figures 20 and 21. Treatment of light-grown seedlings with MeJa resulted in shortening by 24 h the time necessary to detect maximal D4H protein (Fig. 21, MeJa/L at 24h) and this correlated completely with the timing of the highest D4H activity (Fig. 20). In contrast to the effect observed with MeJa, salicylic acid treatment of light-grown seedlings did not transiently activate D4H enzyme activity (Fig. 20) or alter the pattern of accumulation of D4H protein (data not shown).

During the course of etiolated seedling development, *d4h* transcripts were shown to continuously increase to a maximum and then decreased to below detectable levels unless the seedlings were exposed to light (Chapter II). Treatment of seven-day old etiolated seedlings with MeJa or SA in the absence of light did not affect the levels of *d4h* transcripts (Fig. 22). It was exclusively upon illumination that a significant 6-fold

increase in transcript levels was detected (Fig. 22), but the simultaneous application of light and either jasmonate or salicylic acid did not further enhance transcript accumulation (Fig. 22). However, light- and MeJa-treatment increased D4H protein by 100% (Fig. 22, MeJa/L at 24 h) relative to control seedlings (Fig. 21, L at 24 h), whereas the steady-state levels of *d4h* transcript were similar in both cases (Fig. 22). Jasmonate treatment, therefore, also appears to stabilize or to extend the residence time of active D4H in some undetermined manner. For example, ribosomes isolated from jasmonate treated barley plants will preferentially translate defense-related transcripts over those of photosynthetic genes (Reinbothe et al., 1994). One can speculate that this or some other mechanism involving post-transcriptional or post-translational controls could also operate in *Catharanthus roseus*.

The results obtained in this report extend previous observations that MeJa treatment triggers measurable increases in indole alkaloid levels by modulation of both early and late stages of biosynthesis (Aerts et al., 1994). However, jasmonate appears only to modulate the events which are already triggered by development- and environment-specific controls. The timing of the MeJa triggered transient activation of TDC and D4H occurred over a 12 h gap, with the TDC peak of activity occurring first (Figs. 17 and 20). Earlier studies with light-grown developing seedlings have shown that TDC maximum activity precedes the D4H activity peak by 24 to 36 h (De Carolis et al., 1990; De Luca et al., 1986). The results presented here suggest that MeJa differentially regulates the cascade of events leading to the coordinate activation of these genes and that it may modulate some common regulatory control points which govern the kinetics of activation of the early and late stages of vindoline biosynthesis. Jasmonates are involved in several developmental processes (Reinbothe et al., 1994), and while most of their favorable effects on secondary metabolism are associated with a transcriptional activation of genes (Dittrich and Kutcham, 1991; 1992; Kutcham, 1993) they can also exert their regulatory effects at the post-transcriptional and post-translational levels (Reinbothe et al., 1994). This appears to be the case for both TDC (Figs. 18 and 19) and D4H (Figs. 21 and 22).

SA was ineffective in inducing any increase in TDC or D4H activities (Figs. 21 and 20; Aerts et al., 1996). Although it is possible that the applied concentration of SA was below the effective range, we adjusted it to the typical effective concentrations previously reported for other species (Klessig and Malamy, 1994). Since SA is readily mobilized (Klessig and Malamy, 1994), we can assume that it was taken up and transported. In fact, hypocotyls of seedlings treated with salicylic acid were shorter and thicker than the controls, suggesting that the concentrations applied were in the effective range.

In conclusion, the results presented in this work stress the important roles played by development and by light to trigger vindoline biosynthesis in *Catharanthus roseus.* Other inducers of secondary metabolism such as MeJa appear to modulate events already initiated by seedling development and by light. Since MeJa was effective in altering TDC and D4H expression, this signaling molecule may be involved in transmitting development- or environment-triggered events that lead certain cells and tissues to produce alkaloids such as tabersonine or vindoline.

EXPERIMENTAL

Plant materials. Batches of *C. roseus* seeds (*cv.* Tropicana) were sterilized and planted on 3 layers of filter paper in 9 cm Petri dishes as described before (Chapter II). Approximately 100 seeds were used per plate and care was taken to avoid contact between seeds. The plates were sealed with Parafilm and kept in the dark under controlled conditions for 7 days (25°C, 70% RH).

Treatments. Etiolated seedlings were exposed to MeJa or SA and were either kept in the dark or submitted to an 18 h photoperiod (20 μmol m^{-2} s^{-1}) for the periods shown in the figures. MeJa (Firmenich) was diluted in ethanol and 25 µl of this solution were applied in a tiny cup which was placed in the center of the dish, avoiding any direct contact between the seedlings and the jasmonate solution. The final concentration of jasmonate in the internal atmosphere was adjusted to 1.25 ppm (Aerts et al., 1994). SA (BDH), was dissolved in 5% ethanol and 1 ml of the stock solution was applied underneath the paper layers to yield a final concentration of 7000 pmoles SA/petri dish. Samples were collected at the times indicated in the figures, frozen in liquid nitrogen and kept at -80°C until analysis.

Enzyme analysis. Batches of 100 seedlings were ground in 2.5 ml of the extraction buffer containing 200 mM Tris-Cl pH 7.6, 10 mM EDTA, 5 mM dithiothreitol. After centrifugation, the supernatant was desalted on PD-10 columns (Pharmacia) previously equilibrated with 50 mM Tris-Cl pH 7.5 and 28 mM 2-mercaptoethanol. TDC and D4H activities were assayed as described previously (De Carolis et al., 1990; De Luca et al., 1986 respectively).

Immunological analysis. Desalted extracts were mixed with SDS-loading buffer and the protein occurring in a half seedling (8 to 10 µg of protein) were applied and separated by SDS-PAGE (Laemmli, 1970). After electrophoresis, proteins were transferred onto a nitrocellulose membrane for antigen detection (Leary et al., 1983).

TDC antigens were recognized with the anti-TDC antibody (H-95; Alvarez-Fernandez et al., 1988), while D4H was detected with the anti-D4H antibody (Chapter III). Bound rabbit anti-TDC and -D4H antibodies were treated with donkey-anti-rabbit second antibodies coupled to horseradish peroxidase and products were detected by chemiluminescence (Renaissance kit from New England Nuclear).

Nucleic Acid Extraction and Analysis. Total RNA was extracted, separated in agarose gels, and transferred to nitrocellulose membranes as described before [Chapter II]. *tdc* and *d4h* transcripts were detected by hybridization against the cDNA clones TDC-5 (De Luca et al., 1989) and *cD4H-3* (Chapter II), respectively, which had been radiolabelled with [^{32}P]. Conditions for hybridization and autoradiography are described in (Chapter II).

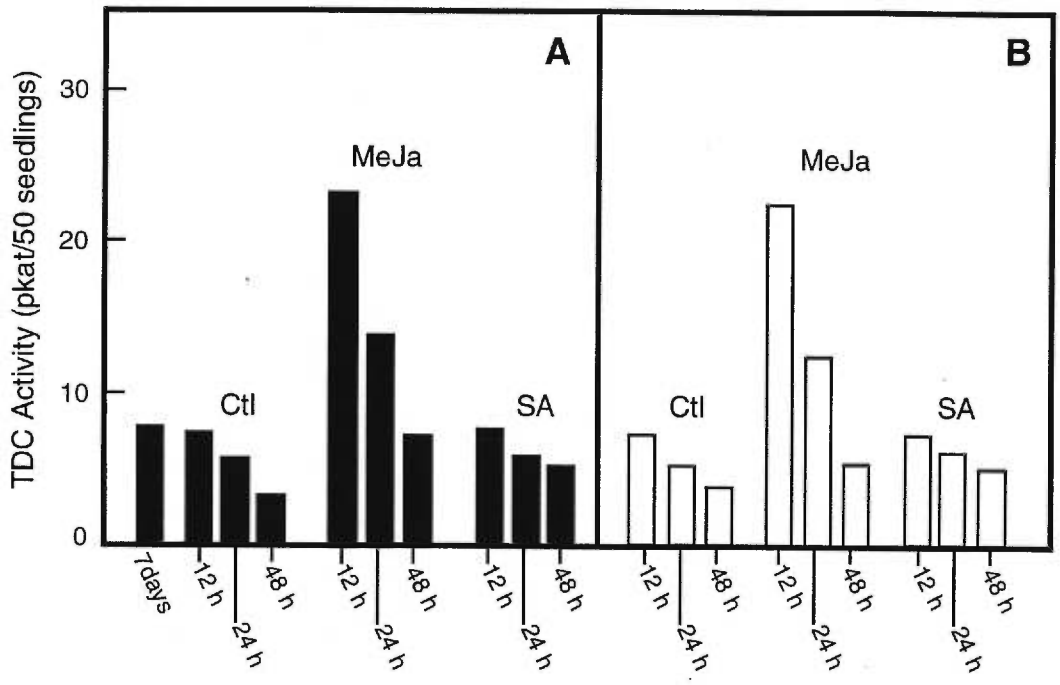
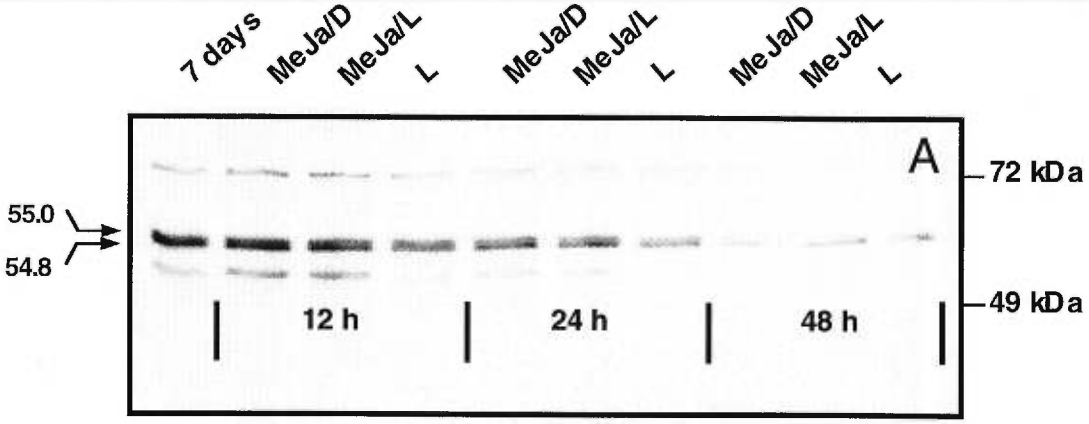


Figure 17: Effects of MeJa and SA on TDC activity during seedling development. Seven-day old etiolated seedlings (**7 D**) were exposed to 1.25 ppm of MeJa vapor, 70 pmoles SA/seedling, or water (Control, **Ctl**). Seedlings were either kept in the dark (**A**), or were exposed to light (**B**) for **12**, **24** and **48** h, respectively. Each data point represents the average of two replicate experiments where the variation was less than 10% of the average.


7 days
MeJa/D
MeJa/L
L
MeJa/D
MeJa/L
L
MeJa/D
MeJa/L
L
A
72 kDa
49 kDa
55.0
54.8
12 h
24 h
48 h

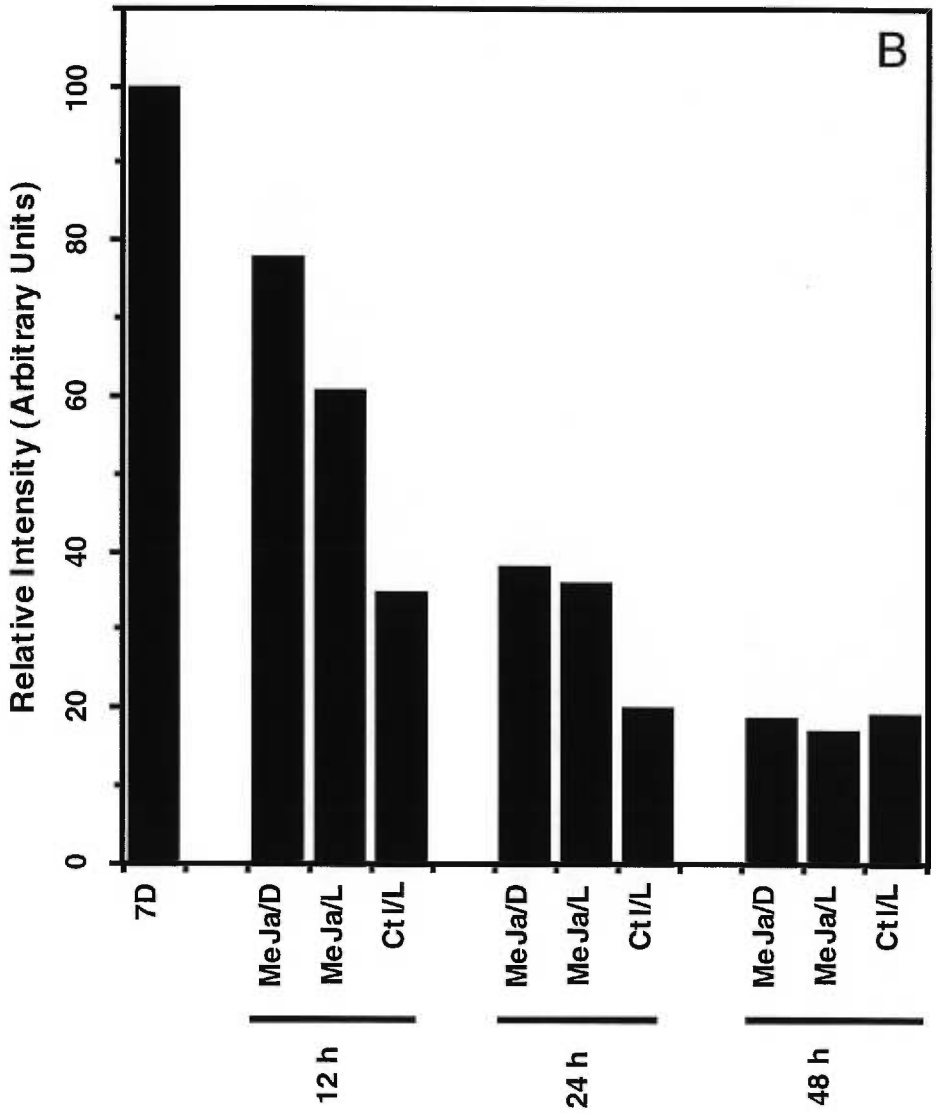

B
Relative Intensity (Arbitrary Units)
0
20
40
60
80
100
7D
MeJa/D
MeJa/L
Ctl/L
MeJa/D
MeJa/L
Ctl/L
MeJa/D
MeJa/L
Ctl/L
12 h
24 h
48 h

Figure 18: Effects of MeJa on TDC protein levels during seedling development. Seven-day old etiolated seedlings (**7 D**) were either exposed to 1.25 ppm of MeJa vapor, or water (Control, Ctl) and were either kept in the dark (**D**), or were exposed to light (**L**) for **12**, **24** and **48** h, respectively. Protein fractions equivalent to half a seedling were submitted to 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and the membranes were probed with anti-TDC antibodies. The immunoblots were scanned by densitometry. The relative joint densities of the 54.6 and 55 kDa immunoreactive proteins are displayed as a bar graph as a percentage of the most intense immunoblot.

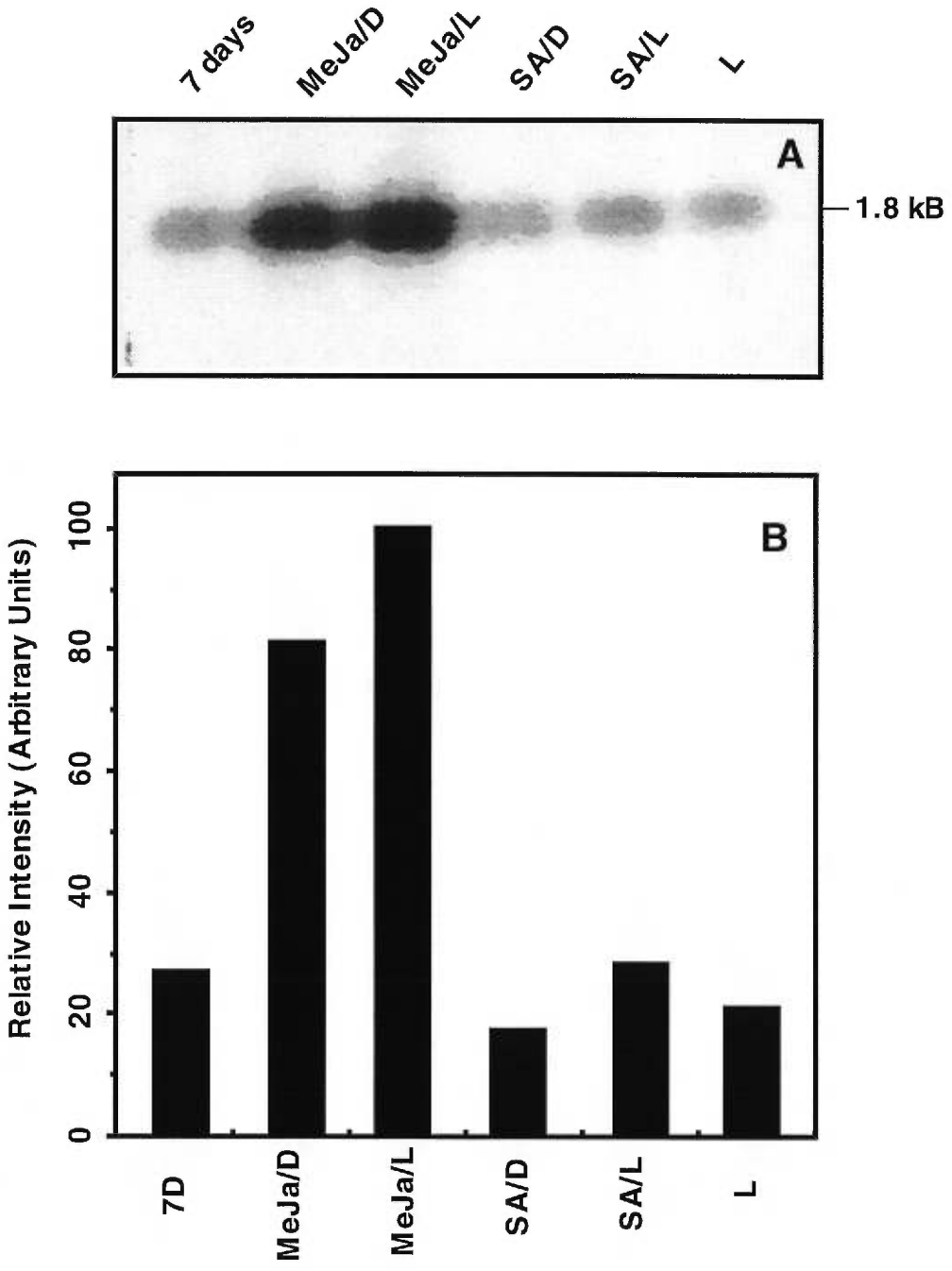


Figure 19: Effects of MeJa and SA on TDC transcript levels during seedling development. Seven-day old etiolated seedlings (**7 D**) were either exposed to 1.25 ppm of MeJa vapor, 70 pmoles SA/seedling, or water (Control, **Ctl**) and were either kept in the dark (**D**), or were exposed to light (**L**) for 24 h. Ten µg of total RNA from each treatment were separated on formaldehyde containing agarose gels, transferred onto a nitrocellulose membrane, and probed with the cDNA clone TDC-5 radiolabelled with [^{32}P]. Northern blots were scanned by densitometry. The relative intensities of transcripts are expressed as a percentage of the most intense blot.

