Light-induced cytochrome P450-dependent enzyme in indole alkaloid biosynthesis: tabersonine 16-hydroxylase[1]

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Abstract **Vinblastine and vincristine are two medically important bisindole alkaloids from *Catharanthus roseus* (Madagascar periwinkle). Attempts at production in cell cultures failed because a part of the complex pathway was not active, i.e. from tabersonine to vindoline. It starts with tabersonine 16-hydroxylase (T16H), a cytochrome P450-dependent enzyme. We now show that T16H is induced in the suspension culture by light and we report the cloning of the cDNA. The enzyme was expressed in *Escherichia coli* as translational fusion with the P450 reductase from *C. roseus*, and the reaction product was identified by mass spectrometry. The protein (CYP71D12) shares 47–52% identity with other members of the CYP71D subfamily with unknown function. The induction by light was strongly enhanced by a nutritional downshift (transfer into 8% aqueous sucrose). We discuss the possibility that the entire pathway to bisindoles can be expressed in suspension cultures.**

Key words: Alkaloid biosynthesis; Cytochrome P450; Indole alkaloid; Light induction; Tabersonine hydroxylase; *Catharanthus roseus*

1. Introduction

Catharanthus roseus (Madagascar periwinkle) produces a large number of medically interesting indole alkaloids, the most important ones being the bisindoles vinblastine and vincristine (Fig. 1), which are used in the treatment of leukemia [1]. However, their concentrations are very low (about 0.0005%) and the complex structures render the chemical synthesis difficult. Many efforts to use cell suspension cultures failed. One of the major problems was that the cultures produced catharanthine and tabersonine, but not vindoline [2,3] and thus lacked one of the essential precursors for the formation of the bisindoles (Fig. 1).

Investigations with seedlings showed that the formation of catharanthine and vindoline is under complex and differential control. One example is that vindoline biosynthesis is light-induced, while catharanthine is also found in etiolated seedlings [4,5]. Differential and tissue-specific expression is also indicated by the finding that vindoline and the bisindoles are restricted to aboveground organs, while catharanthine is found in all tissues [6–8].

The pathway from tabersonine to vindoline requires six reactions (Fig. 2) and tabersonine 16-hydroxylase (T16H) is the first enzyme. It was identified as membrane-bound cytochrome P450-dependent enzyme (P450), and the activity was low in etiolated seedlings, but strongly induced by light [9]. We investigated the expression of T16H because it may be representative for expression of the entire pathway to vindoline. We now show that T16H is induced by light in the suspension cultures and thus is under the same control as in seedlings. This finding was the basis for the cloning strategy, and we identified the T16H cDNA by functional expression in a heterologous host. Induction of the vindoline pathway enzymes by light in combination with other factors had not been investigated in suspension cultures and we discuss the possibility that the entire pathway to complex indole alkaloids can be expressed under appropriate conditions.

2. Materials and methods

2.1. Cell culture and induction

The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L.G. Don, line CP3a) and its maintenance on MX growth medium in continuous dark with subcultures every week have been described [10]. The experiments were carried out with 7 day old cultures. The induction by light was performed with a light field from six white light lamps (Philips 40W/18) and four UV-A fluorescent lights (Osram, 40W/73). The light field was positioned 45 cm above the culture flasks (8 W m^{-2}). The nutritional down-shift by transfer of the cells into a solution of 8% sucrose in water has been published [10].

2.2. cDNA library and screening

Poly(A)-rich RNA was isolated from cell cultures induced by a nutritional down-shift (transfer into 8% aqueous sucrose) and irradiation for 1.5 or 3 h. The construction of the cDNA libraries in phage NM1149 has been described [10].

2.3. Blots with RNA and genomic DNA

The isolation of RNA and DNA and the hybridizations with the cDNA were carried out under hybridization and washing conditions which result in strong signals at more than 70% sequence identity [11].

2.4. Heterologous expression

We used a novel strategy which uses translational fusions between the P450 and P450 reductase (P450Red) from *C. roseus* and expression of the fusion protein in *Escherichia coli* [12]. The cDNA was modified in two steps: (a) extension of the 5′-end to create a membrane anchor (17 amino acids) which is suitable for expression in the

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[1] The nucleotide sequence newly reported in this paper has been deposited in the GenBank/EMBL data banks with the accession number AJ238612.

