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

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This report is dedicated to Prof. Rodney Croteau on the occasion of his 60th birthday.

Abstract

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

Keywords: *Catharanthus roseus*; Apocynaceae; Metabolic and enzymatic profiling; Monoterpenoid indole alkaloids; Low vindoline cultivar

1. Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is a perennial tropical plant belonging to the family Apocynaceae that produces more than 100 monoterpenoid indole alkaloids (MIAs) including two commercially important cytotoxic dimeric alkaloids used in cancer chemotherapy. These two bis-indole alkaloids, vinblastine (**9**) (Fig. 1) and vincristine (**10**), accumulate in trace amounts in leaves and are formed from the oxidative coupling of catharanthine (**8**) and vindoline (**7**) (van der Heijden et al., 2004). The pharmacological and therapeutic value of these low yield indole alkaloids has prompted efforts to improve their production and accumulation in plants and cell suspension cultures. Intensive research efforts have highlighted the complexity and strict regulation of the MIA biosynthetic pathway (St-Pierre and De Luca, 1995; St-Pierre et al., 1999; van der Heijden et al., 2004).

The pathway leading to the production of vindoline (**7**) (Fig. 1) in particular is even more complex since different cell types appear to participate in regulation in addition to developmental, environmental and tissue specific cues (St-Pierre et al., 1999; De Luca and St. Pierre, 2001; Murata and De Luca, 2005). Studies with *C. roseus* cv. Little Delicata have shown that transformation of tabersonine (**1**) to vindoline **7** (Fig. 1) requires six strictly ordered enzymatic reactions, involving hydroxylation of tabersonine by a cytochrome P450-dependent monooxygenase, tabersonine 16-hydroxylase (T16H), *O*-methylation by a cytosolic *S*-adenosyl-L-methionine (AdoMet)-16-hydroxytabersonine *O*-methyltransferase (16-OMT), an uncharacterized hydration of the 2,3-double bond, a thylakoid associated AdoMet: 2,3-dihydro-16-hydroxytabersonine-*N*-methyltransferase (NMT), a cytosolic 2-oxoglutarate

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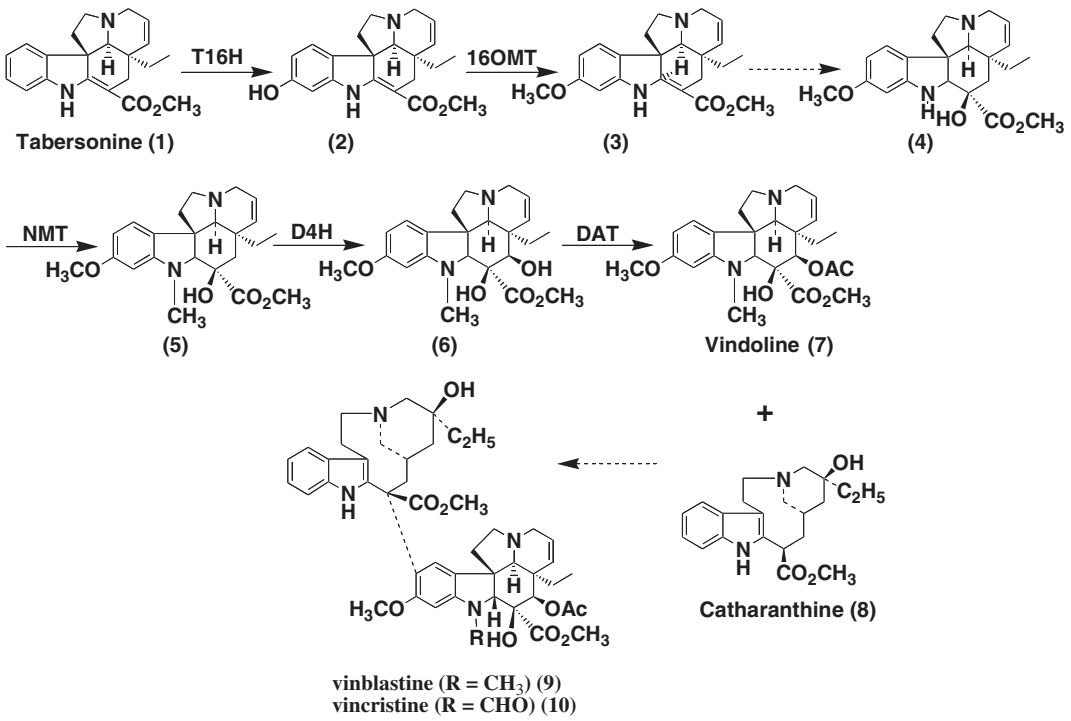