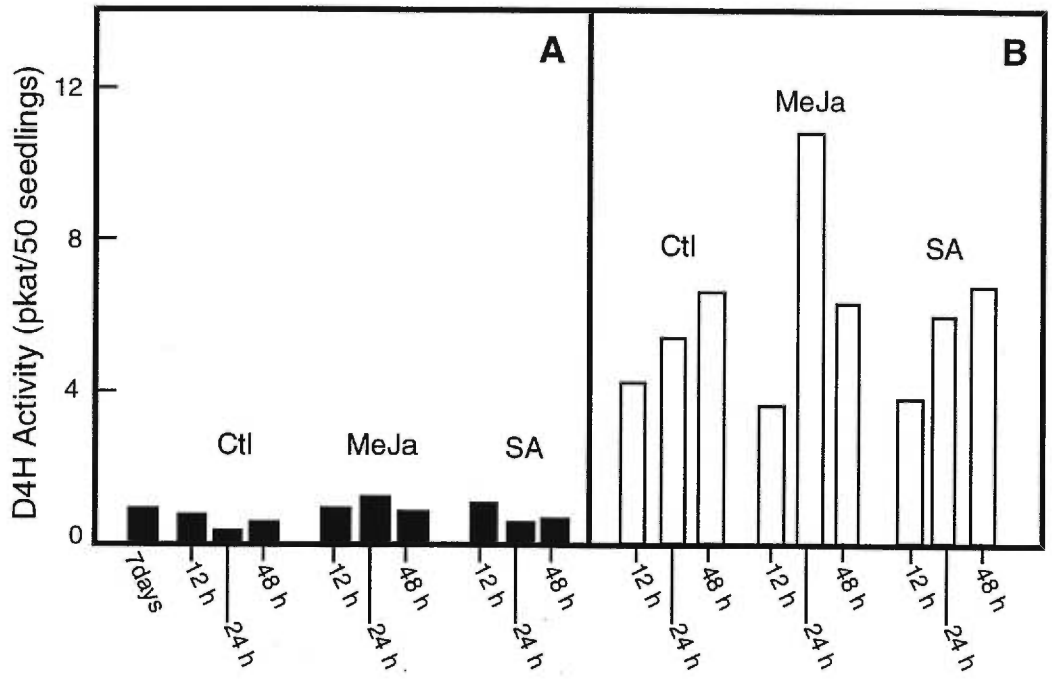


Figure 20: Effects of MeJa and SA on D4H enzyme activity during seedling development. The treatments and symbols are described in Figure 17. Each data point represents the average of two replicate experiments where the variation was less than 15% of the average.

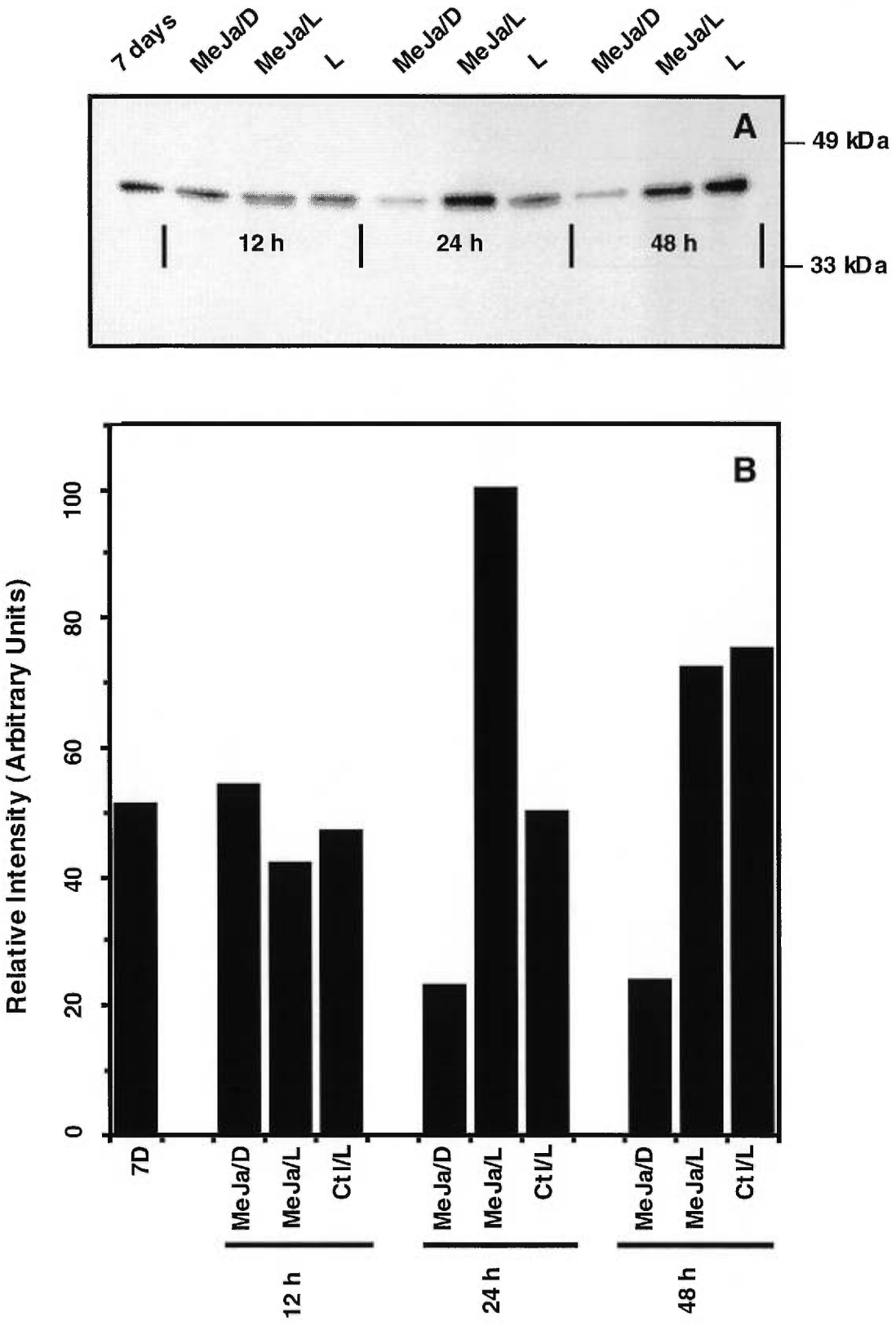

7 days
MeJa/D
MeJa/L
L
MeJa/D
MeJa/L
L
MeJa/D
MeJa/L
L
A
49 kDa
33 kDa
12 h
24 h
48 h
B
Relative Intensity (Arbitrary Units)
0
20
40
60
80
100
7D
MeJa/D
MeJa/L
Ctl/L
MeJa/D
MeJa/L
Ctl/L
MeJa/D
MeJa/L
Ctl/L
12 h
24 h
48 h

Figure 21: Effects of MeJa on D4H protein levels during seedling development. The treatments and symbols are described in Figure 18. The relative densities of the D4H immunoreactive protein are displayed as a bar graph as a percentage of the most intense immunoblot.

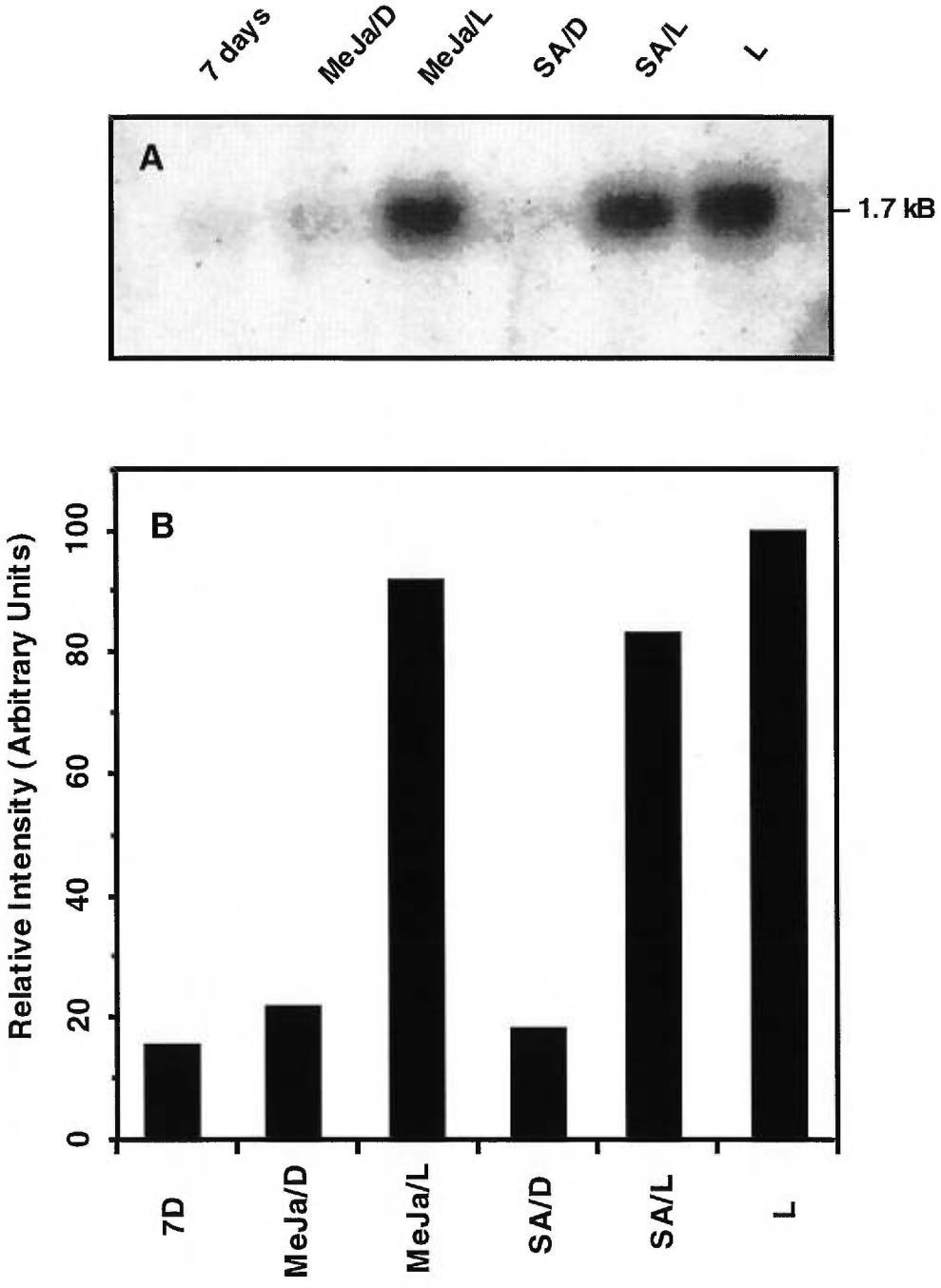


Figure 22: Effects of MeJa and SA on D4H transcript levels during seedling development. The treatments and symbols are described in Figure 18.

Chapter V. Immunocytochemical Analysis of Monoterpenoid Indole Alkaloid Biosynthesis in *Catharanthus roseus*: Involvement of Different Cell-Types in the Expression of TDC and D4H.

Objectives.

General objective.

1) To identify the cell type(s) participating in the biosynthesis of vindoline in *Catharanthus roseus* plants and in developing seedlings.

Specific objectives.

1) To identify plant tissues which express the highest levels of TDC and D4H in mature plants.

2) To immunolocalize cell types within tissues of mature plants which express TDC and D4H.

This work was done in collaboration with Dr. Benoit St-Pierre, who prepared the plant tissues for microscopy, sectioned them and assisted with the photography of the microscopic sections.

Publication of Chapter V: To be submitted for publication.

IMMUNOCYTOCHEMICAL ANALYSIS OF MONOTERPEPENOID INDOLE ALKALOID BIOSYNTHESIS IN *CATHARANTHUS ROSEUS*: INVOLVEMENT OF DIFFERENT CELL-TYPES IN THE EXPRESSION OF TDC AND D4H

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ABSTRACT

The tissue- and cell-specific distributions of tryptophan decarboxylase (TDC) and desacetoxyvindoline 4-hydroxylase (D4H) were studied by Western immunoblot, Northern blot analysis, enzyme assay and immunocytochemistry in *Catharanthus roseus* plants. TDC was found in above and under-ground tissues, while D4H was restricted to leaves and stems. The highest activity for both enzymes occurred in actively growing tissues, which correlated with the presence of TDC and D4H RNA transcripts and proteins. Immunocytochemical analysis suggests that TDC is expressed exclusively in the upper and lower epidermal cells of leaves and in epidermal cells of stems. In contrast, D4H expression was restricted to the laticifers and idioblasts of stems and leaves. The abundance of both antigens decreased following a basipetal gradient in leaf sections and these results were confirmed by immunoblot and RNA blot analyses. Further studies with roots revealed that only TDC is expressed in the root cortical cells around the apical meristem, but not in root epidermal cells. These results suggest that several cell-types are involved in the biosynthesis of the monoterpenoid indole alkaloids. The early biosynthetic reactions, which are common to all these alkaloids, may take place in the epidermis of aerial tissues or in the cortical cells of the root apex, while the late reactions specific to vindoline biosynthesis occur exclusively in laticifers and idioblasts of aerial tissues. The implications of such a differential cellular distribution of pathway gene expression are discused in relation to the regulation of vindoline biosynthesis.

INTRODUCTION.

The organs forming the plant body consist of several different cell-types which are organized in relation to one another and which confer specific functions to the resulting organ. Each cell-type emerges from an undifferentiated meristem according to a sophisticated and partially understood developmental program. Plant cells in the early stages of embryogenesis, unlike animal cells, do not migrate, and developmental information to produce plant organs is primarily acquired from the plane in which cell division takes place (Sylvester et al 1996; von Arnim and Deng, 1996). The commitment to differentiate into specialized structures involves the assimilation by cells in the meristem, of a complex array of signals which communicate cellular age, position in relation to other cells and hormonal balance. Environmental factors, such as light and temperature, also play a critical role in modulating these signals throughout the process of organogenesis (Bernier, 1988; Dale et al., 1988; Salysbury, 1982; Sylvester et al. 1996).

The same morphogenetic processes also yield specialized cells with distinctive biochemical features to biosynthesize and/or accumulate secondary metabolites, such as phenylpropanoids (Jahnen and Halbrock, 1988; Marchant et al., 1987), monoterpenoids (Fahn, 1988; McCaskill et al., 1992) and alkaloids (Eilert et al., 1986; Hashimoto et al., 1991; Facchini and De Luca, 1995; Nessler and Mahlberg, 1978; Robinson, 1974; 1981). Studies with germinating seedlings have suggested that alkaloid biosynthesis and accumulation is associated with seedling development (Aerts et al., 1992; Mothes et al., 1965; Weeks and Bush, 1974), while a developmental gradient of alkaloid distribution occurs throughout the accumulating tissues of mature plants (Frischnecht et al., 1986; Westekemper et al., 1980). Furthermore, alkaloid biosynthesis in cell suspension cultures appears to be coordinated with cytodifferentiation (Kutchan et al., 1983; Lindsey and Yeoman, 1983).

Vindoline biosynthesis in *Catharanthus roseus* also appears to be under this type of developmental and morphogenetic control (Constabel et al., 1982; Mothes et al., 1965; Westekemper et al., 1980). In the leaves of *C. roseus*, vindoline is enzymatically coupled with catharanthine to produce the powerful cytotoxic dimeric alkaloids, vinblastine and vincristine (Svoboda and Blake, 1975). Vindoline, as well as the dimeric alkaloids, are restricted to leaves and stems, while catharanthine is equally distributed througout the above- and under-ground tissues (Farnsworth et al., 1964, Balsevich and Bishop, 1989; Deus Neumann, 1985; Westekemper et al., 1980). The developmental regulation of vindoline biosynthesis has been well documented in *Catharanthus* seedlings where it is light inducible, in contrast to catharanthine which also accumulates in etiolated seedlings (Balsevich et al., 1986; De Luca et al., 1986; Mothes et al., 1965; Scott, 1970). Furthermore, cell cultures which accumulate catharanthine but not vindoline (see Moreno et al., 1995 and van der Heijeden et al., 1989 for recent reviews) recover this ability upon re-differentiation of shoots (Constabel et al., 1982). These observations suggest that the biosynthesis of catharanthine and vindoline is differentially regulated and that vindoline biosynthesis is under a more rigid cell-, tissue-, development- and environment-specific control than that of catharanthine.

Over 100 *Catharanthus* alkaloids that have been identified share many common biosynthetic steps. The early stages of alkaloid biosynthesis in *C. roseus* involves the formation of tryptamine from tryptophan and its condensation with secologanin to produce the central intermediate strictosidine, the common precursor for the monoterpenoid indole alkaloids (Figs. 2 and 3). The enzymes catalyzing these two reactions are tryptophan decarboxylase (TDC) and strictosidine synthase (SS), respectively (see De Luca, 1993 for a review). It is has been proposed that strictosidine is transformed into stemmadenine, which may represent the branch point in the biosynthesis of both the iboga (catharanthine) and aspidosperma (vindoline) type alkaloids (Figs. 2 and 3; Parry, 1975; Scott, 1970). According to this biosynthetic scheme, further enzymatic reactions would transform stemmadenine into tabersonine (Fig. 2 and 3; Parry, 1975; Scott, 1970) and hydroxylation at C-16, 16-*O*-methylation,

hydration of the 2,3-double bond, *N*(1)-methylation, hydroxylation at C-4, and 4-*O*-acetylation would convert tabersonine into vindoline (Fig. 4; De Luca et al., 1986; 1988). The first pair of reactions are catalyzed by tabersonine 16-hydroxylase (T16H), and by AdoMet:16-hydroxytabersonine-*O*-methyltransferase (16-OMT) respectively (St-Pierre and De Luca, 1995). The third to last reaction is catalyzed by an AdoMet:2,3-dihydro-3-hydroxytabersonine-*N*-methyltransferase (NMT; De Luca et al., 1987; Dethier and De Luca, 1993), whereas the 4-hydroxylation is catalyzed by desacetoxyvindoline 4-hydroxylase (D4H), a 2-oxoglutarate dependent dioxygenase (De Carolis et al., 1990; De Carolis and De Luca, 1993). The final reaction is catalyzed by Acetyl CoA: deacetylvindoline-*O*-acetyltransferase (DAT; De Luca et al., 1985; Power et al., 1990). Some of these enzymes are not expressed in cell cultures and in tissues unable of produce vindoline (De Carolis et al., 1990; De Luca et al., 1986; 1988). However, some of the enzymes leading to the production of catharanthine and 16-methoxytabersonine have been detected in in cell suspension and root cultures which explains their ability to accumulate these indole alkaloids. (De Luca et al., 1986; St-Pierre et al., 1995).

It has been suggested that certain tissues lack necessary enzymes required for vindoline biosynthesis and/or the cellular structures necessary for its accumulation (Eilert et al., 1987). Yoder and Mahlberg (1976) used chemical indicators to identify laticifers and "specialized parachyema cells" as the sites of alkaloid accumulation. Direct observation of *Catharanthus* leaves by epifluorescence microscopy showed the random distribution of cells throughout the mesophyll, that displayed distinctive autofluorescent properties (Mersey and Cutler, 1986). Leaf sections and protoplast preparations observed under bright-field, inverted microscopy revealed the presence of larger autoflorescent yellow cells with few chloroplasts, compared to the surrounding red autoflorescent mesophyll cells. The former cells, which are known as idioblasts (Constabel, 1983; Mersey and Cutler, 1986), occur in several plants families, are probably morphologically related to laticifers (Fahn, 1988) and they may be associated with the biosynthesis and accumulation secondary products (Eilert et al., 1986; Platt and Thomson, 1992; Postek and Tucker, 1983).

In the present study, we have used highly specific and immunoreactive polyclonal antibodies against TDC (Alvarez-Fernandez et al., 1988) and D4H (Chapter III) to localize their cell-specific expression in different tissues of *Catharanthus* plants. The results suggest that the early (TDC) and late (D4H) reactions of vindoline biosynthesis occur in different cells.

MATERIALS AND METHODS.

Plant materials. *Catharanthus roseus* (*cv* Little Delicata) seeds were purchased from Sakata Seeds Inc (Salinas Ca) through a local dealer (W.H. Perron, Laval Qc). Seedlings were germinated as described by De Luca et al (1986) and plants were grown from seed to maturity in the greenhouse.

Differential distribution of the enzymes involved in the biosynthesis of vindoline. Leaves at different stages of maturity and various stem internodal regions were collected from 6 month old plants. Roots from the main axis or lateral roots were also collected and harvested tissues were frozen in liquid nitrogen and kept at -80°C until analysis.

Analytical procedures. *Enzyme assays.* The enzyme activity for TDC and D4H were assayed in desalted extracts according to De Luca et al (1986) and De Carolis et al (1990), respectively.