Abbreviations: T16H, tabersonine 16-hydroxylase; P450, cytochrome P450 monooxygenase; P450Red, P450 reductase; SAM, *S*-adenosyl-L-methionine; TLC, thin layer chromatography; D4H, deacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline 4-*O*-acetyltransferase; NMT, *N*-methyltransferase; OMT, *O*-methyltransferase

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bacteria [13]; this step also included the introduction of a *Nco*I cloning site at the start AUG; (b) introduction of a *Sal*I site which removed the stop codon of the P450 and provided a Ser-Thr linker to the P450Red. The details are shown in Fig. 3. The changes were confirmed by sequence analysis. The modified cDNA was then fused with the P450Red as described [12]. The fusion protein (129 kDa) was expressed in *E. coli* strain RM82 containing plasmid pUBS520 [14]. The protein induction, the preparation of the membrane fraction and the identification of the fusion protein by immunoblots with antiserum against *C. roseus* P450Red have been described [12].

2.5. T16H activity

The incubations were performed as described [9], except that we used 0.5 mM NADPH and a NADPH regenerating system (10 mM glucose 6-phosphate, 17.5 mU glucose 6-phosphate dehydrogenase, Boehringer Mannheim, Germany) in the incubations with plant microsomes. The standard assays coupled the T16H performed 16-hydroxylation of tabersonine with the subsequent *O*-methyltransferase reaction (Fig. 2) in the presence of 18 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]SAM), Amersham Life Science, UK) and the product analysis identified the labelled 16-methoxytabersonine. The methyltransferase activity was provided by a preparation of *C. roseus* leaf soluble proteins [9] which contained no T16H activity. Details are given in the figure legends. The assays with *E. coli* membranes were carried out with 1 mM NADPH. The radioactive 16-methoxytabersonine was quantified [10] after thin layer chromatography (TLC) on silica gels with fluorescence indicator ($60F_{254}$, Merck, Darmstadt, Germany). The solvent was ethyl acetate/hexane (1:1, v/v). The R_f values for tabersonine, 16-methoxytabersonine, and 16-hydroxytabersonine were 0.79, 0.72 and 0.48, respectively.

2.6. Analytical HPLC and results

The HPLC system (Waters 600, Milford, MA, USA) was equipped with a 5 μm Nucleosil C18 column (20×4 mm in diameter; Macherey-Nagel, Düren, Germany) and a linear gradient elution system was applied at a flow rate of 1 ml min^{-1}; gradient within 30 min from 45% to 70% solvent B (CH_3CN) in solvent A (0.2% NH_4OH in water). Injections (20 μl) were carried out by an automatic sampler (Waters 717 plus). The compounds were photometrically detected (maxplot between 210 and 450 nm) by a Waters photodiode array detector (DAD). Tabersonine and methoxytabersonine were available as reference substances.

Results for retention times and UV maxima: Tabersonine, 26.7 min, 225, 299 and 328 nm. Product 16-hydroxytabersonine: 13.0 min, 247 and 328 nm; Product 16-methoxytabersonine: 26.2 min, 245 and 326 nm.

2.7. LC/MS and results

The positive ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; CID offset voltage 10 V; heated capillary temperature 220°C; sheath gas nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1×100 mm, Sepserv). HPLC gradient system: H_2O:CH_3CN 4:1 (v/v) to 1:9 within 15 min and then hold at 1:9 for a further 10 min; flow rate 70 μl min^{-1}. All solvents contained 0.2% acetic acid. Collision-induced dissociation (CID) mass spectra of the $[M+H]^+$ ions: collision energy (collision cell) −25 eV; collision gas argon; collision pressure 1.8×10^{-3} Torr. All mass spectra were averaged and background-subtracted.