Fig. 1. The late stages of vindoline (**7**) biosynthesis involves hydroxylation of tabersonine by a cytochrome P450-dependent monooxygenase, tabersonine 16-hydroxylase (T16H), *O*-methylation by a cytosolic *S*-adenosyl-L-methionine (AdoMet)-16-hydroxytabersonine *O*-methyltransferase (OMT), an uncharacterized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

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

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

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

2. Results and discussion

2.1. Accumulation of vindoline and catharanthine in seedlings and young leaves

Seedlings from 50 ornamental cultivars of *C. roseus* were germinated in the dark for 5 days and then exposed to light for a 72 h period before being submitted to alkaloid analysis. Light treatment was required since this activates the terminal stages of vindoline (**7**) biosynthesis (De Luca et al., 1986) and triggers the quantitative conversion of tabersonine (**1**) into vindoline (**7**) (Balsevich et al., 1986). Ten seedlings from each cultivar were separated into cotyledons with hypocotyls and into root tissues before extraction for MIAs in MeOH–H_2O (1:1) and preparation for HPLC analyses. Root tissues from the each cultivar accumulated tabersonine (**1**), horhammericine and catharanthine (**8**) to highly variable extents, but none were shown

to accumulate vindoline (**7**) (data not shown). These data are consistent with what is known about the alkaloid profiles of roots and about the sites of vindoline (**7**) biosynthesis and accumulation in above ground parts of *C. roseus* (van der Heijden et al., 2004). When extracts of cotyledons with hypocotyls were screened for vindoline (**7**) and catharanthine (**8**) content, significant variation in the accumulation of these alkaloids was observed (Fig. 2A and B). For example lines 48 and 49 did not contain any detectable vindoline (**7**) compared to *C. roseus* cv. Little Delicata (Fig. 2A, line 50) whereas other lines (Fig. 2A, lines 1–21) accumulated 2–3 times higher levels of vindoline (**7**) compared to *C. roseus* cv. Little Delicata. Similarly, line 24 (Fig. 2B) had 5 times less catharanthine (**8**), whereas many other lines accumulated 2–3 times more catharanthine than *C. roseus* cv. Little Delicata.