Protein electrophoresis and immunoblotting. Proteins were extracted from the different tissues in 200 mM Tris-Cl pH 7.5, 10 mM dithiothreitol and 5 mM EDTA. Conditions for SDS-PAGE and immunoblotting have been reported before (Chapter III). TDC and D4H antigens were recognized with the polyclonal antisera H95 (Alvarez-Fernandez et al., 1989) and D4H-ab (Chapter III), respectively.

Nucleic acid analysis. Procedures for the isolation, electrophoresis and blotting of RNA, as well as the conditions for hybridization, post-hybridization washes and autoradiography, have been reported elsewhere (Chapter II). *tdc* and *d4h* transcripts were hybridized to the cDNA's clones TDC-5 (De Luca et al., 1989) *cD4H-3* (Chapter II) respectively.

Immunocytochemical localization of TDC and D4H. *Preparation of sections for microscopy*. Different tissues from the mature plant, from dark grown or from light-

treated seedlings (see figures) were fixed overnight at 4°C in FAA (50% ethanol, 5% acetic acid and 10% formalin) and then dehydrated through 1-h intervals in a series of ethanol-*sec*-butanol-water mixtures. After dehydration, the samples were incubated overnight at 60 °C, first in paraplast:*sec*-butanol (1:1) and then in pure paraplast (Oxford Labware, St. Louis Mo). Sections were embedded in paraplast and kept at 4°C until processing (Johansen, 1940). Ten µm sections were cut with a rotary microtome (American Optical 840, Buffalo NY) and mounted on slides pre-treated with 2% (v/v) 3-aminopropyltriethoxysilane (Sigma, St. Louis Mo) in acetone. The embedding resin was dissolved in xylene and the sections were rehydrated by serial incubations in baths of decreasing ethanol:water dilutions and by 2 final washes in pure water (Johansen, 1940).

Immunolocalization of the early and late stages of vindoline biosynthesis. Once rehydrated, the slides were incubated with TBST (TBST is 10 mM Tris base, 150 mM NaCl and 0.05% Tween 20) for 10 min, blocked overnight at 4°C with 3% bovine serum albumin in TBST, and then washed with TBST. The sections were incubated with primary crude antisera diluted in blocking solution (see Figure Legends) for 2-4 h at room temperature in a humid chamber, followed by four 15 min washes in TBST. The sections were then incubated for 2 h at room temperature in a commercial preparation of goat anti-rabbit IgG conjugated with alkaline phosphatase (BioRad, Hercules, CA), diluted 1,000X in blocking solution. Unbound secondary antibodies were removed with two 15 min washes in TBST, followed by two 15 min washes in carbonate buffer ($NaHCO_3$ 100 mM pH 9.8, $MgCl_2$ 1 mM). The sections were incubated with of 0.15 and 0.3 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium respectively, diluted in the carbonate buffer and colour usually developed within an hour of incubation. Sections which were preserved in 7% gelatin, 50% glycerol and 1% phenol, were photographed on Kodak Royal Gold 100 ASA in an Olympus BHS microscope, equiped with a PM-10APS photomicroscopy system (Olympus Optical Co. LTD Tokyo Japan).

RESULTS

Tissue-specific distribution of TDC and D4H. In order to identify the tissues expressing TDC and D4H enzyme activities, different plant organs were analyzed. Six month old *C. roseus* plants were used, which are about 40 cm tall and 60 cm in diameter, and have a bushy growth pattern with several branches emerging around the same internode. Leaves were harvested from a single branch containing a terminal flower or from similar branches located at equivalent positions (Fig. 23). Since the leaf primordia unfold asymmetrically (Stern, 1975), 4 to 5 mm long terminal leaf buds (**TB** in Figures 24 and 25), 8 and 10 mm long unfolding leaves (**1** in Figures 24 and 25) and totally expanded leaves were selected for analysis (Figs. 23-25). The topmost internode which was about 6 mm in length and which occurred between the 2nd and 3rd node was also selected for analysis.

The expression of *tdc* transcripts was restricted to terminal buds, the first two unfolding leaves and to the top two stem internodes (Fig. 24A). Immunoblot analysis and enzyme assay showed that expression of TDC protein (Fig 24B) and enzyme activity (Fig. 24C) was synchronized with the appearance of *tdc* transcripts in leaves and in stem internodes.

The expression of *d4h* transcripts (Fig. 25A) was similar but not identical to that of *tdc* (compare Fig. 24A and 25A), since relatively high levels of *d4h* transcripts were detected in the terminal leaf bud, 1st, 2nd and 3rd leaf pair and in the 2nd and 3rd stem internodes. This transcript expression pattern was corroborated by the presence of corresponding levels of D4H protein (Fig. 25B) and enzyme activities (Fig. 25C) in these tissues.

Primary and secondary lateral roots which represent different stages of developmental growth, were harvested and analysed for expression of TDC and D4H. Developmentally younger lateral roots had high levels of *tdc* transcript, protein, enzyme

activity, whereas much lower levels were detected in the developmentally older primary roots (Fig. 26A, 26B and 26C). No D4H activity, D4H protein or *d4h* transcripts were detected in roots (data not shown, Chapter II).

Immunocytochemical localization of TDC and D4H. Young actively growing leaves with the highest levels of TDC and D4H were prepared for immunocytochemical localization studies. The basal lamina of these leaves curles inwardly towards the abaxial surface and these zones were easily identified, in longitudinal sections (Figs. 27A and 27D). Microscopic observations revealed that leaves contained a layer of upper and lower epidermis, a mesophyll with a file of palisade and several files of spongy parenchyma cells (Figs. 27 and 28; Mersey and Cutler, 1986; Yoder and Mahlberg, 1976). Unbranched unarticulated laticifers (Mersey and Cutler, 1986; Yoder and Mahlbert, 1976) were also identified. These circular structures are usually larger in diameter than the surrounding spongy mesophyll cells and are mainly located at the interphase between the palisade and spongy parenchyma (Figs. 27 and 28). The laticifers have been shown to be cross-connected to major vascular structures (Mersey and Cutler, 1986; Yoder, 1972). In addition, longitudinally oriented subdermal laticifers could also be identified on the abaxial side of leaf cross-sections (Fig. 27A-C; Mersey and Cutler, 1986; Yoder and Mahlberg, 1976). Idioblasts were also identified by epiflorescence microscopy in parallel sections as larger bright yellow cells with few chloroplast and which are generally found in spongy parenchyma, but are not restricted to it (Fig. 28; Mersey and Cutler, 1986).

Differential cellular distribution of TDC and D4H. When longitudinal sections of *C. roseus* leaves were treated with anti-TDC antibodies (H-95), TDC expression was exclusively localized to the upper (**UE**) and lower epidermis (**LE**) of this tissue (Fig. 27A-C). The epidermal layers of the petiole area with its inward curling basal lamina showed the most intense immunological reaction (Fig. 27A), followed by less intense staining of the epidermis in the middle portion of the leaf and virtually no reaction near the tip of the leaf blade (Fig. 27B and 27C). Treatment of the same sections with anti-D4H antibodies

(D4H-ab) showed that D4H is instead, exclusively localized in laticifer and idioblast cells (Fig. 27D-F, 28B and 28C).The most intense reactions for D4H expression were also noticed in the petiole area and in proximal regions (Fig. 27D and 27E; Yoder and Mahlberg, 1976). These results correlate with the denser distribution of laticifers characteristic for the petiole area (Fig 27A and B) than for middle or the tip of the leaf (Fig. 27C and 27D). Observations at higher magnifications (Fig. 28) corroborated the exclusive localization of TDC (Fig. 28A) to the upper and lower epidermis. In constrast, D4H was located in laticifers cross-connected to vascular structures (**CL**) (Fig. 28B), in longitudinal laticifers (**L**) and in idioblasts (**Id**), (Fig. 28B and 28C). Treatment of sections with preimmune serum did not produce any immune reactions to any cell type in these sections even when applied at a low dilutions (Fig. 28D). The differential cellular localization of TDC and D4H suggests that at least two different cell types within the same tissue are involved in the biosynthesis of vindoline in *Catharanthus roseus.*

These results also suggest a basipetal expression gradient for both TDC (Fig. 27A-C) and D4H (Fig. 27D-E). In order to confirm this, the second pair of leaves (Fig. 29A) were dissected into petiole, middle and tip areas and the levels of *tdc*, *ss*, *d4h*, and *dat* transcripts and of TDC, D4H and DAT proteins were analyzed.

Northern blot and Western immunoblot analysis clearly showed the basipetal distribution of all four transcripts and proteins, with the highest, intermediate and lowest levels occurring in the petiolar, middle and tip regions of the leaf, respectively (Fig. 29). These results clearly suggest that the pathway leading to vindoline biosynthesis appears very early in leaf development. *In situ* localization studies of *tdc*, *ss*, *d4h and dat* transcripts have also shown the same distribution pattern (St. Pierre et al., in preparation) reported for TDC and D4H proteins, and analysis of enzyme activity and alkaloid extracts from the different leaf areas are also consistent with such basipetal distribution (St-Pierre et al., in preparation).

Immunocytochemical localization of TDC and D4H in stems and roots. The cellular distribution for TDC and D4H antigens was studied in cross sections of stems and in root logitudinal sections (Figs. 30). Higher antisera concentrations and longer incubations times were required in order to detect TDC and D4H proteins in stem cross sections, but TDC and D4H were still exclusively located in stem epidermis (Fig. 30A) and in subdermal longitudinally oriented laticifers (Fig. 30B), respectively. Stem sections treated with a pre-immune serum (Fig. 30C) did not produce any visible immune reactions.

Longitudinal root sections were reacted with anti-TDC antibodies and revealed that TDC is expressed within cells of the apical meristem region, whereas older cells above this region failed to react to this antibody (Fig. 30D). The results indicate that expression of TDC in roots is restricted to actively dividing cells. Root sections treated with the D4H-ab (Fig. 30E) or with preimmune serum (Fig. 30F) failed to produce any visible immune reactions.

Immunocytochemical localization of TDC and D4H in developing seedlings. TDC and D4H are expressed under a carefully regulated developmental program during seedling development (Alvarez-Fernadez et al., 1989; De Carolis et al., 1990; De Luca et al., 1986; 1988; Chapters III and IV) and both TDC (Alvarez-Fernandez et al., 1989; Fernandez et al., 1989), and D4H (Chapters III and IV) are under the control of complex multilevel regulatory mechanisms. The developmental activation of TDC is not affected by light (De Luca et al., 1986; 1988), whereas D4H enzyme activity is only detected in light-grown seedlings (De Carolis et al., 1990) even if *d4h* transcripts and protein are detected in etiolated seedlings (Chapter II and III).

Sections were prepared from cotyledons of seedlings grown continuously in the dark for 8 days or from cotyledons exposed to light for 36 h after 6.5 days of etiolated growth. Expression of TDC was restricted to the upper epidermis (Fig. 31A) while D4H was localized in cross connecting laticifers, as well as in longitudinally oriented laticifers

(nomenclature according to Yoder and Mahlberg, 1976) in the sub-dermal region of the abaxial side of cotyledons (Fig. 32B-D). The detection of D4H in laticifers of dark (Fig. 31C) and light grown (Fig. 31D) seedlings confirm that inactive forms of D4H may exist in etiolated seedlings, and that light may indeed play a role in the activation of enzyme activity through an undetermined post-translational modification (Chapter III). Control sections probed with the pre-immune serum did not produce any visible immunological reaction (Fig. 31E and 31F).

DISCUSSION.

The distribution of TDC in actively growing shoots and roots of *C. roseus* plants (Fig. 24 and 26; Alvarez-Fernadez et al., 1989), suggests that meristems of different organs are capable of making tryptamine and monoterpenoid indole alkaloids. The actual presence of different alkaloids in both above and underground tissues suggests that distinct tissue-specific biosynthetic pathways are expressed (Deus-Neumann et al., 1987; Reda et al., 1978; Westekemper et al., 1980). The root distribution of high levels of catharanthine has been clearly documented in mature plants (Balsevich and Bishop, 1989; Deus-Neumann et al., 1987). In contrast, leaves and stems, which accumulate low levels of catharanthine, appear to be the exclusive sites of vindoline accumulation. This conclusion is clearly supported by the apical meristem-, leaf- and stem-specific distribution of D4H (Fig. 25; Chapter II).

The accumulation of alkaloids in actively growing tissues may be a defensive and protective strategy against predators (Frischnecht et al, 1987). Some antifeeding properties have been associated with vindoline accumulation (Frischnecht et al., 1987; Guillet et al., unpublished results), and the cytotoxic effects of the dimeric alkaloids also point to such a role. However, the bisindole alkaloids are generally absent in young shoots (Balsevich and Bishop, 1989; Naaranlathi et al., 1991) suggesting that the ability to synthesize and accumulate dimers appears with leaf maturation (Westekemper et al., 1980).

The immunocytochemical localization of TDC and D4H revealed that both enzymes followed a basipetal distribution in young expanding leaves (Figs 26, 27 and 29). These results were confirmed by immunoblot and RNA blot analysis of dissected leaf segments (Fig. 29). In dicots, cell division frequently occurs until the lamina has reached about 90% of its final leaf area (Dale et al., 1988). The size of young leaves used in this study represents 20% of the final surface area of a mature fully expanded leaf and were, therefore, probably under active cell division. However, the petiolar

region appears to undergo a more active cell division than those in the leaf tip, where cells are more developmentally mature and cellular volume increase is the more predominant growth process (Dale et al., 1988). The distribution of gene expression observed confirms that alkaloid biosynthesis is initiated in very young rapidly growing tissues.

The immunocytochemical analysis also showed a spatial separation of TDC and D4H gene expression within leaf and stem tissues (Figs. 27, 28, and 30). Expression of TDC was restricted to the epidermis of leaves and stems (Figs. 27A-C and 28A), while expression of D4H was associated with laticifers and idioblasts (Figs. 27D-E and 28B-C). In contrast, expression of TDC in roots was restricted to the cells of the root meristem (Fig. 30A) and there was no detectable D4H expression in these tissues (Fig. 30B). These results indicate that part of this pathway is restricted to the epidermal layers of leaves and stems, whereas late biosynthetic steps commited exclusively to vindoline formation are located in laticifers and idioblasts. This raises questions about the proportion of the pathway which is expressed in the epidermis and how specific intermediates are mobilized into laticifers and idioblasts for elaboration of vindoline.

Tabersonine and other post-tabersonine intermediates have been detected in *Catharanthus* roots (Farnsworth et al., 1964) as well as in root and cell cultures (Bhadra and Shanks, 1997; Kurz et al., 1980; Kutney et al., 1980; Parr et al., 1990; Toivonen et al., 1989), none of which apparently contain laticifers (Eilert et al., 1985). These results suggest that 1) laticifers are not required for tabersonine biosynthesis; 2) that tabersonine or a later intermediate may be the transport form which is translocated to laticifers and idioblasts for enzymatic elaboration into vindoline. Since laticifers are closely associated with the vascular system of the plant, transport of catharanthine and vindoline pathway intermediates from roots seems possible. However, the ability of stem and leaf epidermis to participate in alkaloid biosynthesis suggest, but do not prove that tabersonine and catharanthine could be made in these tissues as well.

The role of laticifers and idioblasts in catalysing the final steps of vindoline biosynthesis raises the question of where the coupling reaction to make the highly toxic dimeric indole alkaloids occurs. It is conceivable that these specialized cells are required to accomodate and sequester the toxic dimers from normal cellular processes. However the close association of idioblasts and subdermal longitudinal laticifers to epidermal cells (Fig. 28) could easily allow the movement of small molecules between these cell-types. Cell to cell mobilization has been well documented for flavonoids in parsley seedlings (Reinhold and Halbrock, 1998).

The immunocytochemical location of TDC and D4H reported here have been corroborated by *in situ* hybridization studies of their corresponding transcripts (St-Pierre et al., in preparation). In fact, both *tdc* and *ss* transcripts are expressed in root meristem, stem epidermis and leaf epidermis whereas *d4h* and *dat* transcripts are expressed exclusively in idioblast and laticifer cells (St-Pierre et al., in preparaton). The cummulative data strongly suggest that vindoline is formed in idioblast and laticifer cells, while other corynanthe, iboga and aspidosperma alkaloids may be elaborated in the epidermis of aerial tissues and in root meristems.

It is interesting to notice that enzymes involved in phenylpropanoid biosynthesis, such as phenylalanine ammonia-lyase and chalcone synthase, have also been localized in epidermal cells (Jahnen and Halbrock, 1988). Nectaries, trichomes and other secretory structures, which originate from epidermal cells (Fahn, 1988) also represent major sites of synthesis for various terpenoids (Keene and Wagner, 1985). The occurrence of alkaloid biosynthetic enzymes in specific cells has been reported in other species. In *Papaver somniferum* the expression of tyrosine decarboxylase, which catalyzes the first reaction in the formation of morphine and benzylisoquinoline alkaloids, was found in laticifers associated with pholem cells in stems (Facchini and De Luca, 1995). The enzyme, hyoscyamine 6ß-hydroxylase (H6H) was localized in the pericyle of young roots (Hashimoto et al., 1991). This pericycle location has been associated with the translocation the H6H reaction product, scopolamine, from its site of

synthesis to the leaves where it is accumulated (Hashimoto et al., 1991). The present study suggests that the precise cell-, tissue- and organ-specific compartment of monoterpenoid indole alkaloid biosynthesis, and of secondary metabolism in general, is regulated by differential expression of biosynthetic pathways and by a controlled transport of intermediates to the most appropriate sites for accumulation.

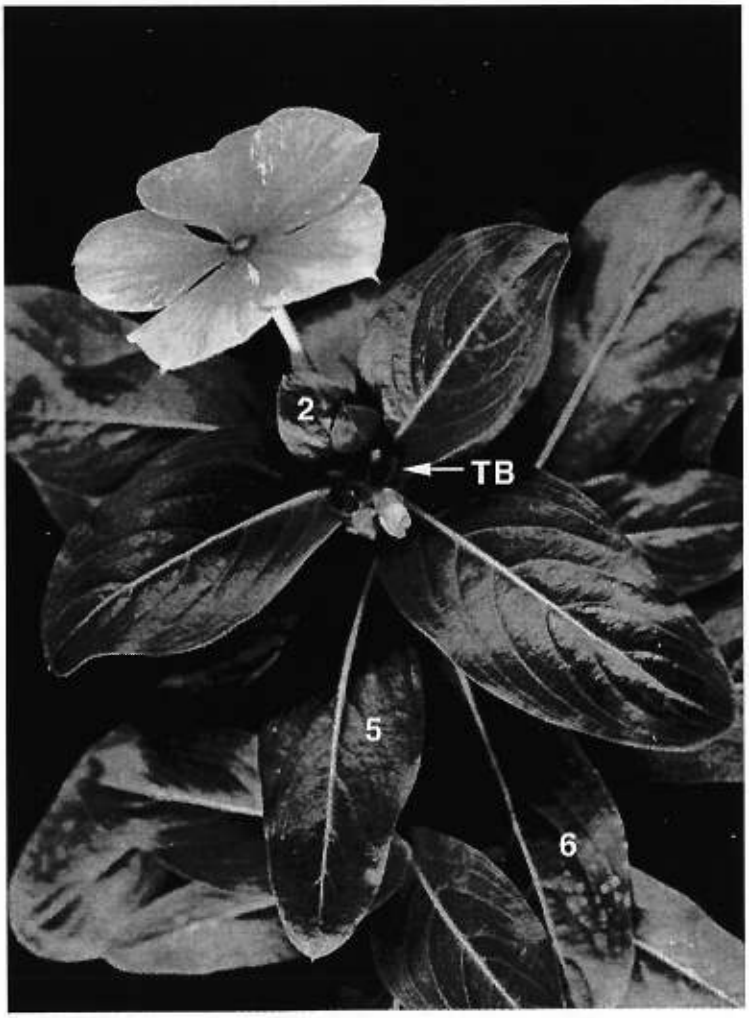


Figure 23. Photo of a 6 month old *Catharanthus roseus* plant. Numbers represent leaf pairs used in the experiments described in Figures 24 and 25. **TB** represent the terminal bud.

