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Citation: Liscombe, David K., Aimee R. Usera, and Sarah E. O'Connor. "Homolog of Tocopherol C Methyltransferases Catalyzes N Methylation in Anticancer Alkaloid Biosynthesis." Proceedings of the National Academy of Sciences 107.44 (2010) : 18793 -18798. Copyright ©2010 by the National Academy of Sciences

As Published: <http://dx.doi.org/10.1073/pnas.1009003107>

Publisher: National Academy of Sciences (U.S.)

Persistent URL: <http://hdl.handle.net/1721.1/64435>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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Homolog of tocopherol C methyltransferases catalyzes N methylation in anticancer alkaloid biosynthesis

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Edited* by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved September 17, 2010 (received for review June 23, 2010)

Madagascar periwinkle (*Catharanthus roseus*) is the sole source of the anticancer drugs vinblastine and vincristine, bisindole alkaloids derived from the dimerization of the terpenoid indole alkaloids vindoline and catharanthine. Full elucidation of the biosynthetic pathways of these compounds is a prerequisite for metabolic engineering efforts that will improve production of these costly molecules. However, despite the medical and commercial importance of these natural products, the biosynthetic pathways remain poorly understood. Here we report the identification and characterization of a *C. roseus* cDNA encoding an S-adenosyl-L-methionine-dependent N methyltransferase that catalyzes a nitrogen methylation involved in vindoline biosynthesis. Recombinant enzyme produced in *Escherichia coli* is highly substrate specific, displaying a strict requirement for a 2,3-dihydro bond in the aspidosperma skeleton. The corresponding gene transcript is induced in methyl jasmonate-elicited seedlings, along with the other known vindoline biosynthetic transcripts. Intriguingly, this unique N methyltransferase is most similar at the amino acid level to the plastidic γ -tocopherol C methyltransferases of vitamin E biosynthesis, suggesting an evolutionary link between these two functionally disparate methyltransferases.

medicinal plant | specialized metabolism | gene discovery | tabersonine

Madagascar periwinkle (*Catharanthus roseus*) plants produce over 100 monoterpenoid indole alkaloids derived from tryptamine and the iridoid terpene secologanin (1). These products include the bisindole alkaloids vinblastine **9a** and vincristine **9b**, which are derived through the dimerization of vindoline **7a**, an aspidosperma-type alkaloid, and catharanthine **8** monomers (Fig. 1). Vinblastine **9a** and vincristine **9b** are microtubule disruptors that have been used extensively in the treatment of several types of cancer, including leukemia and lymphoma (2). Although yields of vincristine **9a** and vinblastine **9b** are approximately 0.0003% and 0.01%, respectively, from dried leaves of *C. roseus* (3), this plant nevertheless remains the only commercial source for these drugs. No other known plant species produces these compounds, and total syntheses of vinblastine **9a** and vincristine **9b** (for representative examples, see refs. 4 and 5) have not yet been adopted as industrial production methods.

Despite the medicinal and commercial importance of these compounds, several aspects of bisindole alkaloid biosynthesis are still not understood. Identification of the biosynthetic genes of this pathway is required for rational metabolic engineering efforts to improve production levels in the plant, explore heterologous production platforms, and to reprogram the pathway to make useful alkaloid derivatives. Although a number of the genes encoding vindoline **7a** biosynthetic enzymes have been cloned and characterized (6–9), genes encoding the hydrating enzyme or N methyltransferase (NMT) involved in converting tabersonine **1** to vindoline **7a** have not been identified (Fig. 1). Many of the validated biosynthetic enzymes are represented in the collection of approximately 20,000 ESTs for *C. roseus* available in GenBank (10, 11). Additional genes corresponding to unknown steps might also be represented in this dataset. Pioneering work by De Luca and colleagues demonstrated that the NMT of vindoline **7a** biosynthesis is associated with thylakoid membranes