Results for the CID spectra of the $[M+H]^+$ ions (*m/z*, relative intensity). Tabersonine, 337 ($[M+H]^+$, 90), 305 ($[M+H-MeOH]^+$, 100), 276 (17), 228 (51), 144 (38), 136 (5). 16-Methoxytabersonine, 367 ($[M+H]^+$, 100), 335 ($[M+H-MeOH]^+$, 80), 307 (10), 279 (7), 258 (38), 226 (9), 136 (6). 16-Hydroxytabersonine, 353 ($[M+H]^+$, 100), 321 ($[M+H-MeOH]^+$, 87), 293 (8), 265 (12), 244 (32), 184 (15), 136 (21).

3. Results

3.1. Tabersonine 16-hydroxylase activity is inducible in C. roseus suspension cultures

Microsomes from cultures grown in MX-medium in the dark revealed no detectable T16H activity (< 0.05 μkat/kg). T16H is induced by light in seedlings and we investigated whether the suspension culture had retained this regulation. Irradiation of the cultures accompanied by a nutritional down-shift (transfer into 8% sucrose) indeed revealed high T16H activity after 15 h (1.6 μkat/kg), comparable to that described for induced *C. roseus* seedlings [9]. The results provided suitable conditions for the isolation of the cDNA.

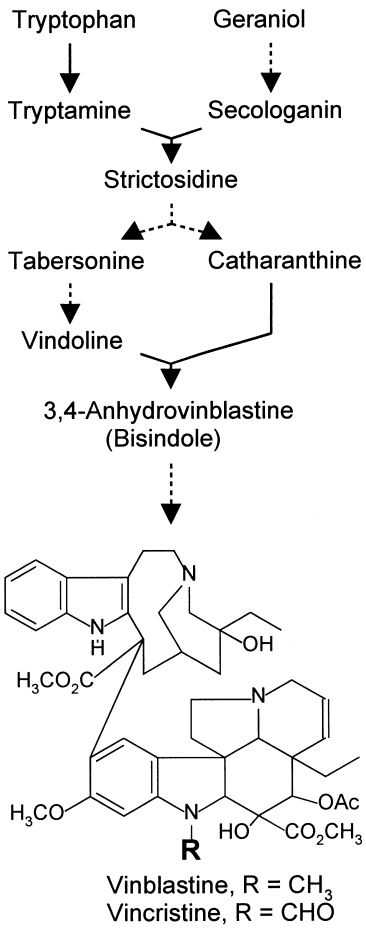

Fig. 1. Principle of the biosynthesis of bisindole alkaloids in *C. roseus*. Dashed arrows indicate multiple enzymatic reactions.

3.2. cDNAs for light-induced P450

A cDNA library from the light-induced cells was established and P450-specific sequences were amplified by using a previously established strategy which used degenerate oligodeoxynucleotide primers for conserved sequences around the conserved heme-binding site close to the C-terminus of the protein [15]. Several clones were sequenced and none was closely related to known P450 sequences. The cDNAs were hybridized in Northern blots to RNA from induced and non-induced cells. The hybridization with one of the cDNAs (obtained six times independently) followed the induction kinetics of T16H activity.

Overlapping cDNA clones for the 5′ and 3′ regions of this mRNA were obtained from the library by using specific oligodeoxynucleotides and phage primers. The sequences of all cDNAs were identical in the overlapping regions, indicating that they represented transcripts from the same gene. The deduced protein was named CYP71D12 according to the standardized system of P450 nomenclature [16]. The compar-

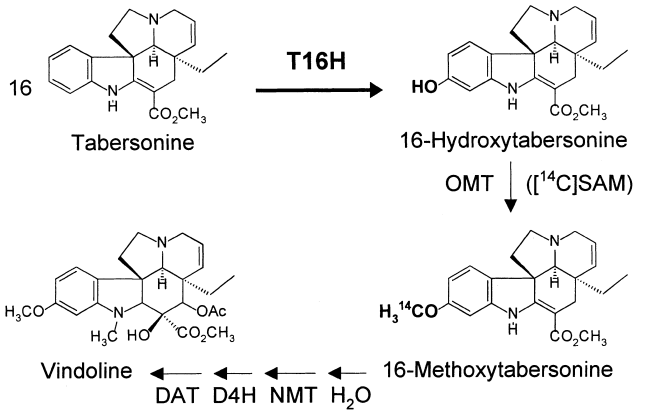

Fig. 2. Pathway from tabersonine to vindoline. The reactions of T16H and of the subsequent *O*-methyltransferase (OMT) are shown in detail to illustrate the labelling of 16-methoxytabersonine by [^{14}C]SAM in the standard T16H incubations. The further reactions: H_2O, hydration; NMT; D4H; DAT.

ison with other members of the CYP71D subfamily indicated that it lacked about five amino acids of the N-terminal membrane anchor. They were not obtained in several unsuccessful attempts, but this was not important for the purpose of this work because the expression of the protein in *E. coli* required the restructuring of the membrane anchor anyway.