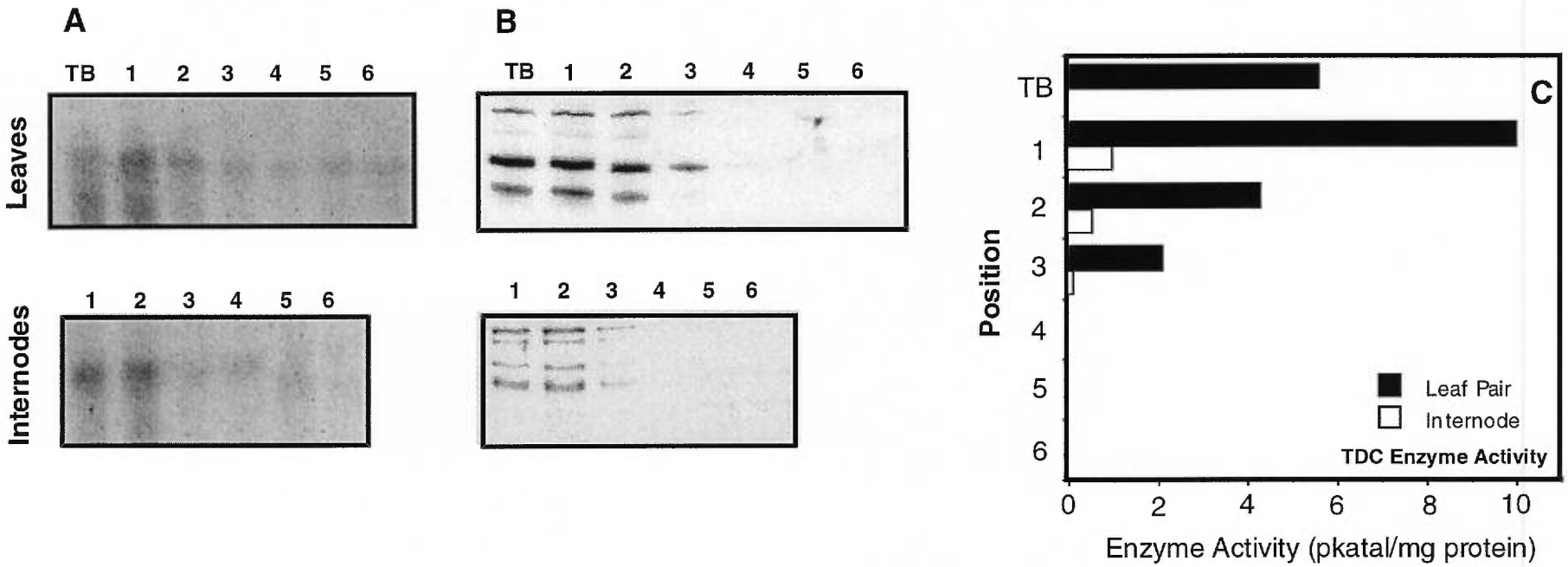


Figure 24. Expression of TDC in the leaves and stem internodes of *Catharanthus roseus* plants. (**A**) Distribution of *tdc* transcripts in leaves (upper panel), and internodes (lower panel). (**B**) Distribution of TDC immunoreactive proteins in leaf (upper panel) and internodes (lower panel). (**C**) TDC enzyme activity in different leaves pairs (closed bars) and in stem internodes (open bars). **TB** terminal buds; **1** to **7** (leaf) position on stem after the TB; or internodal segments of stems, beginning from the one in between the second and third pair of leaves.

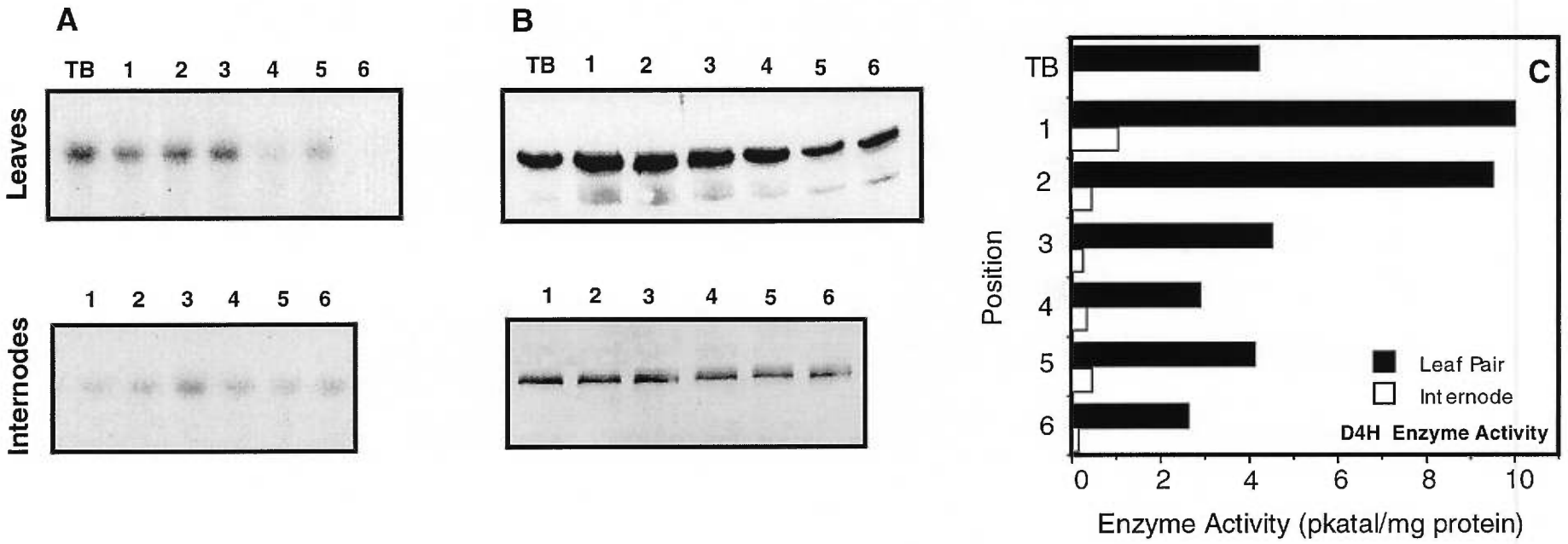


Figure 25. Expression of D4H in leaves and stem of *Catharanthus roseus* plants. (**A**) Distribution of *d4h* transcripts in leaves (upper panel), and internodes (lower panel). (**B**) Distribution of D4H immunoreactive proteins in leaves (upper panel) and internodes (lower panel). (**C**) Enzyme activity in different leaf pairs (closed bars) and in stems internodes (open bars). **TB** terminal buds; **1** to **7** (leaf) position on stem after the TB; or internodal segments of stems, beginning from the one in between the second and third pair of leaves.

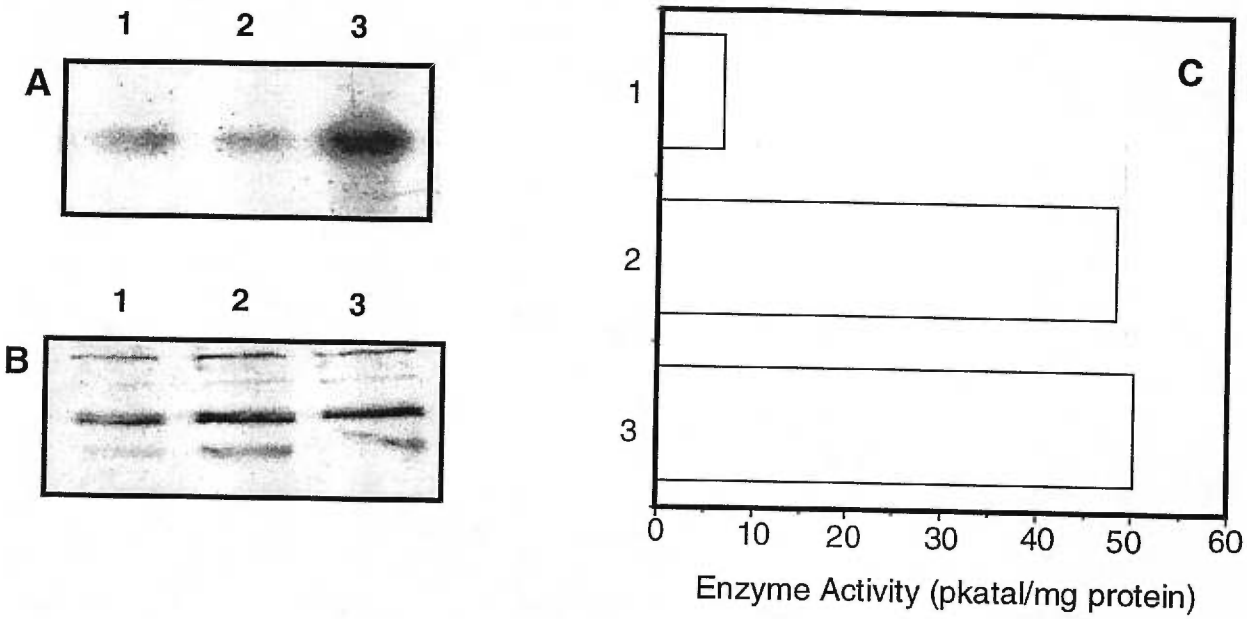
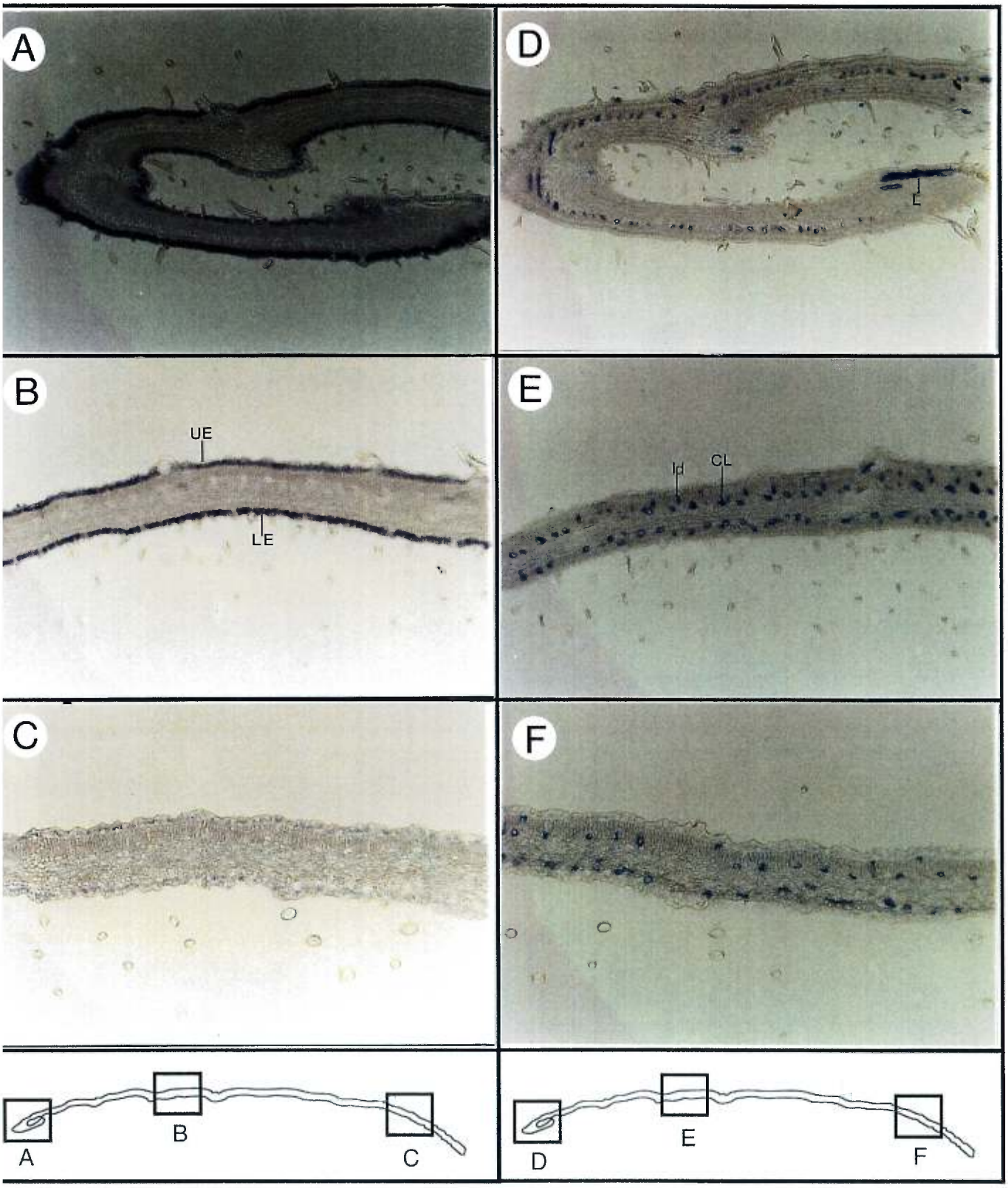


Figure 26. Distribution *tdc* transcripts (**A**), TDC immunoreactive proteins (**B**), and enzyme activity (**C**) in roots at different developmental stages. **1**, Main axis root; **2**, lateral secondary roots, **3**, lateral tertiary roots.


A
D
E
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UE
LE
Id
CL
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F
A
B
C
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E
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Figure 27. Immunocytochemical detection of TDC (**A-C**) and D4H (**D-F**) in longitudinal sections of *C. roseus* leaves. TDC antiserum was diluted 1,000-fold in blocking solution whereas D4H antiserum was diluted 2,500-fold. (**A** and **D**) petiole region, (**B** and **E**) middle region, (**C** and **F**) tip region. Lower panels, diagramatic representations of a longitudinal section showing the different regions photographed; (**CL**) cross oriented laticifers, (**L**) longitudinally oriented laticifer (**Id**) idioblasts, (**LE**) lower epidermis, (**UE**) upper epidermis. Objective 10X.

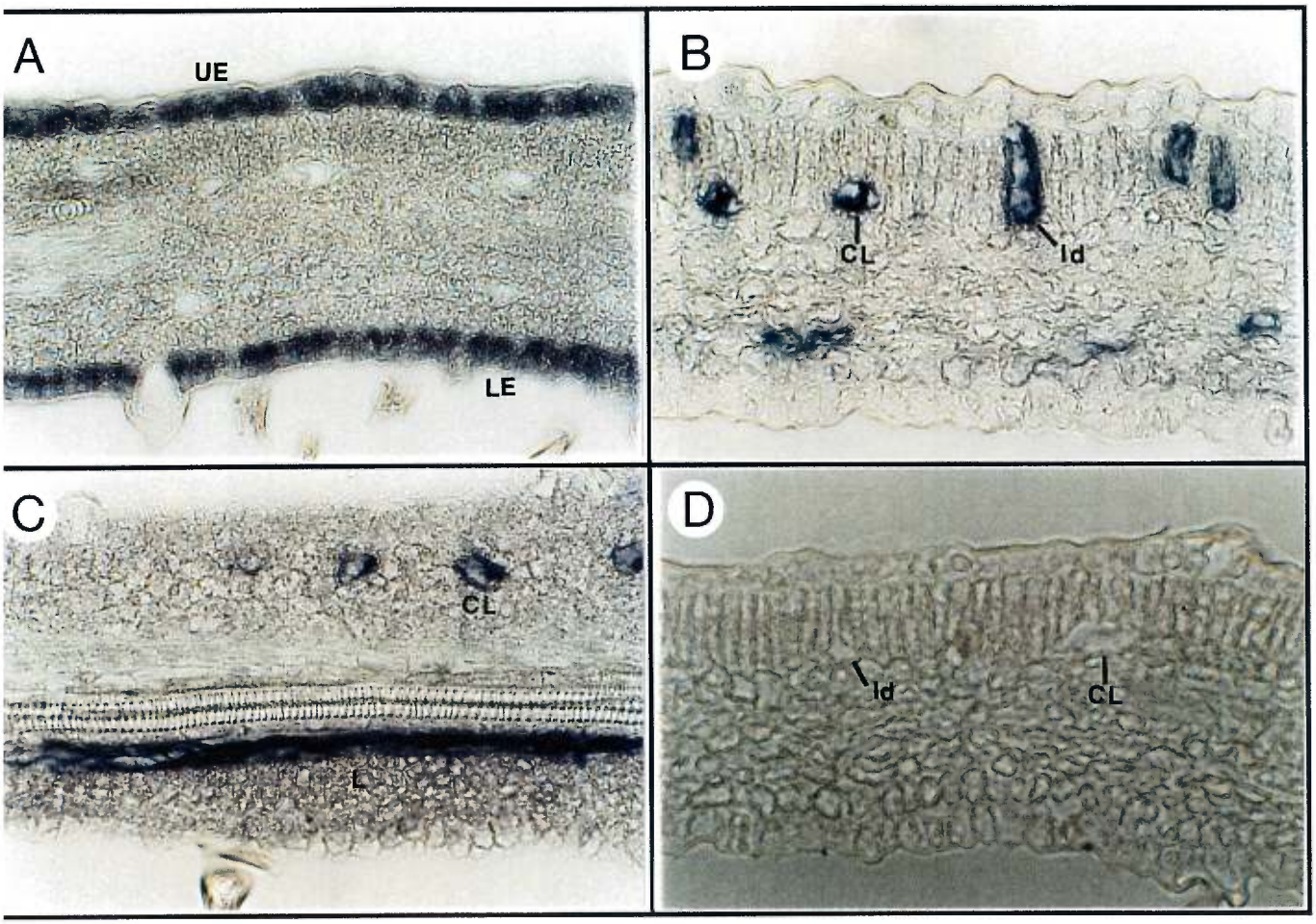

Figure 28. Magnification (objective 40X) of leaf section probed with (**A**) anti-TDC antiserum (Fig. 27B); (**B**) and (**C**) anti-D4H antiserum (Figs. 27E and 27B respectively) or (**D**) preimmune serum (diluted 500-fold). (**CL**) cross oriented laticifers; (**L**) longitudinally oriented laticifers; (**LE**) lower epidermis, (**UL**) upper epidermis, (**Id**) idioblasts.

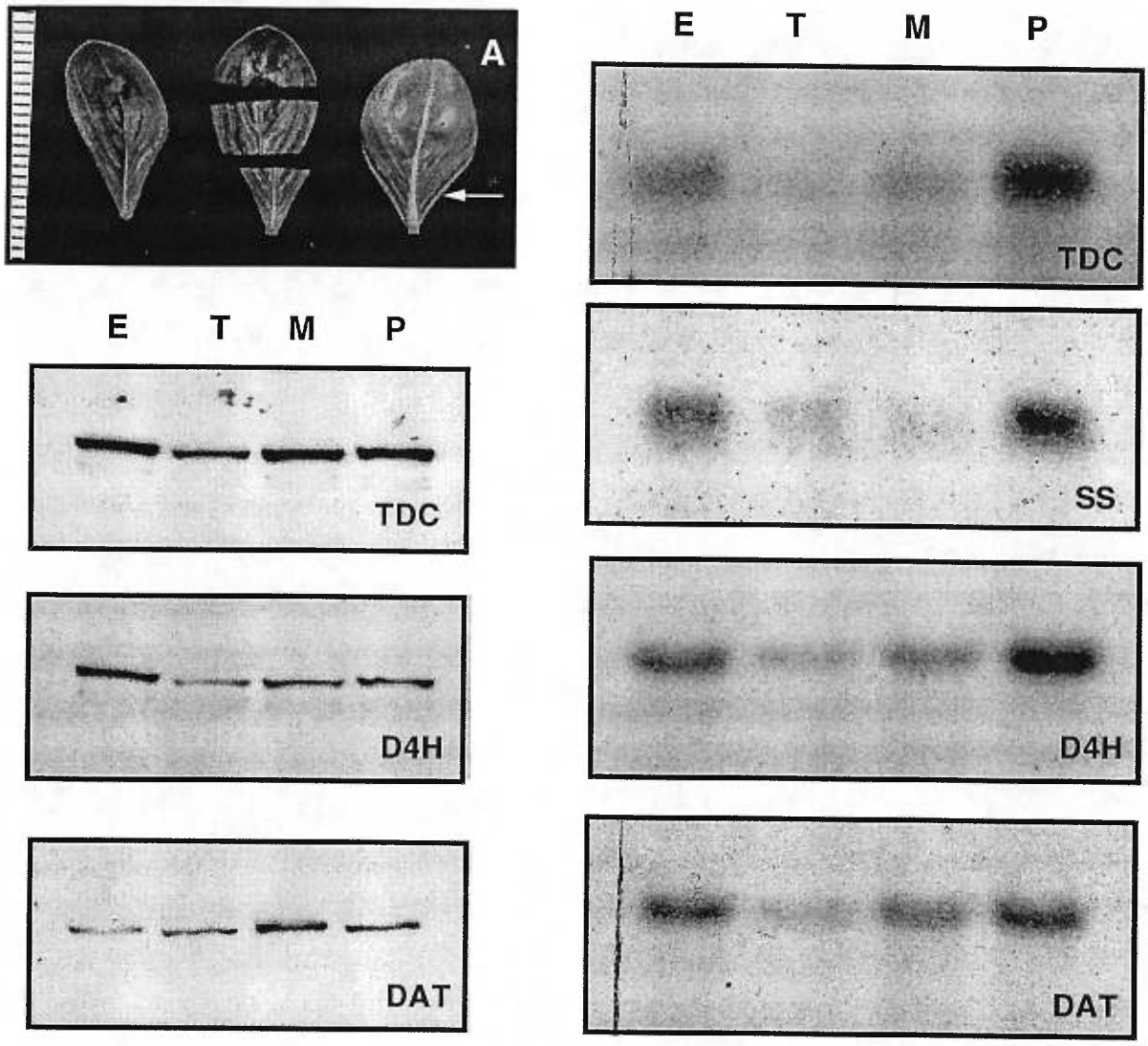

Figure 29. Dissection of leaf 2 (Fig. 23) into petiole-, middle- and tip-regions. (**A**) Photography of a dissected leaf showing (arrow) the curled edges at the petiole region. Immunoblots (left) and RNA blots (right) of extracts from each regions of leaf 2. **E**, entire leaf; **T,** tip region; **M**, middle region; **P**, petiole region. (**D4H**) desacetoxyvindoline 4-hydroxylase, (**DAT**) deacetylvindoline 4-*O*-acetyltransferase, (**SS**) strictosidine synthase, (**TDC**) tryptophan decarboxylase.