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

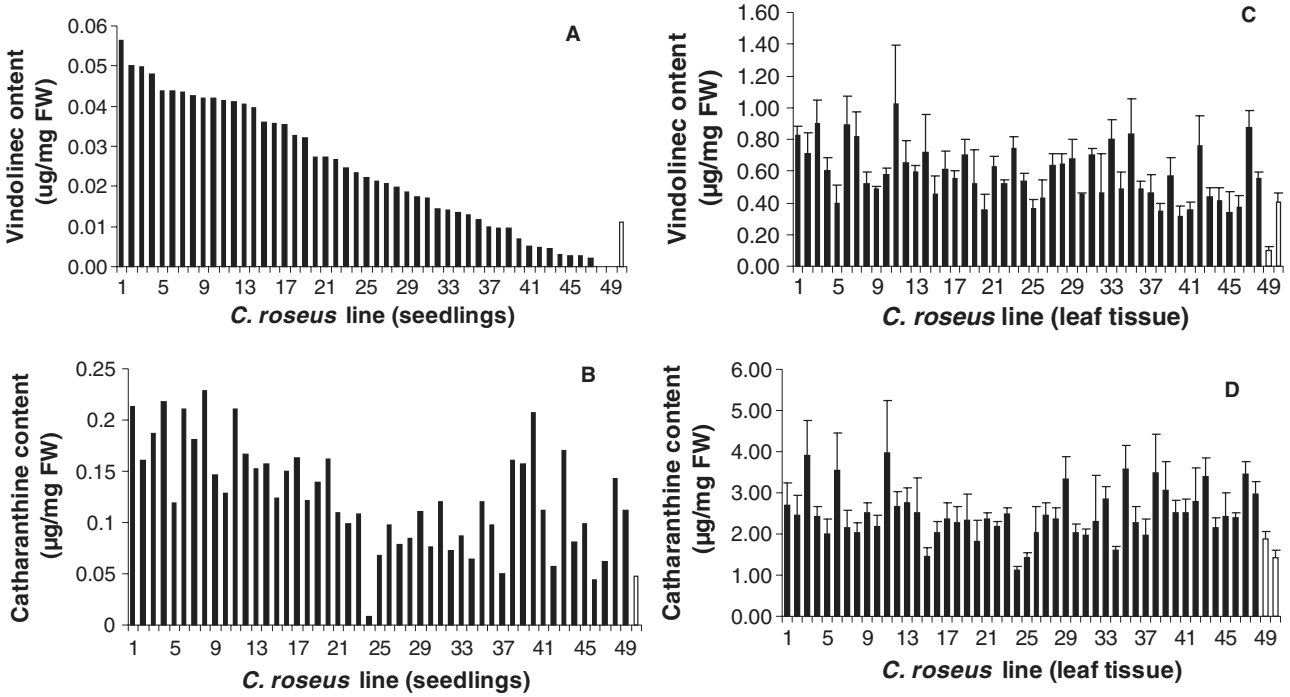

Fig. 2. Vindoline (A) (**7**) and catharanthine (B) (**8**) accumulation in cotyledons with hypocotyls of 50 flower cultivars of *C. roseus*. Vindoline (C) (**7**) and catharanthine (D) (**8**) accumulation in young leaves of 50 cultivars of *Cathatanthus roseus*. The data obtained for the *Catharanthus roseus* cv. Little Delicata reference cultivar and the low vindoline accumulating line # 49 are highlighted in white bars compared to the rest of the cultivars that are in black. The cultivars are numbered as follows: 1. Vinca Cooler Coconut; 2. Vinca Blue Pearl; 3. Vinca Pacifica Peach; 4. Vinca Pacific Red; 5, Vinca Cooler Red; 6. Vinca Pacifica Blush; 7. Vinca Cooler Grapes; 8. Vinca Cooler Rose; 9. Vinca Pacifica Apricot; 10. Vinca Pacifica Pink; 11. Vinca Pacifica Cherry Red; 12. Vinca Pacifica Lilac; 13. Vinca Pacifica Burguoy; 14. Vinca Cooler Apricot; 15. Vinca Stardust Orchid; 16. Vinca Cooler Orchid; 17. Vinca Pacifica White; 18. Vinca Cooler Raspberry Red; 19. Vinca Pacifica Polka Dot; 20. Vinca Sunglow ScarletW/Eye; 21. Vinca Sunstorm Bright Red; 22. Vinca Sunshower Pink; 23. Vinca Cooler Icy Pink; 24. Vinca Cooler Peppermint; 25. Vinca Sunshower Orchid W/Eye; 26. Vinca Sunstorm Blush; 27. Vinca Sunstorm Pink; 28. Vinca Sunshower White W/Eye; 29. Vinca Pacifica Coral; 30. Vinca Sunshower Lilac; 31. Vinca Cooler Pink; 32. Vinca Sunstorm Orchid; 33. Vinca Mediterranean Polka DT; 34. Vinca Cooler Strawberry; 35. Vinca Sunstorm Violet W/Eye; 36. Vinca Pacifica Deep Orchid; 37. Vinca Sunstorm White W/Eye; 38. Vinca Pacifica Icy Pink; 39. Vinca Sunstorm Apricot; 40. Vinca Pacifica Orchid Halo; 41. Vinca Apricot Delight; 42. Vinca Mediterranean Lilac; 43. Vinca Pacifica Punch; 44. Vinca Sunstorm Rose W/Eye; 45. Vinca Suoress White W/Eye; 46 Vinca Sunstorm Lilac; 47. Vinca Mediterranean Deep Rose; 48. Vinca Mediterranean WH Broad; 49. Vinca Mediterranean DP Orchid; 50. *Catharanthus roseus* cv. Little Delicata.

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

In order to determine if the low vindoline (**7**) phenotype of line 49 was due to changes in specific enzyme activities in the vindoline pathway, the activities of TDC, T16H, NMT, 16-OMT and DAT measured in comparison to those found in *C. roseus* cv. Little Delicata (Fig. 3). The results obtained show that while the specific activities of TDC, NMT, 16-OMT and DAT were very similar between these two lines, those of T16H were at least 10 times lower in line 49 than those of *C. roseus* cv. Little Delicata (Fig. 3). This low T16H activity of line 49 suggested that this reaction was rate limiting in the conversion of tabersonine (**1**) to 16-hydroxytabersonine (**2**) leading to the lower levels of vindoline (**7**) found in this cultivar.