The genomic blot suggested the presence of at least two closely related genes in *C. roseus*, taking into account the number of strongly hybridizing bands and the position of restriction sites in the cDNA (Fig. 4).

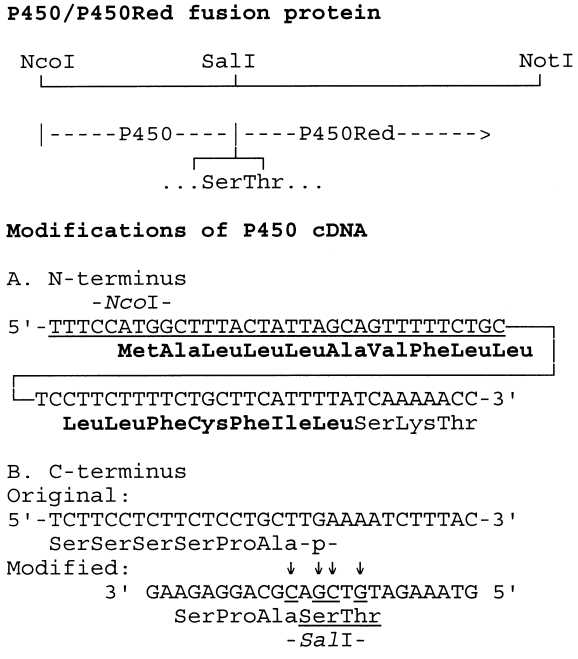

Fig. 3. Construction of a translational fusion between the P450 and P450Red in *E. coli*. Principle of the fusion protein and details of the cDNA modifications performed to create a suitable *N*-terminal membrane anchor containing a *Nco*I site and a *Sal*I-linker for the junction between the P450 and P450Red. A: The sequences added to the cDNA to create the membrane anchor are underlined and the amino acids of the membrane anchor are in bold letters. B: Note that the oligodeoxynucleotide is from the complementary strand of the cDNA.

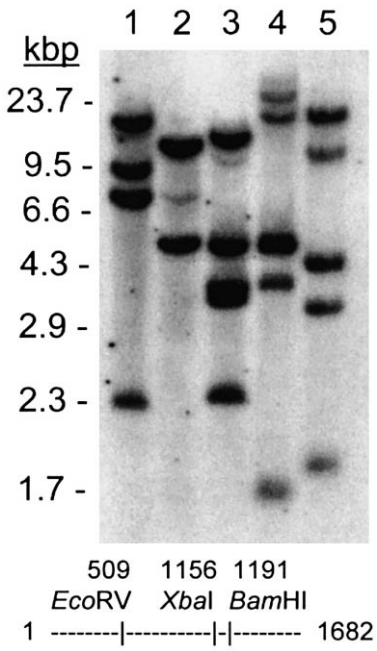

Fig. 4. Genomic Southern blot of *C. roseus* DNA. The hybridization was carried out with the complete cDNA. Restriction digests: 1, *Xba*I; 2, *Eco*RI; 3, *Hin*dIII; 4, *Bam*HI; 5, *Eco*RV. Bottom: Scheme of the cDNA and the position of restriction sites for enzymes used in the digestion of the DNA.

3.3. CYP71D12 is T16H

The cDNA was modified at the 5′ end to create a membrane anchor suitable for expression of P450s in *E. coli* and the protein was expressed as translational fusion with the P450Red from *C. roseus* (details in Section 2). Immunoblots with antiserum against the P450Red showed that the *E. coli* cells synthesized a protein of the predicted size (129 kDa, Fig. 5, top) after induction of protein expression. As previously noted [12], the weakly cross-reacting protein of even larger size is from *E. coli*. The P450/P450Red fusion was also detectable as induced protein band in gels stained with Ponceau S.