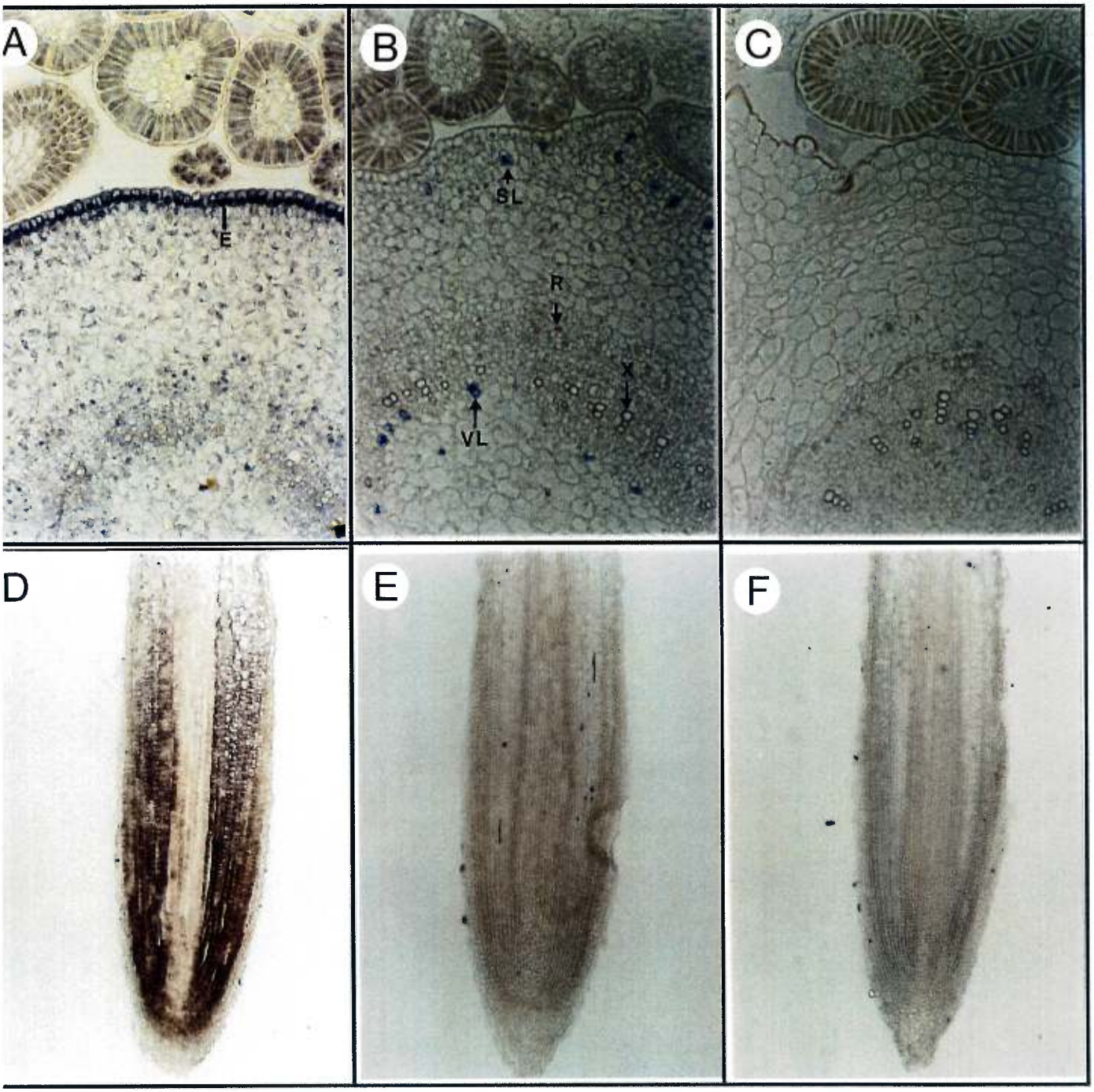

Figure 30. Immunocytochemical analysis of stems and roots. Cross sections from *C. roseus* stems obtained from first internode. Sections were probed with (**A**) anti-TDC antiserum (diluted 300-fold), (**B**) anti-D4H antiserum (diluted 1000-fold), or (**C**) preimmune serum (diluted 500-fold). Longitudinal sections of *C. roseus* roots were probed with (**D**) anti-TDC antiserum (diluted 1000-fold), (**E**) anti-D4H antiserum (diluted 300-fold) or (**F**) preimmune serum (diluted 500-fold). (**L**), subdermal laticifers; (**VL**) vascular associate latificer (**P**), Phloem; (**X**), Xylem. (Objective 20X)

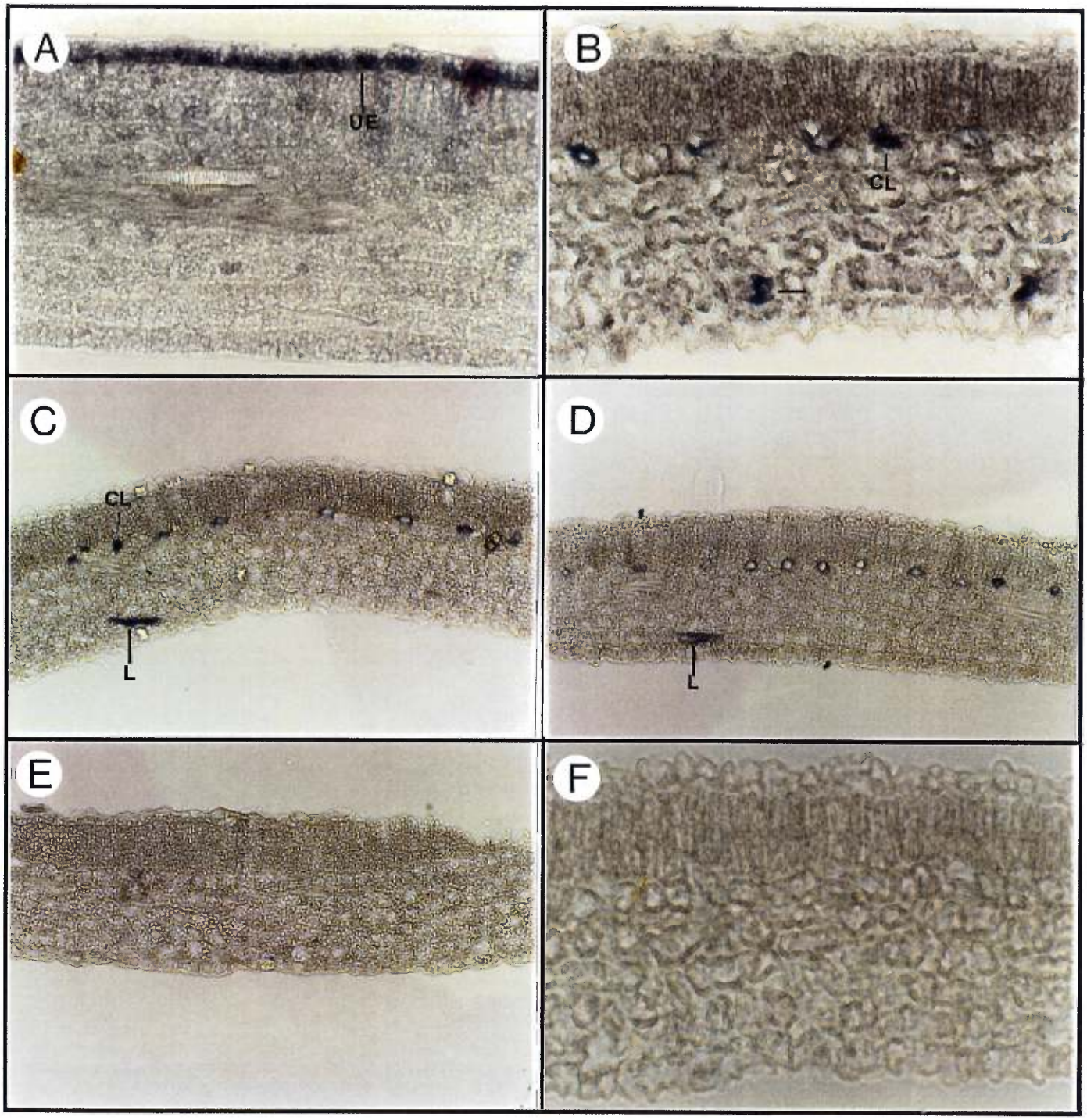

Figure 31. Immunocytochemical analysis of logitudinal sections of *C. roseus* cotyledons. (**A**) Cotyledons from dark grown seedlings probed with TDC antiserum (500-fold dilution). (**B-C**) Cotyledons from light grown seedlings probed with D4H antiserum (1000-fold dilution). (**D**) Section of dark grown seedlings treated with D4H antiserum. (**E** and **F**) Sections treated with a preimmune serum (300-fold dilution). Legends as in Figure 27. (Objective 20X in **C**, **D** and **E**; objective 40X in **A**, **B** and **F**).

Chapter VI. Discussion and Future Perspectives.

Although *Catharanthus roseus* produces more than a hundred monoterpenoid indole alkaloids, only ajmalicine, serpentine, catharanthine and vindoline accumulate as major constituents. Among these alkaloids, only vindoline biosynthesis appears to be regulated by additional tissue-, development-, and environment-specific factors. The present work corroborates previous findings which suggested that D4H plays a major role in conferring regulatory specificity to the vindoline pathway (De Carolis et al., 1990; De Luca et al., 1992). The data presented documents that, unlike earlier steps in this pathway, both light and development play important synergistic roles in the molecular regulation of D4H. These differences in regulation may also be due to the epidermis-specific expression of the early part of the pathway (TDC and SS) compared to the laticifer- and idioblast-specific expression of the terminal steps of vindoline biosynthesis (D4H and DAT) (St. Pierre et al. 1998) in *Catharanthus* leaves. Additional detailed studies have also shown that expression of D4H appears to be under transcriptional, translational and post-translational control which can be modulated at different levels by development and light. These results increase our understanding of the complex biological processes which appear to be functioning in the operation of the vindoline pathway.

D4H belongs to an α-ketoglutarate dependent dioxygenase family which is responsible for a large number of oxidative reactions in plants and which is involved in the elaboration of hormones and many different secondary metabolites (De Carolis and De Luca, 1994; Prescott and John, 1996). Similarly, DAT appears to belong to the chloramphenicol-*O*-acetyltransferase family of genes, which may also be responsible for a large number of acylation reactions in plant secondary metabolism (St. Pierre et al., 1998). In both cases, D4H and DAT contained recognition domains which were responsible for binding ferrous ions and Acetyl CoA, respectively. The total lack of similarity between these proteins which could be used to identify a common alkaloid binding site, suggests that D4H and DAT evolved independently. The common cellular and tissue distribution, as well as the similar control exercised by plant development and light for expression of both D4H and DAT (Chapter V; St-Pierre et al., in preparation)

suggests that common genetic features conferring this type of expression may exist in both sequences. Whether these control elements were acquired prior to after they were recruited for the synthesis of vindoline remains an open question. The lack of obvious similarities between these sequences leads to the suggestion that the tertiary structure of each protein may share common features which bind very similar alkaloid substrates. Structural analysis of both proteins may be useful to identify which common amino acid residues are involved in the binding of alkaloid substrate to the active site of the enzyme and may help to resolve the catalytic domain.

Besides these structural considerations, the regulation of D4H by light and development emphasizes that enzymes involved in the synthesis of indole alkaloids appear to be under the same sophisticated control as enzymes involved in primary metabolism. Multilevel regulation allows fine tuning control of the enzyme in order to cope with the changing cellular and environmental conditions occurring throughout plant growth (Kaiser and Huber, 1994). The ability of seedlings to express D4H involves modulation of the steady state levels of transcripts and immunoreactive protein, which varied with the developmental stage at when *Catharanthus* seedlings were exposed to light (Fig. 11; Chapter III). These results suggest that *Catharanthus* seedlings can adjust, to a certain extent, the rate of vindoline biosynthesis by adjusting either the level of *d4h* transcript or active protein. Additional fine control of D4H expression was discovered when light was shown to induce the appearance of a new and slightly more acidic isoform of the D4H protein (pI 4.6), which was coupled to the loss of the more basic D4H isoform (pI 4.7) occurring in etiolated seedlings. These results suggest that light may trigger a post-translational modification leading to the activation of an inactive form of D4H protein which exists in etiolated seedlings (Fig. 12; Chapter III). The nature of this post-translational modification and the reasons for this further type of regulation remain to be established.

Light is probably the most important external factor in promoting plant development. At this stage it is not possible to distinguish if the effects of light on D4H expression are related to the de-etiolation process triggered by photomorphogenesis (von Arnim and Deng, 1996). The biochemical and morphological changes occurring as

part of photomorphogenesis are related to the change from a mainly oxidative to a predominantly reductive metabolism. These changes include the optimization of light perception mechanisms and its capture, as well as adaptative responses to protect the cell against excessive irradiation (Nick et al., 1993; van Arnim and Deng, 1996). The close relationship between the perception of light and development-mediated activation of D4H expression which is documented in Chapter III, suggests that the terminal steps in vindoline biosynthesis may also be regulated by this process in *Catharanthus roseus* seedlings. The minimal dose of red light required for the induction of D4H does not allow its classification as a low fluence response. However, the relatively short time span between reception of the light stimulus on the induction of D4H activity and its photoreversibility (Fig. 16; Chapter III), strongly suggest that vindoline biosynthesis is closely associated with the photomorphogenetic process in *Catharanthus roseus*.

Alternatively, activation of D4H may follow a partially or totally independent sequence of genetic and biochemical events than those leading to the de-etiolation process. In this case, vindoline biosynthesis should have been fully active in etiolated seedlings and the characteristic photomorphogenetic phenotypes (Chapter III) should not have been observed. In this context, it is relevant that external application of chemical inducers like jasmonate and salicylic acid failed to activate D4H in dark grown seedlings (Chapter IV).

Studies with tomato seedlings have shown that red light activation of phytochrome mobilizes at least two independent signal transduction pathways which appear to be responsible for triggering photomorphogenesis. The earliest common components for both signal transduction pathways, after phytochrome, involve the activation of heterotrimeric G proteins (Bowler et al., 1994; Neuhaus et al., 1993). The G-proteins in turn activate two individual signal transduction pathways which use either cGMP or Ca_2^+/Calmodulin as second messengers. The former has been shown to induce anthocyanin formation within vacuoles, whereas the latter leads to chloroplast development. It has been shown, however, that interaction between these pathways also occurs, since parts of both pathways appear to be required for the assembly of photosystem I (Bowler et al., 1994; Neuhaus et al., 1993). It is reasonable that similar

pathways may be involved in transmission of the light signal resulting in the induction of D4H activity and of vindoline biosynthesis in *Catharanthus* seedlings.

The early (TDC and SS) and late (D4H and DAT) stages of vindoline biosynthesis were shown to coexist in meristems, leaves and stems, but *in situ* immunological (Chapter V) and RNA localization (St-Pierre et al., in preparation) studies revealed that they do not coexist in the same cell types. TDC and SS are exclusively expressed in the upper and lower epidermis of leaves and in the epidermis of stems. In constrast, expression of D4H and DAT were clearly localized to laticifers and idioblasts of leaves and stems. TDC and SS were expressed in cortical root cells near the root tip but not in the epidermis, whereas D4H and DAT were not detected in any root cells (Chapter V; St-Pierre et al., in preparation).

The participation of different organs and/or cells in the synthesis and accumulation of secondary metabolites may be a common characteristic in plants. The reciprocal grafting between nicotine-producing and non-producing tobacco plants showed that it is mainly synthesized in roots and is then translocated *via* xylem to the leaves (reviewed by Hashimoto and Yamada, 1994). Similarly, the localization of hyoscyamine 6ß-hydroxylase (H6H) in the pericycle (parenchyma cells surrounding root vasculature) of young roots of *Hyoscyamus niger*, converted hyoscyamine to scopolamine, prior to its translocation to stems and leaves for final storage (Hashimoto et al., 1991). In opium poppy, the first committed step in the synthesis of the morphine-type and other benzylisoquinoline alkaloids involves the decarboxylation of tyrosine by tyrosine decarboxylase (TyDC). Transcripts of the different members of the *TyDC* gene family were mainly detected in the vascular tissues (metaphloem and protoxylem) of young stems and roots (Facchini and De Luca, 1995). The specific localization of TyDC in phloem elements is in agreement with the proposed origin of laticifers, where TyDC enzyme activity and morphine are mainly detected (Facchini and De Luca, 1995 and references therein). It has been speculated that morphine biosynthesis in laticifer cells of the stem may facilitate translocation of this alkaloid to the seed capsules, where it accumulates (Facchini and De Luca, 1995 and references therein).

It is not clear if a similar root-to-shoot alkaloid transport system exists in *Catharanthus roseus* (Section **I.2.2.3**). Catharanthine and advanced precursors of vindoline biosynthesis, such as tabersonine, may be synthesized in roots (Section **I.2.2.1**; Chapter V) to be transported to laticifers and idioblasts in leaves and stem, for elaboration into vindoline and dimeric alkaloids, respectively. However, the ability of *in vitro* leaf cultures, which have no roots, to make vindoline suggests that roots are not absolutely required to make this possible (Section **I.2.3.3**). The available evidence suggests that tabersonine and catharanthine may be produced in more than one location and it remains to be elucidated how substrates for vindoline and dimer production are made available.

The clear differential cell localization of the early and late stages of vindoline biosynthesis in leaves (Chapter V; St-Pierre et al., in preparation) strongly suggests that undetermined post-strictosidine compounds are mobilized from the epidermis to laticifers and idioblasts, where at least the last two reactions of vindoline biosynthesis take place (Chapter V; St-Pierre et al., in preparation). Differential cell-specific expression and intercellular transport of metabolites have also been suggested for the phenylpropanoid pathway (Jahnen and Halbrock, 1998; Reinold and Hahlbrock, 1997; Schmelzer et al., 1988; Wu and Halhbrock, 1992). Flavonoids and furanocoumarins are two major products derived from different branches of the phenylpropanoid pathway, downstream from 4-coumaryl-CoA. In parsley leaves, the enzymes involved in "general phenylpropanoid metabolism" such as phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase (4CL) are mainly expressed in the vascular bundle and in epithelial cells surrounding oil ducts. Lower levels of PAL and 4CL transcripts were also detected in the leaf epidermis, palisade cells, spongy mesophyll cells and within the oil duct surrounding cells (Jahnen and Halhbrock, 1988; Reinold and Halhbrock, 1997). However, the locations of the pathways leading to the formation of flavonoids or furanocoumarins were mutually exclusive. Chalcone synthase (CHS), which is specifically involved in flavonoid biosynthesis, was restricted to the epidermis, spongy mesophyll and oil duct surrounding cells, whereas *S*-adenosyl methionine: bergaptol *O*-methyltransferase, which is involved in furanocoumarin biosynthesis, occurred in the vascular bundle, palisade parenchyma and the oil duct ephitelial cells (Jahnen and

Halhbrock, 1988; Reinold and Halhbrock, 1997). Interestingly, flavonoids were detected in vascular bundles and oil duct ephithelial cells which do not express CHS, suggesting the mobilization of these compounds from other expressing cell (Reinold and Hahbrock, 1997).