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

The results obtained with mature plants suggested that the lack of vindoline (**7**) production observed with 8-day old light-treated seedlings of line 49 (Fig. 2A) might also be due to low T16H enzyme activity. To test this, 5-day old etiolated seedlings were grown for a further 48 h in the presence or absence of light and whole seedlings were harvested at different time points in triplicate to perform MIA analysis and T16H enzyme assays. Alkaloid analyses clearly showed that no vindoline (**7**) was detected in etiolated or light-treated line 49, whereas tabersonine (**1**) levels dropped as vindoline (**7**) accumulated in *C. roseus* cv. Little Delicata upon light treatment (Table 1, $T = 24$ and 48 h). The tabersonine (**1**) levels also dropped in light-treated seedlings of line 49 suggesting that these were converted to other products, but not into vindoline (**7**) (Table 1). Enzyme assays for T16H clearly showed the importance of light on the induction of this activity and on the appearance of vindoline (**7**) in light-treated developing seedlings of *C. roseus* cv. Little Delicata (Fig. 4). These results corresponded with previous studies describing the light induction of T16H activity (Schröder et al., 1999; St-Pierre and De Luca, 1995) in this cultivar. In contrast, light treatment of line 49 resulted in virtually no increases in T16H activity in either etiolated or light treated seedlings as compared to those of *C. roseus* cv. Little Delicata. Enzyme activity in 48 h light treated seedlings of *C. roseus* cv. Little Delicata was 10-fold higher than those of line 49. The similar results obtained with light treated seedlings and with young leaves

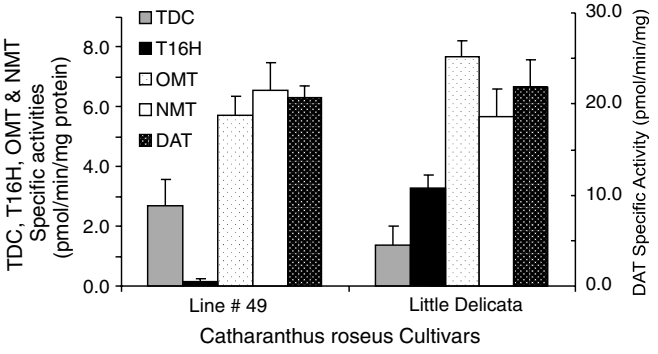

Fig. 3. Specific activity of TDC, T16H, NMT, OMT and DAT enzyme activities in young leaves of line 49 and of *Catharanthus roseus* cv. Little Delicata.

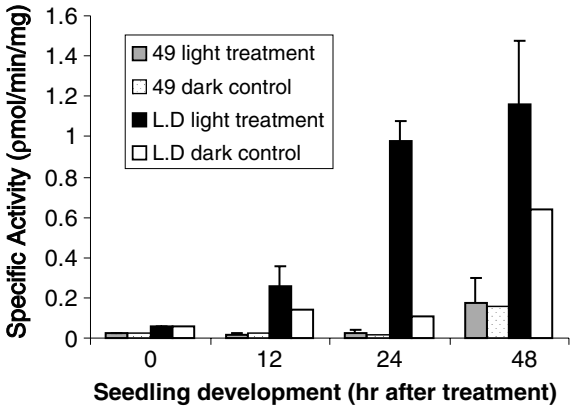

Fig. 4. Specific activity of T16H enzyme activity in young seedlings of line 49 and *Catharanthus roseus* cv. Little Delicata (L.D.). Five-day old etiolated seedlings were exposed to light or dark conditions and were harvested after an additional 0, 12, 24, and 48 h of growth, respectively.

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

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

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

of mature plants, suggest that the low vindoline (**7**) levels found in line 49 are caused by a decreased T16H activity.

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

3. Experimental

3.1. Plant materials

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

3.2. Culture medium and culture conditions

3.2.1. Cultivation of seedlings for alkaloid analysis and enzyme assay

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

3.2.2. Cultivation of seedlings for growing plants

Seeds from the 50 flowering cultivars were treated as described above but these Petri dishes were maintained under light in a growth chamber with a 16-h photoperiod and at 25 °C, unless otherwise mentioned. Fourteen days after seed germination seedlings were removed from the growth cabinet and were transferred to gardening soil. *C. roseus* plants were grown under greenhouse conditions for one month prior to replanting to an outdoor flowerbed during the months of May to September.