Fig. 5 shows an example of the TLC analysis carried out after the coupled assay which synthesized radioactive 16-methoxytabersonine. A radioactive product was only synthesized with membranes from cells expressing the P450/P450Red fusion protein. The formation was dependent on a methyltransferase source (leaf soluble proteins) and time courses of the reaction indicated that up to 45% of the [^{14}C]SAM were incorporated in extended incubations. The product was isolated by HPLC and the mass spectrometric comparison with the authentic substance identified it as 16-methoxytabersonine (data in Section 2).

We noted in these experiments that the TLC plates contained not only 16-methoxytabersonine (R_f 0.79), but also an unlabelled fluorescing product at R_f 0.48. Further studies showed that its formation was independent of the methyltransferase source. *C. roseus* suspension cultures are known to contain tabersonine derivatives which could be synthesized through a P450 activity performing an epoxidation of a double bond, e.g. lochnericine and derivatives [17], and such a product would be no substrate for the methylation reaction. The identification by HPLC/DAD and LC/ES MS-MS

showed, however, that it was 16-hydroxytabersonine (data in Section 2). This was confirmed by using the isolated substance as substrate for 16-methoxytabersonine formation with the leaf soluble proteins. The recombinant protein apparently synthesized more 16-hydroxytabersonine than could be processed to 16-methoxytabersonine. Even high concentrations of the leaf proteins could not overcome the problem that the coupled assay underestimated the T16H activity. The small differences between the spectra of substrate and product did not permit a precise quantification of the synthesized 16-hydroxytabersonine with an optical assay. These reasons precluded an accurate kinetic analysis of the T16H activity.

3.4. T16H is induced by light in suspension cultures

The induction of T16H activity used in the initial experiments involved simultaneous irradiation and a nutritional down-shift. We investigated the contribution of each of the two factors and also included the control (MX growth medium in the dark) in order to identify the conditions essential for the induction. The results of the four kinetics are summarized in Fig. 6.

Cultures kept in the dark, either in the standard MX growth medium (D+MX) or with the nutritional down-shift (D+Su), revealed no T16H activity (detection limit <0.05 μkat/kg), indicating that the nutritional downshift was not sufficient for T16H induction. The D+Su kinetics revealed throughout the time course a low level of a transcript hybridizing with the T16H cDNA, but no T16H activity was detected. The genomic blot suggested at least two closely related genes (Fig. 4) and the RNA may reflect transcripts from such a related gene which encodes a different enzyme function.

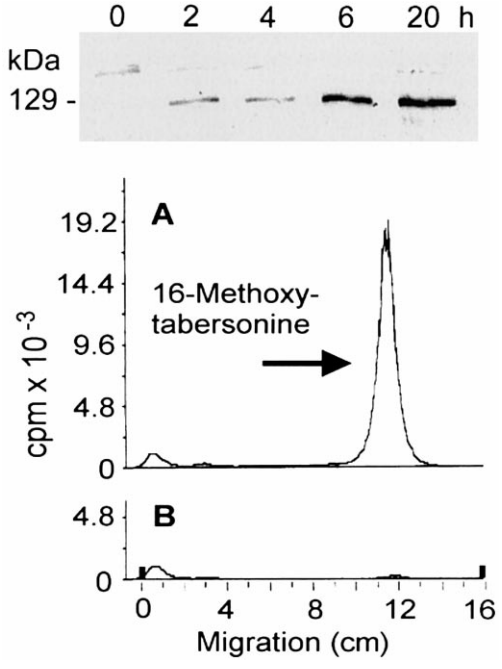

Fig. 5. Expression of the P450/P450Red fusion protein in *E. coli* (top) and TLC analysis of T16H activity (bottom). Top: Immunoblot of membrane fractions isolated at various time points after begin of protein induction. The fusion protein (129 kDa) was immunodecorated with antiserum against the P450Red. Bottom: Enzyme assays with membrane fractions isolated from *E. coli* 6 h after protein induction (A) and from non-induced bacteria (B). Assay conditions: 50 μg membrane protein, 80 μg *C. roseus* leaf soluble proteins (*O*-methyltransferase source), 30 μM tabersonine, and 18 μM [^{14}C]SAM; incubation for 20 min. The small amount of radioactive substance at the origin of the chromatogram is [^{14}C]SAM carried over from the aqueous phase of the ethyl acetate extraction.