A similar cellular distribution to those of leaves was also found for TDC, SS, and D4H (Chapter IV, St. Pierre et al., 1998) in cotyledons of developing seedlings. No differences in the cellular distribution of D4H were found in the cotyledons of dark or light grown seedlings. The presence of inactive (Chapter III) D4H protein in idioblasts and laticifers of etiolated seedlings (Chapter V), indicates that this isoform of D4H is properly expressed in laticifers and idioblasts from the early stages of seedling development. The results suggest that light activates D4H and DAT expression (St. Pierre et al, 1998) rather than to induce the production of particular cells like idioblasts or laticifers. It remains to be established, however, whether light triggers the formation/proliferation of particular subcellular structures required for vindoline biosynthesis (De Luca et al., 1992).

Since no clear physiological roles for vindoline have been described, this alkaloid could be considered both as a final product as well as a precursor in the formation of the dimeric alkaloids. In the latter event, the suggested spatially separate origin of the catharanthine subunit may play a critical role in regulating the formation of dimers which have been shown to be toxic when exogenously applied to cell suspensions of *Catharanthus* (De Luca et al., unpublished results; McCaskill et al., 1988; Chapter V).

Finally, the present work illustrates the importance of development and cellular differentiation for the biosynthesis of one particular alkaloid in *Catharanthus* plants. Specific requirements should be satisfied for the synthesis of vindoline to take place. The complex regulatory mechanism of D4H seems to operate from the early stages of seedling development to the later phases of vegetative growth, and suggests an important role for vindoline in *Catharanthus* plants. Its distribution in young actively growing tissues (Chapter V; Westkemper et al., 1980) indicates that it may have some protective role (Frischknetch et al., 1987). Recent studies in our laboratory indicate that

crickets preferred to feed on etiolated *Catharanthus* seedlings rather than on those grown in light, where vindoline is the main alkaloid (Guillet et al., unpublished results).

Future Studies

Future studies on the regulation of D4H may take different directions. The identification of the regulatory elements conferring the cell-, tissue-, development- and environment-specific expression of this enzyme in *Catharanthus* plants will help to understand the genetic basis for the specificity of vindoline biosynthesis. These results will further help to explain why cell cultures fail to express this pathway, even under conditions leading to increased secondary metabolism (van der Heijden et al., 1988; Appendix III). The characterization of the post-translational modifications leading to the formation of D4H proteins with different isolectric points represents another interesting project. This study may provide some insight on the specificity and cellular distribution of the factor(s) involved in this modification and its importance in the overall regulation of vindoline biosynthesis. Finally, the identification of the events leading from the perception of light to the activation of D4H could provide valuable information to determine the nature of the relationship between the light trigger and the developmental regulation of vindoline biosynthesis. The molecular tools and insights generated in the present and previous studies (De Carolis, 1994) for D4H (Chapter III, IV and V) for D4H and for DAT (St-Pierre et al., 1998) represent important steps towards the achievement of such objectives.

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Appendices

Appendix I. Suppementary Data to Chapter II

1. Map of 3 D4H cDNA clones containing unrelated sequences.

The structure of these clones (Fig. 32A) was determined from sequence analysis and restriction mapping. Figure 32B displays the organization of the construct used to express active heterologous D4H protein.

2. Primer Extension Analysis.

Primer extension analysis was carried out to determine if *cD4H-3*, the longest among the cDNA clones isolated, represented the entire *d4h* transcript. This method uses radiolabeled oligonucleotide primers to map the 5' terminus of the transcript under analysis (Sambrook et al., 1989) and the product size is then estimated by polyacrylamide gel electrophoresis. The predicted ORF of *cD4H-3* encodes a protein of 386 amino acids with a *Mr* of 43.96 kDa which was very close to the *Mr* of the purified protein. However this clone did not include a typical ATG start codon and surrounding consensus sequences (Joshi, 1987a) or a 5' untranslated region. The first in frame methionine in the amino acid sequence of *cD4H-3* would encode a 376 amino acid protein with a *Mr* of 42.61 kDa, which is slightly smaller than the 44.70 kDa *Mr* obtained by gel filtration chromatography and by SDS-PAGE for the pure enzyme isolated from the plant (De Carolis, 1994).

Oligonucleotide primers. A 24 mer oligonucleotide primer was designed based on the complementary sequence between nucleotides 54 and 77 (included) of the clone *cD4H-3*. The sequence of this oligonucleotide was: 5'TTC TTC TTC TGA GAG TGT AGC AGC3'

Assay. Primer extension was performed according to Sambroock et al (1989): Fourteen pmol oligonucleotide primer were mixed with 16 pmol of [^{32}P]ATP (50 nCi; ICN pharmaceuticals, Costa Mesa CA) and 10 U of T_4 polynucleotide kinase (New England Biolabs, Beverly, MA) in a final volume of 20 µl. The reaction was incubated for

30 min at 37° C and then stopped by heating at 65° C for 10 min. The products of the reaction were precipitated with 10 volumes of *n*-butanol for 15 min at -20° C. The radiolabeled primer was mixed with either 20 µg of total RNA or with 2 µg of Poly(A^+) RNA, respectively, in a total volume of 100 µl of water. The mixture was precipitated by incubation for 15 min with 5 volumes of ethanol at -20° C. and by centrifugation. The pellet was dissolved in 10 µl of 0.4 M KCl and allowed to anneal for 60 min at 37° C. Once primed, the hybrid RNA/oligonucleotide was mixed with 2 mM dNTP's (Promega, Madison WI), 30 U of RNasin (Promega) and with 500 U of M-MLV reverse transcriptase (Promega) in a final volume of 50 µl. The mixture was incubated for 60 min at 37° C and the reaction was stopped by heating the mixture at 65° C for 10 min followed by phenol/chloroform extraction and ethanol precipitation. The synthesized *c*DNA was separated from the RNA template by incubation in 100 mM NaOH and 1 mM ETDA for 30 min at 37° C in a volume of 20 µl. Finally, the cDNA was ethanol precipitated, resuspended it 2 µl of water and mixed with an equal volume of loading dye. Product was submitted to electrophoresis on 6% polyacrylamide gels containing 9 M urea and its size was estimated by comparison with a sequencing reaction between clone *cD4H-3* and the oligonucleotide primer 4OH-3C-UP (nt's 219 to 236 of *cD4H-3*). The longest products indication the transcription start site were163 nt (Fig. 33A and 33B). Shorter products could be detected, with the most abundant occurring at 88 nt, which probably resulted from incomplete reactions.

3. Sequence of the *d4h* gene.

The sequence of the *d4h* gene was determined from the genomic clone *gD4H-8*. Figure 34 displays the complete sequence, as deposited in GenBank under the accession number AF008597. The sequence of the oligonucleotide primers used are displayed in Table 3

4. Comparison of the intron insertion site in *d4h* with other dioxygenases.

The conservation in the sequences around the site of intron insertion was first noticed by Kanegae and coworkers (Kanegae et al., 1995). Figure 35 compares intron

insertion sites of *d4h* with *e8* gene from tomato and and the hyoscyamine 6ß-hydroxylase gene from *Hyoscyamus niger*.

5. PCR of reverse transcribed RNA (RT-PCR).

Since Southern blot anlaysis suggested the presence of a single copy of the *d4h* gene, it was possible that *cD4H-1* resulted from a cloning artifact. This technique is used to reverse transcribe RNA with Moloney murine leukemia virus reverse transcriptase (MMLV-RT) using oligo-dT as primer. The resulting *c*DNA is then specifically amplified with primers targeted to each transcript of interest (Frohman, 1990) which was used to confirm the presence of the *cD4H-1* and *cD4H-3* transcripts in *Catharanthus* tissues.

Total RNA was extracted from both dark and light grown seedlings, as well as from mature leaves. Reverse transcription was performed in a total volume of 25 µl by mixing 5 µg of total RNA with 50 pmoles of a 17mer oligo-dT primer (0.125 µg; Promega) and incubating for 30 min at 37° C. The primed RNA was mixed with 100 U of MMLV (Promega), 1 mM NTP's, 12 mM $MgCl_2$ and 10 U of Rnasin (Promega), and the reaction was allowed to proceed for 60 min at 37° C before stopping it by heating at 65° C for 5 min.

One µl of the reaction product was mixed with 50 µmoles of primers (1R-4OH and 3U-4OH as sense and anti-sense, respectively); 2 mmoles of each NTP, $MgCl_2$ to a final concetration of 1 mM and 3 units of Taq polymerase (Boehringer Mannhein, Laval, Qc). A total of 35 amplification cycles were performed at 96, 50, and 72° C for template denaturation, primer annealing, and amplification, respectively, except for the first cycle, when the denaturation step was performed for 10 min (GeneAmp Perkin Elmer Cetus system from Norwald CT). PCR products were separated on 1% agarose gels in TAE (TAE is 40 mM Tris-Acetate pH 8.0, 1 mM EDTA; Sambrook et al., 1989).

After electrophoresis, PCR products were transferred to nylon membranes and probed with the radiolabeled oligonucletotide 2R-4OH (Table 3). Prehybridization and

hybridization were carried out at 45° C in 200 mM phosphate buffer containing 0.5% SDS, Denhardt's solution at 5X (5X Denhardt's solution is Ficoll 0.1 g, 0.1 g polyvinylpyrrolidone and 0.1 g of BSA per 100 ml), and 10 µg/ml of shreaded salmon DNA' Sambrook et al., 1989). Post-hybrydization washes were at 42° C for 30 min in 4X SSC with 0.1% SDS and 0.2X SSC with 0.1% SDS.

Both *cD4H-1* and *cD4H-3* transcripts are expressed in *Catharanthus*. The 3'UTRs of *cD4H-1* and *cD4H-3* which are shown in Figure 36A share 64% identity. RT-PCR of RNA from dark or light grown seedlings produced two products identical to the 460 bp product expected for *cD4H-1* or the 520 bp product expected for *cD4H-3* or both products expected fom the cDNA library (Fig. 36B top panel). Negative controls (Fig. 36B; water, RT-RNA from roots, or non RT-RNA from leaves) were not amplified when using the D4H primers. However, RT-RNA from roots, amplified with a specific primer for TDC resulted in a product, identical in size to the one obtained by amplifying TDC-5 cDNA clone. Genomic DNA (either from the plant, the EMBL genomic library, or clone *gD4H-8*) gave a single 2.1 kB fragment, as expected since these also contained a 1.7 kb intron (Fig. 36B top panel). The identities of the amplified fragments were confirmed by the Southern blot analysis since the 460 and 520 bp products both hybridized with the oligonucleotide 2R-40H (Fig. 36B, botton panel). These results suggest that both *cD4H-1* and *cD4H-3* transcripts are produced in dark and light grown seedlings as well as in leaves, but not in roots.

Table 3. Sequence and position of the oligonucleotide primers used in the sequencing reactions of the cDNA and genomic D4H clones.

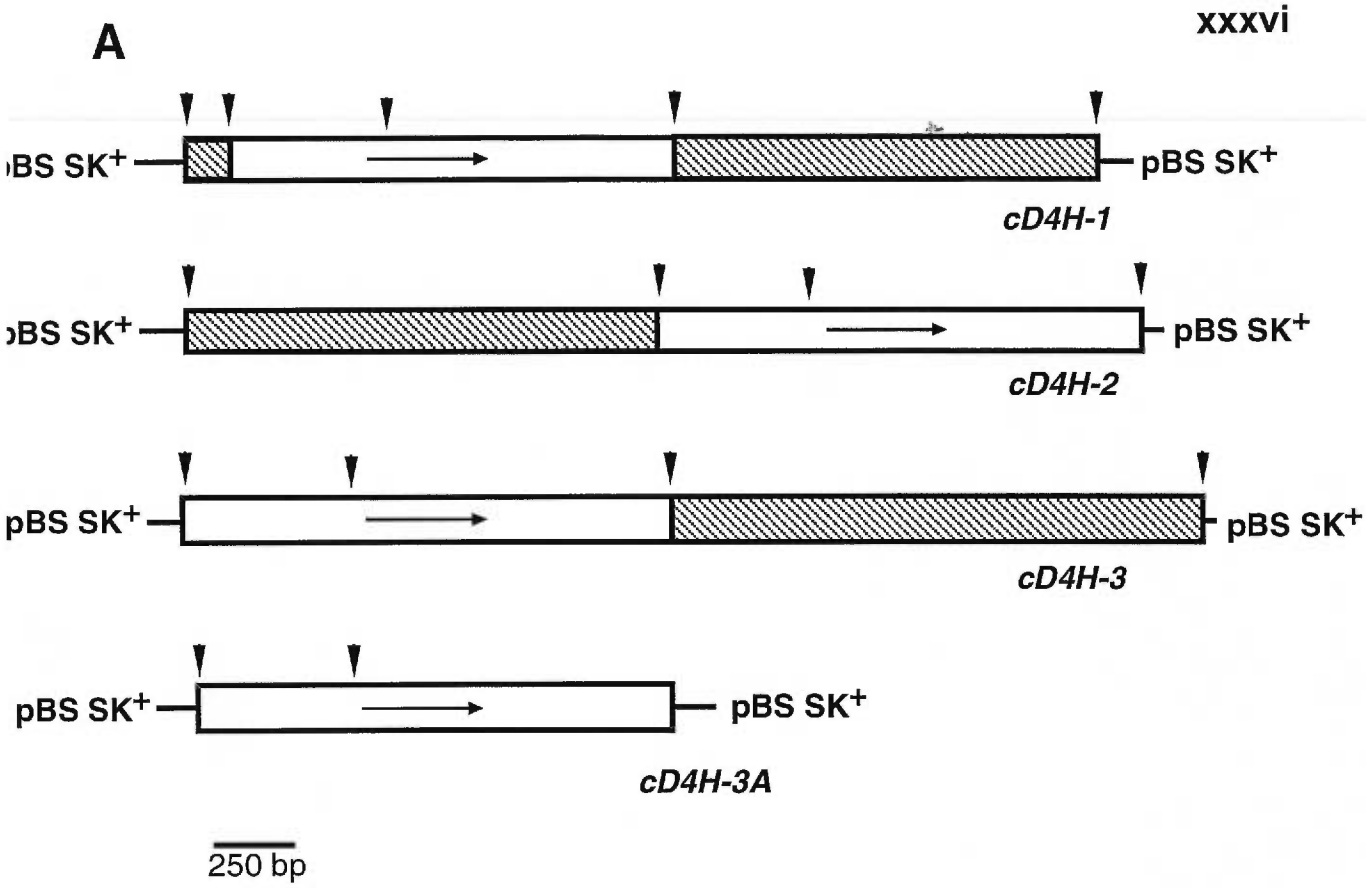
ANTISENSE OLIGONUCLEOTIDE PRIMERS

OLIGONUCLEOTIDE	SEQUENCE	POSITION	
		cD4H-3	*gD4H-8*
4OH-RT-UP	GTCATATTGATTGGAATCC	NP	358- 379
4OH-RT	TTCTTCTTCTGAGAGTGTAGCAGC	55 - 78	667- 690
G1-4OH	TCCCTTTTACACCAGCC	114- 129	725- 741
4OH-3C-UP	CTATAGTTCTAAGGTCA	219- 236	832- 848
40H-3C	GAGTACTTCATCAATAT	576- 595	1394-1402
2R-4OH	CTGCAATGTTAATAACT	866- 883	1684-1700
40H-7CUP-F	GGCCGAAGATTGAGTTTG	NP	2826-2843
4OH-7CUP	TCACATTTCGACTTAATC	NP	3107-3124
4OH-7C	GAAATCCTCGGACCAAC	948- 965	3486-3504
3U-4OH	TTCACCCGAAGATCTCTC	1376-1394	3916-3932

IN SENSE OLIGONUCLEOTIDE PRIMERS

OLIGONUCLEOTIDE	SEQUENCE	POSITION	
		cD4H-3	*gD4H-8*
4OH-5S	TTCCAGTTATAAACTTG	229- 246	841- 858
40H-6S	CTATGGAATGCATTATG	512- 527	1124-1140
1R-4OH	ATCAATGGATTGATGTCC	829- 847	1647-1634
4OH-1R-UP	CTCGCACATATTTCATGTATTC	NP	1907-1140
40H-1R-UP-F	GCCTCTCTGCATGTGCC	NP	2216-2232
4U-4OH	AAATGGTCTGCTAGAGAG	1315-1326	3844-3864

NP Not present in the sequence


A
pBS SK+
pBS SK+
cD4H-1
pBS SK+
pBS SK+
cD4H-2
pBS SK+
pBS SK+
cD4H-3
pBS SK+
pBS SK+
cD4H-3A
250 bp

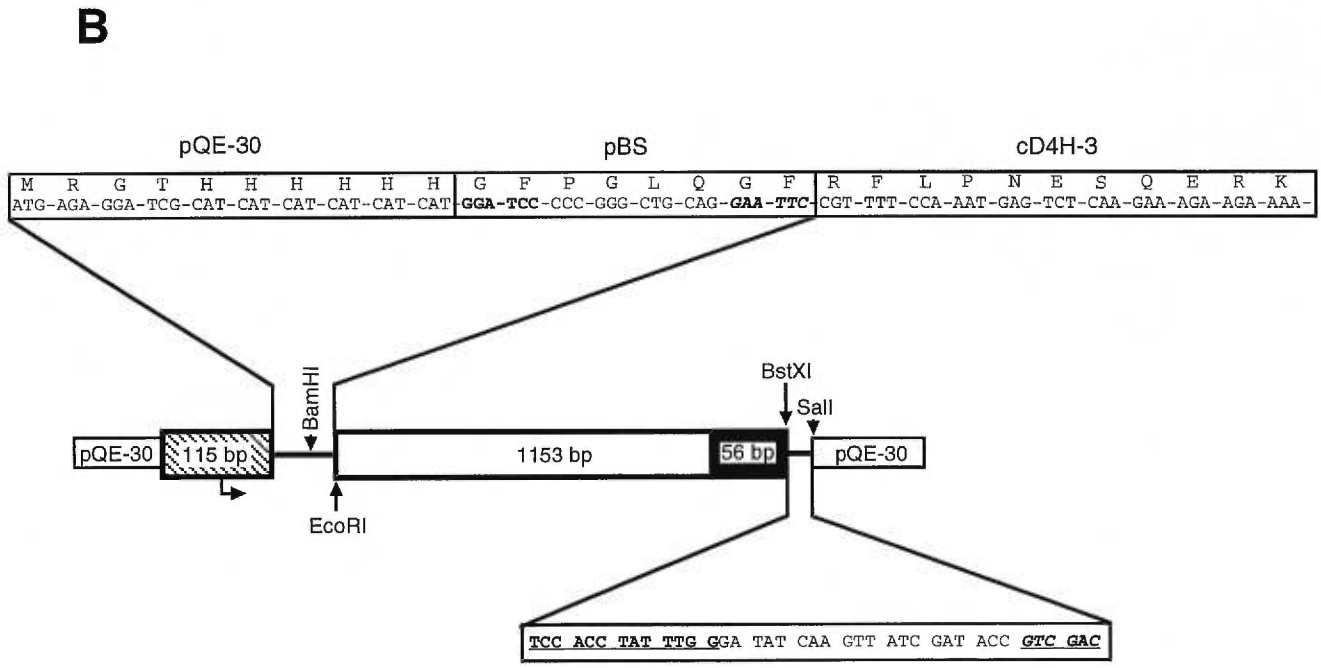
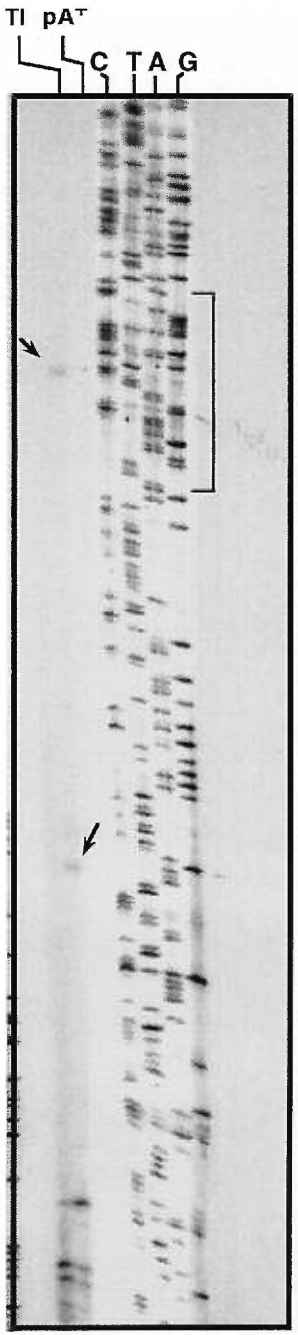

B
pQE-30
pBS
cD4H-3
M R G T H H H H H H
ATG-AGA-GGA-TCG-CAT-CAT-CAT-CAT-CAT-CAT
G F P G L Q G F
GGA-TCC-CCC-GGG-CTG-CAG-GAA-TTC-
R F L P N E S Q E R K
CGT-TTT-CCA-AAT-GAG-TCT-CAA-GAA-AGA-AGA-AAA-
BamHI
BstXI
SalI
pQE-30
115 bp
1153 bp
56 bp
pQE-30
EcoRI
TCC ACC TAT TTG GGA TAT CAA GTT ATC GAT ACC GTC GAC

Figure 32. Map of the D4H cDNA clones. **(A)** Map of the 3 D4H clones isolated from the *C. roseus* cDNA library. White boxes represent the D4H cDNA. Dashed boxes represent unrelated cDNA. Arrows within the boxes indicate the 5' to 3' orientation of the D4H sequence. Arrow heads indicate the position for *EcoR*I restriction sites. **(B)** Non-scale map of the pQD4H-19 expression construct, engineered with clone *cD4H-3A* into the expression vector pQE-30 (from QiaGen). The white box represents the coding region for D4H while the black box represents the 3' UTR. The dashed box respresent the control regions of expression vector and the translation start site is indicated by the arrow. The sequences around the cloning regions of the vector are displayed in the boxes above and below the vector. The *BamH*I site is in bold characters; the *EcoR*I site is in bold italic characters; the *BstX*I site is in bold underlined characters and the *Sal*I site is in bold italic underlined characters.