3.3. Preparation of crude protein extract

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

3.4. Protein determination

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

3.5. Alkaloid extraction and analyses

Seedlings that had been grown for 5 days in the dark followed by 3 days in a 16 h photoperiod as well as young leaves (50 mg fresh weight) were harvested. Ten seedlings (fresh weight about 150 mg) were separated into cotyledons with hypocotyls and into roots before processing for alkaloid extraction. Each of these tissues were extracted by transferring them to 1.5 mL Eppendorf tubes containing MeOH–H_2O (1:1; 400 μl) and by homogenizing with a Kontes pellet pestle (Fisher Scientific, Canada). After homogenization, samples were centrifuged in an Eppendorf centrifuge for 5 min at 15,000 rpm and the supernatant was filtered through a 0.45 μ Pall filter. The filtrate was analyzed by HPLC as described previously (Tikhomiroff and Jolicoeur, 2002; Murata and De Luca, 2005). In additional developmental time course experiments, 5-day old etiolated seedlings were grown for a further 48 h in the presence/absence of light and whole seedlings were harvested at different time points. Ten whole seedlings (about 150 mg Fresh weight) from each time point were extracted by transferring them to 1.5 mL Eppendorf tubes containing MeOH–H_2O (1:1; 400 μl) and were homogenized with a Kontes pellet pestle (Fisher Scientific, Canada). To the homogenate was added 100 μl of 10% sulfuric acid followed by extraction with an equal volume of EtOAc. After centrifugation as described above, and discarding the organic phase, the aqueous phase was treated with 10 N NaOH (50 μl), and alkaloids were extracted with an equal volume of EtOAc. The organic phase was collected and evaporated to dryness using an SPD Speed Vac (Thermo Savant, Holbrook, NY); the residue was resuspended in MeOH (300 μl) and filtered through a 0.45 μ Pall filter. The filtered alkaloid extracts were analyzed by HPLC as described above.

3.6. Enzyme assays

Young leaves (0.8 g) or seedlings (10 seedlings) were ground in 3 mL of extraction buffer (0.1 M Tris, pH 8, 14 mM Mercaptoethanol) using a mortar and pestle. The extract was filtered through mira cloth and desalted directly by PD10 Sephadex G25 column chromatography (GE Healthcare) and this extract was used directly for enzyme assays. TDC, T16H, 16OMT, NMT and DAT enzyme activities were assayed as described previously (De Luca et al., 1986, 1987, 1989; St-Pierre and De Luca, 1995; St-Pierre et al., 1998). All assays were performed in 100 μl reaction volumes containing desalted crude protein extract together with various substrates and cofactors related to each enzyme assay: TDC assay [20.8 μM (0.1 μCi) L-tryptophan (side chain-3-^{14}C), Moravek, Brea, CA, USA]; T16H assay [30 μM tabersonine, 8.33 μM *S*-adenosyl-L-(methyl-^{14}C)methionine and 1 mM NADPH]; 16OMT assay [approximately 30 μM 16-OH tabersonine and 8.33 μM (0.1 μCi) *S*-adenosyl-L-(methyl-^{14}C)Met, GE Healthcare, Buckinghamshire, UK]; NMT assay [30 μM 2,3-dihydro-3-hydroxy-tabersonine and 8.33 μM *S*-adenosyl-L-(methyl-^{4}C)Met]; DAT assay [30 μM deacetylvindoline and 16.6 μM (1-^{14}C)acetyl-coenzyme A, GE Healthcare]. Unlike the other four enzyme assays, the T16H assay is a coupled assay that detects the production of 16-methoxytabersonine (**3**) from tabersonine (**1**) by coupling it with endogenous 16-OMT activity, as described previously (St-Pierre and De Luca, 1995). Standard assays were conducted at 37 °C for 60 min, except for the DAT assay that was incubated for only 30 min. The products from each assay were treated with base (10 N NaOH) to facilitate the extraction of labeled alkaloids into an equal volume of EtOAc and the organic phase that was harvested after separation from the aqueous phase by centrifugation and was taken to dryness by vacuum centrifugation using an SPD Speed Vac (Thermo Savant, Holbrook, NY) system. Each dried sample was dissolved in MeOH (5 μl) and reaction products were separated by analytical thin layer chromatography (TLC) [Polygram Sil G/UV254 (Macherey-Nagel)]. TLC plates were developed in various solvent systems depending on the assay: T16H and 16OMT [Et_2O–hexane (1:1, v/v)]; NMT and DAT [EtOAc–MeOH (9:1, v/v)]; TDC [$CHCl_3$–MeOH–25% NH_4OH (5:4:1, v/v)]. The radioactivity was visualized and quantified by exposure of the TLC to a storage phosphor screen (GE Healthcare, Piscataway, NJ, USA) for 16 h and emissions were detected using a Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge ver. 3.0 (Fujifilm, Tokyo, Japan).

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