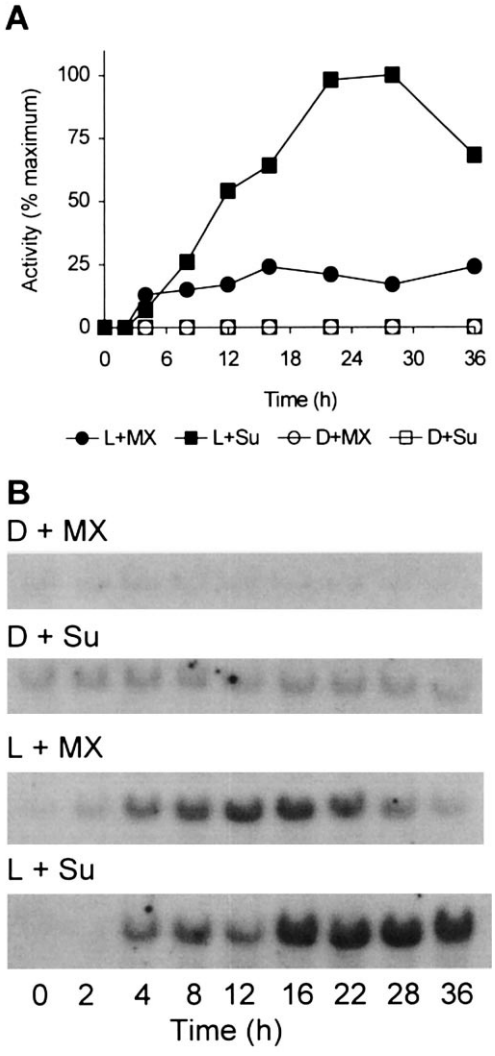

Fig. 6. T16H is a light-induced enzyme in *C. roseus* cell cultures. A: Enzyme activities. The assays contained 50 μg microsomes and 100 μg leaf soluble proteins. The highest enzyme activity (100%) was 2.8 μkat/kg. The difference between duplicate assays was smaller than 10%. B: RNA blots (16 μg total RNA per lane). L, light; D, dark; MX, MX growth medium; Su, nutritional downshift (transfer from MX medium to an aqueous 8% sucrose solution).

Irradiation of the suspension culture in MX growth medium (L+MX) led to induction of T16H activity, accompanied by a transient increase of the transcript. However, much higher activities (2.8 μkat/kg) were obtained by irradiation and simultaneous nutritional downshift (L+Su). The maximum was between 22 and 28 h after onset of the induction and the enzyme activity increases were accompanied by large increases in the transcripts. The discrepancy between enzyme activity and mRNA at the latest time point (36 h) was probably caused by a metabolic disarrangement due to the nutritional downshift. The results showed that light was essential for T16H expression in the suspension culture but that other factors influenced the extent of the induction.

4. Discussion

T16H is the first P450 in the complex pathway to indole alkaloids that is now characterized by cDNA cloning and functional identification. It belongs to the CYP71D subfamily which at present contains 12 other members. The sequences available from the databases are from plants not known to synthesize indole alkaloids. A fragment for 101 amino acids of a putative P450 from almond (*Prunus dulcis*, accession number AF107765) shared 70% identity with T16H. The others were 47–52% identical with T16H, i.e. CYP71D3 from *Arabidopsis thaliana* (fragment, accession Z27299), CYP71D6 and CYP71D7 from *Solanum chacoense* [18], CYP71D8, CYP71D9 and CYP71D10 from *Glycine* max [19,20], and CYP71D11 from *Lotus japonicus* [21]. The functions of these proteins are unknown. Predictions based on T16H are not possible because a few amino acid exchanges may cause drastic differences in the substrate specificities of P450s [22].