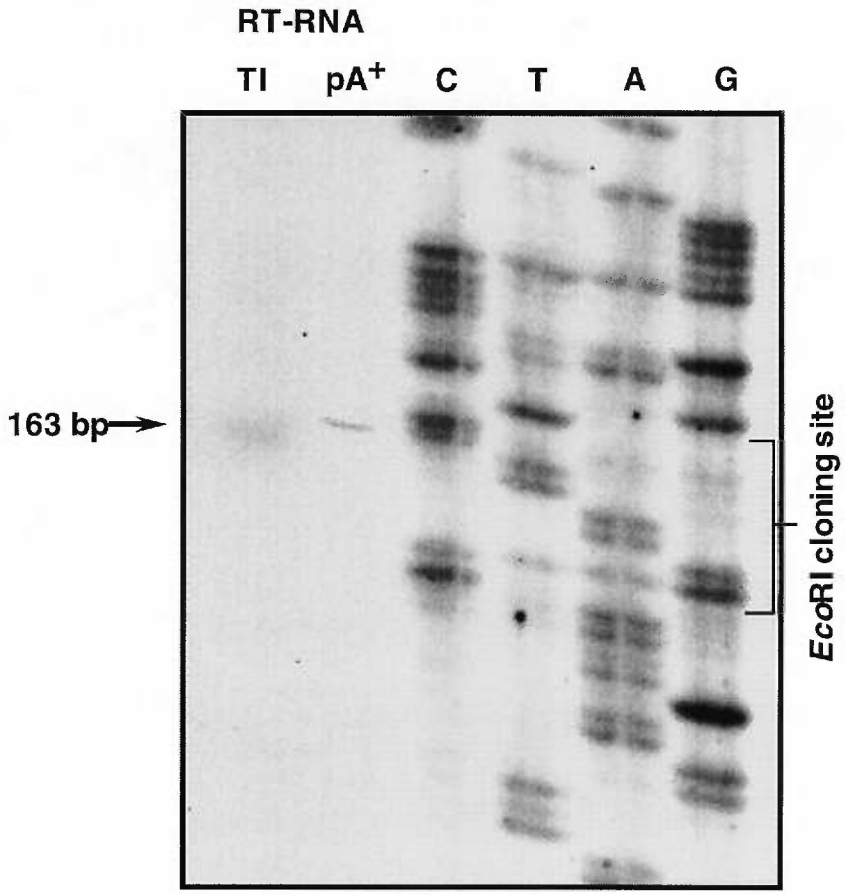


Figure 33. Primer extension analysis of the *d4h* transcript. **(A)** Autoradiogram showing the 88 and 163 bp products; **(B)** magnification of the area where the 163 bp product was resolved. RT-RNA, reverse transcribed RNA; **TI**, total RNA; **pA**, poly(A^+) RNA.

```
   1 acagtcgtct gacagtctag acgtatcaac agtttgagta gcataaattc tattgtattc
  61 attgagatct gttcttgtat acattccatt gtaaataaac gatcaacatt ggtctaagat
 121 tgaagtgaag agtcttagag agtgtttagg gaattgtttt acacacacaa aattttcaca
 181 tgaaaataaa atttttaaaa ttttttcgag ttatccttat taatttccta attgtcttta
 241 ttaactctct tttttaagaa ggcatggaga gaaatttgaa tttttttacg actataattt
 301 gattttacgt tattctgaat attatgctcc taaaagtata gttattattg tctataaagg
 361 attccaatca tatatgacat tttgggtaac catctatact aacatattaa tataatatct
 421 ttttattata aaaatttaat aaaaaataaa tattaaGGAA AAAATAAATT ATAAAAAAAA
 481 TATATTATAG AAGGAAAATT TTAAACAGAT AATACGGTTA TTATATTTGA CTGGCAGATT
 541 TAGACTGTCC GCAGTCTGTA TCCTATAAAA ATGCCTAAGT CTTGGCCAAT TGTGATATCA
 601 TCTCATAGTT TCTGTTTTCT TCCAAATTCT GAGCAAGAAA GAAAAATGAA GGACTTGAAC
 661 TTTCATGCTG CTACACTCTC AGAAGAAGAA TCTTTAAGGG AATTGAAGGC TTTTGATGAG
 721 ACAAAGGCTG GTGTAAAAGG GATTGTAGAT ACTGGGATAA CCAAAATTCC ACGTATCTTT
 781 ATCGATCAAC CAAAAAATCT TGACCGGATT TCAGTGTGTA GAGGAAAATC CGATATCAAG
 841 ATTCCAGTTA TAAACTTGAA TGGCCTCAGT AGCAATTCAG AAATACGGCG TGAGATTGTG
 901 GAGAAAATTG GAGAAGCGAG TGAGAAATAT GGATTCTTCC AGATAGTTAA TCATGGGATT
 961 CCACAAGATG TTATGGATAA AATGGTAGAT GGAGTTCGTA AGTTTCATGA ACAAGATGAT
1021 CAAATCAAGA GACAATATTA CTCCCGTGAC CGCTTCAACA AAAATTTTCT ATATAGCAGT
1081 AATTACGTTT TGATTCCAGG AATTGCTTGC AATTGGAGGG ATACTATGGA ATGCATTATG
1141 AATTCTAATC AACCTGATCC TCAGGAATTC CCAGATGTAT GCAGAgtatt ttttataact
1201 ctctccatcc gtttttttatt atttgtgaaa tttcggatat aggaattttc aaattttaag
1261 ttttatattt tttgttaatt tttaatttag agattcatat cttttctcac aaataattta
1321 tatttttaca ataattattg tttacatctt cgatttatga aattgaaact attacattga
1381 tgacatgcag GACATATTGA TGAAGTACTC AAATTATGTA AGAAATTTGG GGCTTATTCT
1441 CTTTGAATTA CTATCAGAAG CTTTGGGGCT CAAACCAAAT CATCTTGAAG AAATGGATTG
1501 TGCTGAAGGA CTTATACTTC TTGGTCATTA CTACCCTGCA TGCCCTCAAC CAGAGTTGAC
1561 ATTTGGGACA AGCAAGCACT CAGACAGTGG TTTCTTAACC ATACTTATGC AGGATCAAAT
1621 TGGCGGCCTT CAAATTCTTC TTGAGAATCA ATGGATTGAT GTCCCCTTCA TTCCTGGAGC
1681 TCTAGTTATT AACATTGCAG ATCTTTTACA GCgtcaagta ataaatgacc taattaagta
1741 ctataattaa gataattgta ctagcattct ttttagttta taaattttca tactctattt
1801 ttcacttttt tcataatttt ttttattttg ttttaggtac cctaattcta ttttttgaca
1861 ccataaatcc ctaatttttt tatacacaat agaatctaac aatttaactc gcacatattt
1921 catgtattct gtttcataaa atatttttgt ccgtttatac taataaaata tataaatata
1981 tgtaatttct ttttttttt taatacgcag actttcctat acctgaggaa acttgacatt
2041 ggtggtgact aagaatagtt catggataaa caaagtaggg aaaatggtca ttctagtcct
2101 aaaatacaag cctttctatt tggttggcaa caaatttttt caaaactttt ttatttgcct
2161 ataattccta ctaaattcca tatttctaaa tcaatctcta ggcaccaaaa ttaatgcctc
2221 tctgcatgtg ccattatttt aagttgtaaa acatgaaaaa acaagcaaaa ttcaagaaca
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3001 ccatgaattg aagattttgt taaatgaatt aaatgagcta tattgattaa atatcttgtt
3061 ttcccttcaa ataatgatac gagaggggca caatttttg gtctttgatt aagtcgaaat
3121 gtgaaatgtg ataatgagat taaatatgga tgaaaaaaaa tttctccgta atgattgaaa
3181 aaaaaaaaag aaaaaaagaa ctttctggaa tcaaaaaaaa tagaagggtc tctgttttgg
3241 accataatga gcatttttc acaaaattaa tgctaaaact ctaactgtta attaaactca
3301 agcattaaga tttatgaaag gaatattaca taaagaaaat attaggatcg gttaaattga
3361 ttatctcaat tacaatgtga ccctcttgct attgctaatg aaagaactca tgtgctttgt
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3481 AACAAAGTTG GTCCGAGGAT TTCTGTTGCT GTTGCTTTTG GTATAAAAAC ACAAACTCAA
3541 GAAGGGGTTT CGCCAAGATT GTACGGACCA ATTAAAGAGT TAATATCAGA GGAAAATCCA
3601 CCCATTTACA AGGAGGTCAC TGTTAAAGAT TTTATAACAA TTCGATTTGC CAAACGCTTT
3661 GACGATAGCT CTTCCTTATC TCCTTTCAGG TTAAACAATT AGATCGGGAG AAGCTAATAC
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3781 ATGTTGGACT AAATAAGCGT CGGCGAATAT TTGTTTCGTC TCATTTTCAA TAATCAAATA
3841 AAGCAAATGG TCTGCTAGAG AGTTTGGCCA AACTATACTT GCGATTCGAG TAGCCTTTCT
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4021 TCTTAATTTT CCTTTATTTT TCTACCTCGT ACCTTGTTTT CTTTATGGAT TAAGGTAAGT
4081 TTTATGCACT GCCTTTTGTA ATTTATTTCA CTTGATTATT AATAAAACCG AGCATCGCCC
4141 gactagttct atccttgata tatatatata tatacatata tatacatata tatatatata
4201 tataaaagaa tactcatgac tggtatgaga ct
```

Figure 34. Sequence of a complete *d4h* gene, *gD4H-8*. Lower case and capital characters are nontranscribed and transcribed sequence, respectively. Capital underlined characters represent transcribed but not translated sequences. Bold and bold italic characters represent the positions of sense and antisense oligonulceotide primers used for sequencing. The boxed region indicates a shared sequence for a sense (4OH-5S; Table 3) and antisense (4OH-3C-UP; Table 3) oligonucleotide.

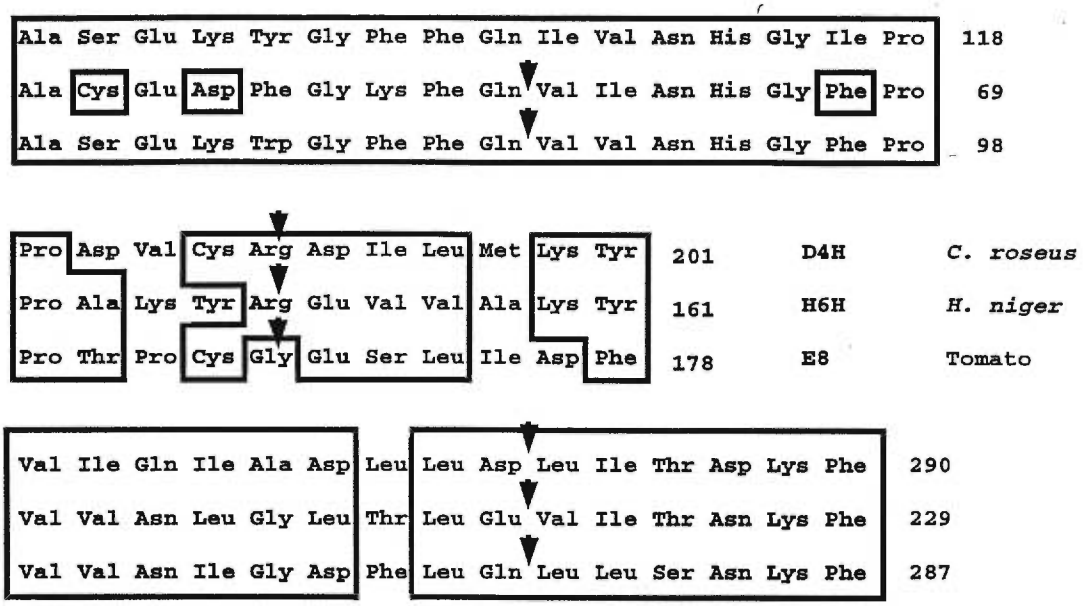


Figure 35. Intron insertion positions of the *d4h* gene product from *C. roseus* compared with hysocyamine 6ß-hydroxylase (H6H) from *Hyoscyamus niger* and E8 protein from tomato. Arrow heads indicate the insert positions (Modified from Kanegae et al., 1995).

A

```
                1164                                                1210
3 GTTAAACAATTAGATCGGGAGAAGCTAATACTAGCTTAAGTGTTTCTATATAAATCTCCA
1 **************--**--*-*--**-------*---*--*------*-*---*-*---
                                                    1270
3 CCTATTTGGCGCTGAAACTGATAATTTTTGCATGTTGGACTAAATAAGCGTCGGCGAATA
1 --*------**-**----*--*-------*-*-------**************T*****
                                                    1330
3 TTTGTTTCGTCTCATTTTCAATAATCAAATAAAGCAAATGGTCTGCTAGAGAGTTTGGCC
1 **-***-***************--**********************************G*
                                                    1390
3 AAACTATACTTGCG-ATTCGAGTAGCCTTTCTATTTTCTACAATTTGAGAGATCTTCCGG
1 **************G*****************************************
                                                    1450
3 TGAACATTAGAGGTAAATATTTCGTGGATATCTCAATTACCCCTTATCATCAGTGTGTTT
1 *************----------*-*-----**---*-*------**--*---*-*----
                                                    1510
3 TGACCCCTTATCATCAGTGTGTTTTGACAGGGTCTTAATTTTCCTTTATTTTTCTACCTC
1 ---**--**-*-**---*-*-**-----*----*---------***--------*------
                                                    1570
3 GTACCTTGTTTTCTTTATGGATTAAGGTAAG-T---TTT-ATGCACTGCCTTTTGTAATT
1 ********************************G*AAG***T*******************
                                                    1630
3 TATTTCACTTGATTATTAATAAAACCGAGCATCGCCCGAAAAAAAAAAAAAAAAAAAAA
1 ************************************ACT********.............
```

B

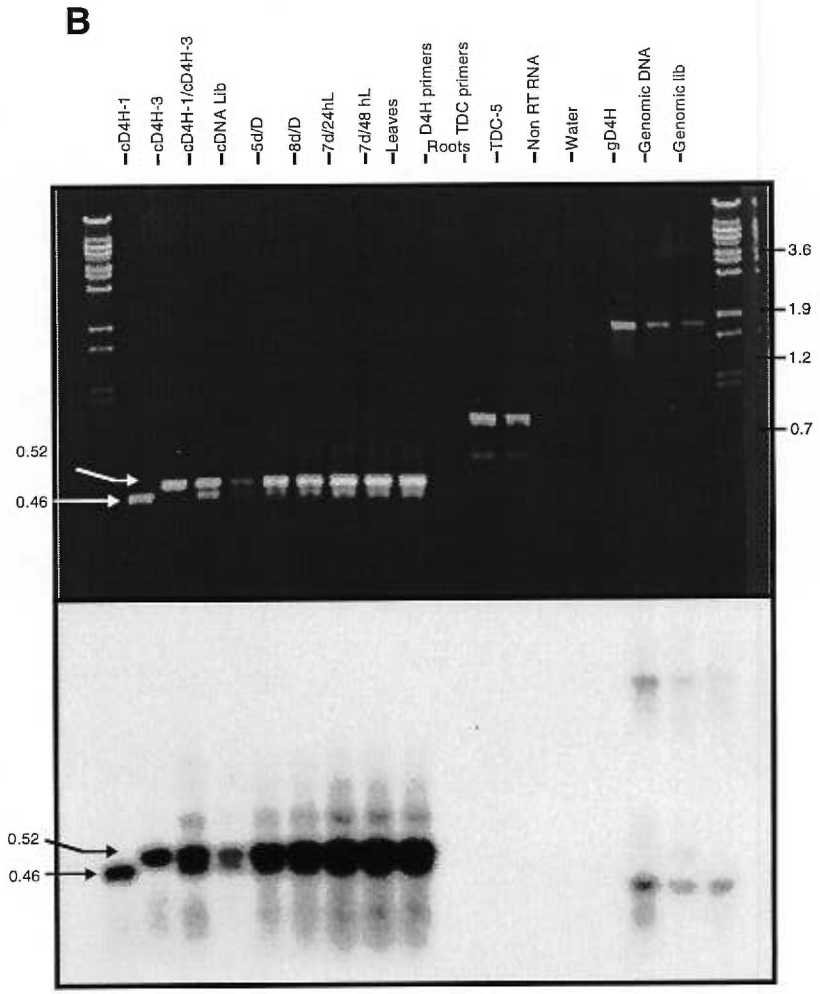


Figure 36. RT-PCR analysis of the clones *cD4H-1* and *-3*. **(A)** Sequences at the 3'UTRs of both clones. Stop codons are bold and underlined. Differences between *cD4H-1* and *-3* are in bold and in italic characters or contain dashes where sequence is missing in relation to the other clone. The underlined sequence represents the position of the antisense oligonucleotide primer 3U-4OH. **(B)** Agarose gel of the PCR products (top panel) and Southern blot analysis of the PCR products (bottom panel). **5d/D** and **8d/D**, reverse transcribed RNA from 5- and 8-day old etiolated seedlings, repectively; **7d/24hL**, **7d/48hL** and **Leaves**, RNA from 7-day old etiolated seedling exposed for 24 and 48 h to white light, respectively; **Roots**, reverse transcribed RNA from roots amplified with either **D4H** or **TDC** primers; **TDC-5**, TDC cDNA clone; **NonRT RNA**, RNA before reverse transcription.

Appendix II. Supplementary Data to Chapter III.

1. Purification of recombinant D4H.

Recombinant protein was purified from *E. coli* cells induced with IPTG for 4 hours. Purification procedures included ammonium sulfate precipitation (fraction 35 to 80%), desalting (a 12 x 2 cm Sephadex G-100 column; Pharmacia) and affinity chromatography (Ni-NTA affinity resine) as described by QiaGen.

Chromatographic procedures were performed with a FLPC system (Pharmacia). Proteins bound to the affinity resin were eluted (0.5 ml/min) with a 30 ml zero to 500 mM imidazole gradient in phosphate buffer (50 mM sodium phosphate, 300 mM NaCl and 10% glycerol; pH 6.0) and 1ml fractions were collected. The highest D4H specific activities eluted in the second half of the gradient (fractions 16 to 28, corresponding to 248 to 421 mM imidazole). A 500 ml bacterial suspension yielded aproximadely 1.52 mg of homogenous recombinant protein as determined by SDS-PAGE (Fig. 37A). The recombinant protein was purified 52 fold from crude extracts and its specific activity was 46.75 pkatal/mg of protein, which is approximately 50% that of pure leaf D4H (De Carolis and De Luca, 1993).

2. Production of D4H antibodies.

Pure D4H protein was supplied to Cocalico Biologicals who proceeded to produce antibodies (Described under the section *Materials and Methods* of Chapter III). The final antiserum reacted mostly against a single protein of the expected *Mr* in immunoblots of crude extracts from *Catharanthus* leaves or from seedling extracts submitted to SDS-PAGE and Western transfer onto nitrocellulose (Fig. 37B).

3. Coordination of the early (TDC; SS) and late stages (D4H) of vindoline biosynthesis.

Expression studies of TDC were followed simultaneously with those of D4H during seedling development in order to compare the ocurrence of the early and late stages of vindoline biosynthesis. TDC was assayed as described by De Luca et al (1986). Briefly, desalted extracted from seedlings, were mixed with 0.5 mM pyridoxal phosphate (Sigma), 50 nCi of L-^{14}[C]H_3 tryptophan (54 Ci/mol; Amersham), and 50 mM Tris-Cl, pH 7.5 in a final volume of 120 µl. The mixtures were incubated at 37 °C for 20 min and the reaction was stopped with 100 µl of 1 M NaOH. Radioactive tryptamine was extracted with 250 µl of EtOAc and quantitated in a scintillation counter. TDC activity appeared transiently during seedling development with maxima ocurring between days 5 to 6, which is 24 to 48 h before maximun D4H activitiy could be observed (Fig. 11B Chapter III). No differences in TDC activity were observed between dark and light grown seedlings (Fig. 38A). Northern analysis showed minimal changes in *tdc* transcript levels with seedling development (Fig. 38B), in contrast to the observed changes in enzyme activity. Nevertheless, maximum transcript accumulation also ocurred between days 5 to 6 and decreased slowly with continued seedling development (Fig. 39B). Transcript levels were similar in dark or light grown seedling (Fig. 38B). The same membranes were also probed for expression of strictosidine synthase. Northern analysis revealed that the pattern of expression of *ss* was identical to that of *tdc* (Fig. 38C).

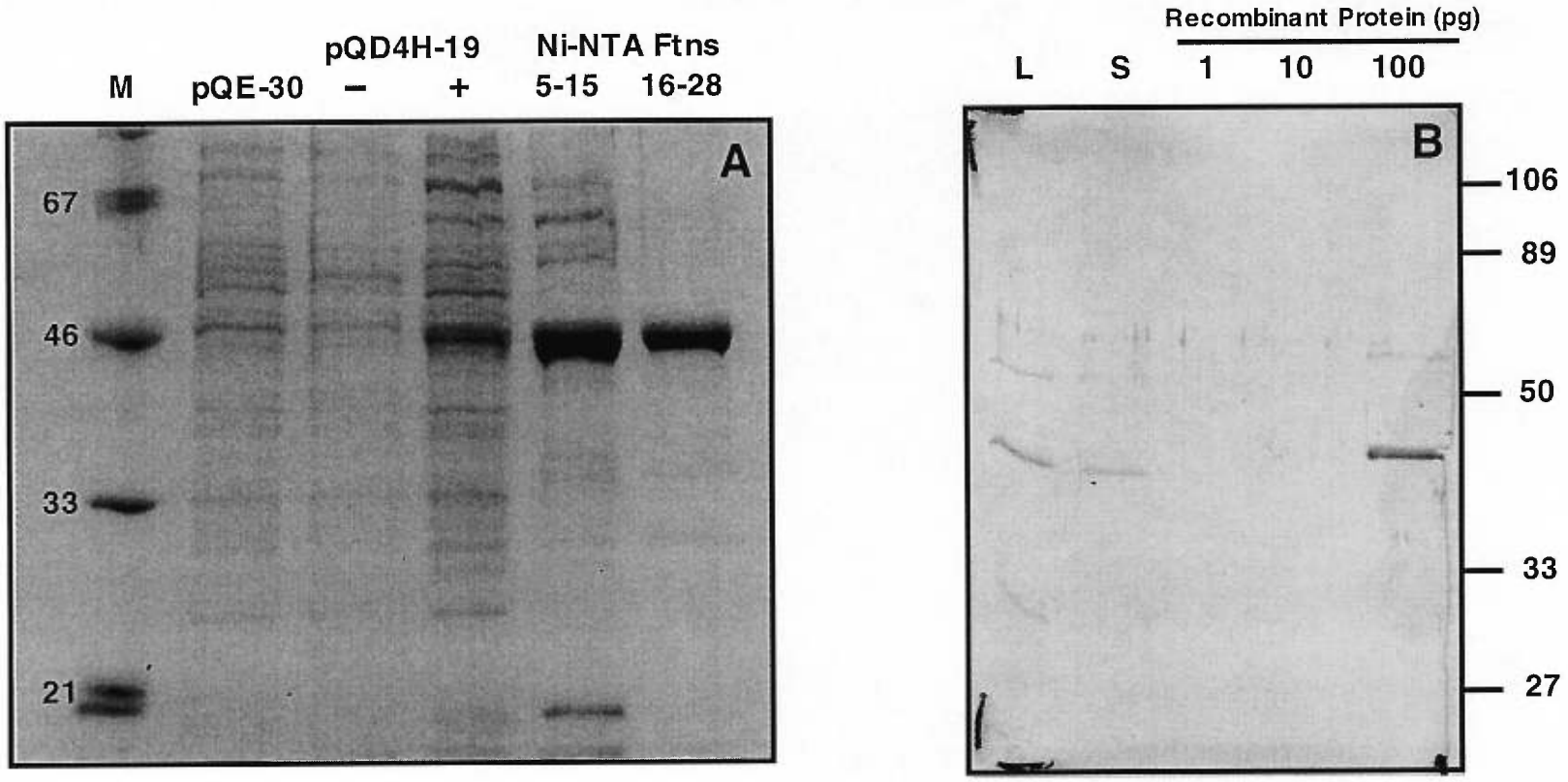


Figure 37. Purification of the recombinant D4H and antiserum specificity. **(A)** Coomasie blue stained SDS-PAGE of different stages of purification of D4H. **pQE-30** and **pQD4H-19** are protein extracts from recombinant *E. coli* BB4 cells transformed with the expression vector minus and plus D4H insert, respectively, before (-) and after (+) induction with IPTG. Affinity resin Ni-NTA chromatography **(Ni-NTA Ftns)** with pooled fractions **5** to **15** and **16** to **28** of the imidazole elution gradient. Each lane was loaded with aproximately 7 µg of protein (Bradford, 1979). (**B**) SDS-PAGE, Western transfer onto nitrocellulose and immunoblot with D4H antiserum (diluted 10 000-fold). Leaves (**L**) or 7-day old etiolated seedlings subsequently exposed to 24 h of light (**S**) were extracted (Chapter III; Materials and Methods) and 5 µg of crude protein was submitted to this analysis. The titer of the antibody was also tested with different amounts of pure recombinant D4H protein (**1**, **10**, or **100** picograms).

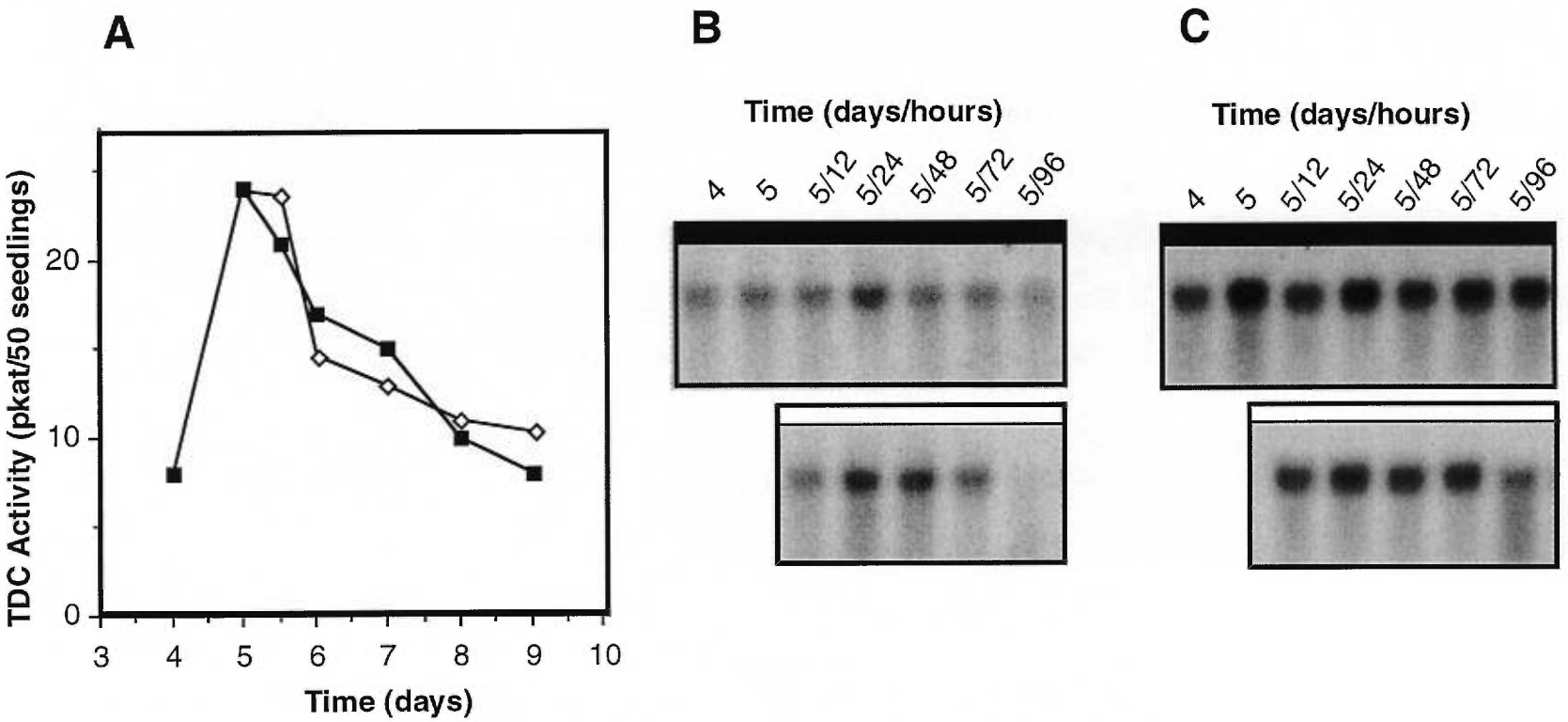


Figure 38. (**A**) Profile of TDC enzyme activity during seedling development in the dark (closed symbols) or after seedlings were transferred to the light (open symbols) after 5days of dark growth. (**B**) Northern blot showing the expression of *tdc* during seedling development in the dark (upper panel) or after seedlings were transferred to the light (bottom panel). Membranes were probed with a ^{32}P-radiolabelled *EcoR*I fragment of clone TDC-5 (De Luca et al., 1989). (**C**) Northern blot showing the developmental accumulation of *ss* transcripts. Upper panel, RNA extracted from seedlings continuosly grown in the dark; bottom panel, RNA from seedling grown in the dark for 5 days and thereafter exposed to light. Membranes were probed with a ^{32}P-radiolabelled *BamH*I fragment from SS clone obtained from McKnight et al (1990).

Appendix III. Effects of Inducers of Secondary Metabolism in Cell Suspension Cultures of *Catharanthus roseus.*

1. Treatments.

Catharanthus cell cultures, grown under normal condition, do not present any detectable D4H enzyme activity. In order to investigate if stress conditions that promote indole alklaoid biosynthesis would also induce expression of this activity, the *Catharanthus roseus* cell line 615 (Constabel et al., 1981) was submitted to one of the following treatments:

Alkaloid production media. Seven day old cell suspension cultures were collected by decanting the spent media and were then transfered to the alkaloid production media formulated by Knobloch et al (1980).

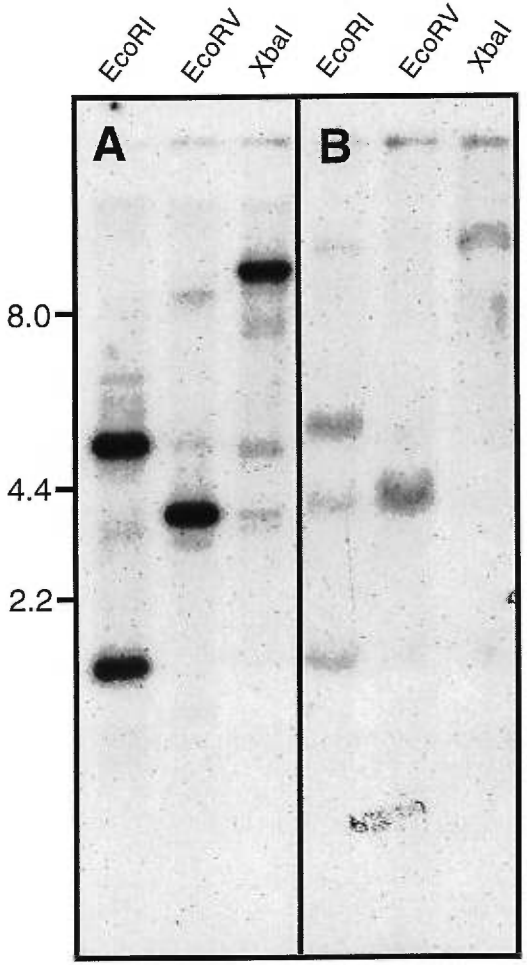
Methyl jasmonate. Seven day old cultures were exposed to 1 ppm of methyl jasmonate diluted in 70% ethanol.

Fungal elicitor. Seven day old cultures were exposed to 5% v/v of a *Pythium aphanidermatum* filtrate preparated as follows. Approximately 1 cm^2 of fungal micellium was taken from a fresh PDA plate (Difco Laboratories, Detroit MI) and cultured in 50 ml of B_5 liquid media (Gamborg et al., 1968) without growth regulators at 27 °C in the dark. Cultures were shaken for one day (100 rpm) and then left without agitation for 6 extra days. Cultures were autoclaved for 30 min at 125 °C and micellium was discarded by filtration. This filtrate was used as the elicitor (Eilert et al., 1987).

2. Effects of different treatments on expression of D4H.

Southern blot analysis confirmed the presence of *d4h* gene in the *Catharanthus* cell cultures (Fig. 39A and B), but neither D4H enzyme activity (Fig. 39C) nor transcript accumulation (Fig. 39D, botton panel) could be detected under any induction conditions. Cell line 615 responded to chemical and biotic elicitors, since both TDC enzyme activity (Fig. 39C) and transcript accumulation (Fig. 39D, upper panel) were transiently induced by methyl jasmonate and by elicitor treatments. These results agree

with the proposed differential tissue-, development- and environment-specific regulation of the early and late stages of vindoline biosynthesis and the requirement for minimal cellular organization or differentiation for vindoline biosynthesis to occur.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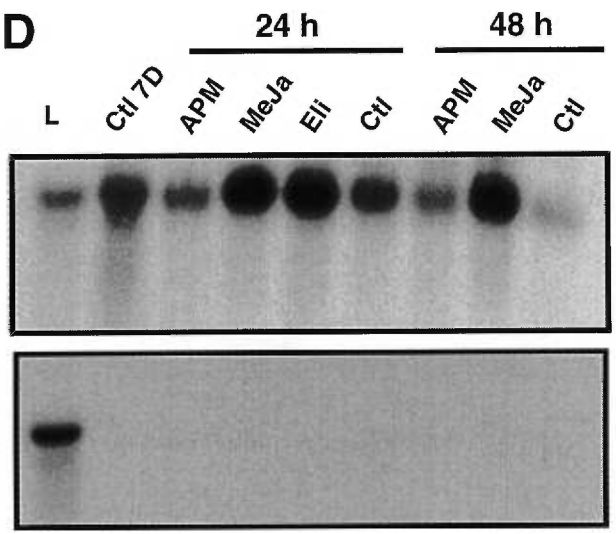
EcoRV
XbaI
EcoRI
EcoRV
XbaI
A
B
8.0
4.4
2.2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48 h
L
Ctl 7D
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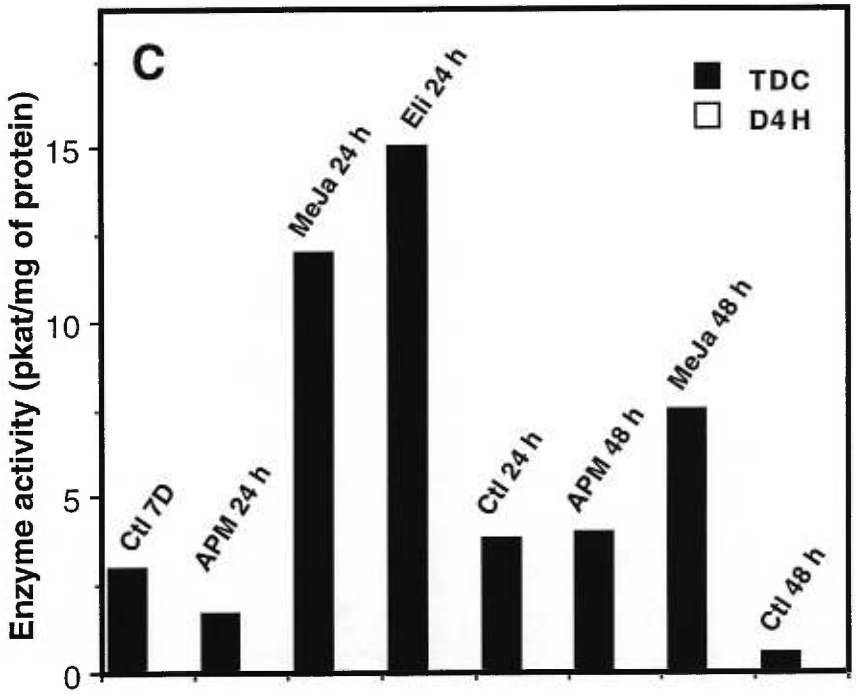
Eli
Ctl
APM
MeJa
Ctl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TDC
D4H
Enzyme activity (pkat/mg of protein)
0
5
10
15
Ctl 7D
APM 24 h
MeJa 24 h
Eli 24 h
Ctl 24 h
APM 48 h
MeJa 48 h
Ctl 48 h

Figure 39. Induction of secondary metabolism in cell cultures of *Catharanthus roseus*. Southern blots of genomic DNA from *Catharanthus* plants (**A**) or cell cultures (**B**) digested with different restriction enzymes. Agarose gels were loaded with 10 and 4 µg of DNA, respectively, and were probed with the entire open reading frame of clone *cD4H-3*. Differences between this blot and the one showed in Fig. 4 of Chapter II include an extra 1. 8 kB hybridization band (observed in the EcoRI lane). These differences are due to the longer probe used in this membrane. (**C**) TDC (closed bars) and D4H (open bars) enzyme activity in cell cultures submitted to different treatments; **Ctl7D**, Control at 7 days (start of the experiment); **APM**, in alkaloid production media (Knobloch et al., 1980); **Meja**, treated with 1 ppm methyl jasmonate; **Eli**, treated with *Pythium aphanidermatum* elicitor (5% v/v). Treatments lasted for **24** or **48 h**. (**D**) Northern blots for *tdc* (upper panel) and *d4h* (bottom panel) transcripts in *Catharanthus* cell cultures. Legends as in (**C**). Total RNA from leaves (**L**) which express both TDC and D4H was loaded as a positive control. The *Catharanthus* line 615 was grown in Gamborg B_5 media (Gamborg et al, 1968) supplemented with 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2% sucrose (1-B_5 media; Constabel et al., 1981).