Earlier reviews indicated that vindoline biosynthesis in plants is restricted to aboveground organs and that the pathway from tabersonine to that intermediate is not expressed in suspension cultures [2,3]. One of the major reasons could be that they lack the differentiation to some of the specific cell-types which are present in plants. A very recent study [23] clearly suggested that the formation of the major leaf alkaloids in plants requires the participation of at least two cell-types and the intercellular translocation of a pathway intermediate: in situ hybridization and immunolocalization experiments showed that genes involved in strictosidine formation were only expressed in the epidermis of aerial tissues and in cortical cells of the root apical meristem, whereas expression of the last two steps of vindoline biosynthesis (deacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline 4-*O*-acetyltransferase (DAT), Fig. 2) occurred exclusively in laticifers and idioblasts of aerial tissues. It is not clear, however, whether the differential cell-type specific expression is essential for vindoline formation in all circumstances. Low levels of vindoline were detected in hairy root cultures obtained by transformation with *Agrobacterium rhizogenes* [24,25]. More importantly, suspension cultures established after leaf disc transformation with either *Agrobacterium tumefaciens* or *A. rhizogenes* revealed a stable production of both catharanthine and vindoline, and the activity of the last enzyme in the vindoline pathway (DAT) was demonstrated [26]. These results indicated that enzymes capable of vindoline biosynthesis were present, but their properties may be different from those in the aerial parts, and their main roles might be in the formation of other products. Recent experiments indeed identified one example, a root-specific DAT homolog [27] and unpublished data) which is distinguished from the enzyme expressed in aerial parts by its different substrate specificity (Laflamme et al., unpublished observations). Although the root-specific enzyme will also *O*-acetylate deacetylvindoline, its major role appears to be in the biosynthesis of *O*-acetylated tabersonine derivatives which accumulate in roots [28]. It could well be that the DAT activity reported in Ref. [26] measured that enzyme. It will be interesting to see whether the vindoline formation in tissue cultures represents true activation of a pathway otherwise active only in aerial parts, or whether it results from a combination of activities which also participate in the biosynthesis of other tabersonine derivatives.

Irradiation of the cell cultures, in particular in the combination with nutritional downshift, had not been investigated systematically with respect to vindoline formation. The one available publication [29] did not investigate vindoline, but reported an increased formation of the indole alkaloid serpentine, suggesting that these conditions may lead to a general increase in alkaloid formation. Previous studies on the presence of vindoline used either continuous dim light [30] or they were performed with photoautotrophic cultures in the absence of sugar [31].

We noticed in our experiments that the cultured cells were not homogeneous in their response to light. Induction experiments with enzymes from the anthocyanin pathway (unpublished results) showed that dark-grown cultures were light-yellow, but gradually turned red 25–30 h after initiating the irradiation. Microscopic inspection showed mostly aggregates of 10–30 cells. Interestingly, only about 30% of the cells were colored and often two neighbors joined by large areas of common cell wall were strikingly different: one contained anthocyanins, while the other did not. These findings indicated the presence of different cell-types in the irradiated cultures.

It is possible that the irradiation induced differentiation processes which include the activation of genes which are not expressed in the dark. Investigations with seedlings showed that T16H [9] and the last two enzymes (D4H and DAT, Fig. 2) in the pathway to vindoline are all induced by irradiation [5,32–35] and there is no evidence for differential regulation of the three enzymes. Experiments in our laboratory showed that the *O*-methyltransferase (OMT, Fig. 2) is expressed in the suspension culture (unpublished work), as suggested by previous reports on the detection of 16-methoxytabersonine in cultures [2]. The enzyme for the next step (formally a hydration) is not yet characterized, and the subsequent *N*-methyltransferase (NMT) does not require light for expression in seedlings [32]. It will be interesting to study how vindoline formation may be obtained under appropriate induction conditions, even if the cultures do not develop the specialized cell-types identified in the aerial parts. Suspension cultures synthesize catharanthine [2,3], the second precursor necessary for the synthesis of the bisindole alkaloids. There is also evidence that enzymes for the formation of the bisindole 3,4-anhydrovinblastine and for further modification reactions are present in suspension cultures [36–38].

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