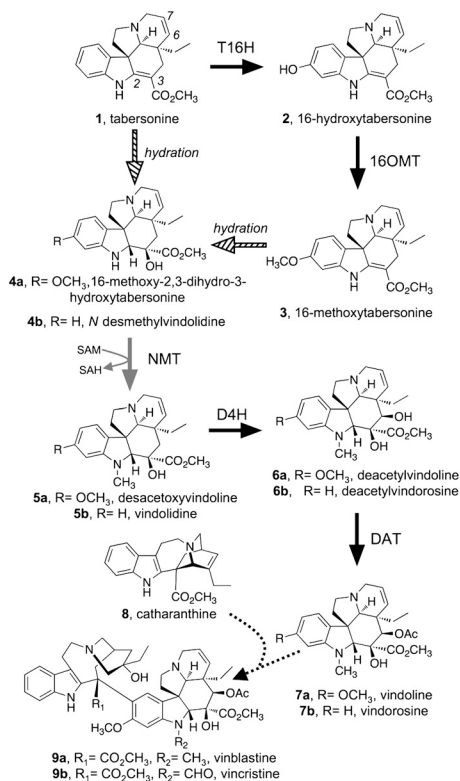


Fig. 1. Proposed pathway for vindoline **7a** and vindorosine **7b** biosynthesis in *Catharanthus roseus*. Solid black arrows indicate steps for which a corresponding cDNA has been isolated and characterized. Previously, 16-methoxy-2,3-dihydro-3-hydroxytabersonine N methyltransferase (NMT, gray arrow) had only been partially purified. An enzyme responsible for the hydration of tabersonine **1** and/or 16-methoxytabersonine **3** (hashed arrow) has not been detected. The dotted arrow between vindoline **7a** and vinblastine **9a** indicates multiple enzymatic steps. Abbreviations: T16H, tabersonine 16-hydroxylase; 16OMT, 16-hydroxytabersonine 16-O-methyltransferase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline acetyltransferase.

of chloroplasts (12–14). Therefore, when we identified a number of candidate cDNAs that encode putative methyltransferases, we paid particular attention to a subset of these genes that also displayed sequence similarity to plastidial γ -tocopherol methyltransferases (γ -TMTs) (15–17). Functional expression of these

Author contributions: D.K.L. and S.E.O. designed research; D.K.L. and A.R.U. performed research; A.R.U. contributed new reagents/analytic tools; D.K.L., A.R.U., and S.E.O. analyzed data; and D.K.L. and S.E.O. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. Cr2270 (HM584929), Cr1196 (HM584930), and Cr2551 (HM584931)].

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009003107/-DCSupplemental.

candidate cDNAs led to the identification of a γ -TMT homolog that methylates the aromatic nitrogen of a model aspidosperma substrate and can selectively methylate proposed native substrates present in seedling extracts.

Results and Discussion

Identification of *N*-Methyltransferase Candidates. We relied on two distinguishing features of the NMT of vindoline **7a** biosynthesis to guide the gene discovery process. First, this enzyme is associated with thylakoid membranes of chloroplasts (12, 13, 18), and, thus, we expected the translation product of the NMT gene to possess a transit peptide, or to resemble known plastid-localized proteins. It is also well established that vindoline **7a** biosynthesis occurs primarily in the leaves (7, 11), so we focused on those candidates that were expressed in this biosynthetically active tissue. *C. roseus* EST assemblies on the PlantGDB server (<http://plantgdb.org/cgi-bin/blast/PlantGDBblast>) were mined using tBLASTn, and all annotated plant NMT sequences in GenBank were used as queries. This initial list of NMT candidates derived from BLAST results was manually edited, where contigs that were identical or nearly identical to methyltransferases of known function (i.e., protein methyltransferases) were removed, to provide the master candidate list presented in Table S1. A phylogenetic tree (Fig. 2) illustrates the relationship between *C. roseus* NMT candidates and selected, functionally characterized *S*-adenosyl-L-methionine (SAM)-dependent enzymes from plants and bacteria. Predicted translation products of Cr5804, Cr6424, Cr8458, and Cr3910022 were too short to be included in the tree. We noted that five of our candidates (Cr1196, Cr2270, Cr7756, Cr3710017, and Cr3910022) were homologous to γ -TMTs, enzymes that are known to reside in plastids, where they participate in tocopherol biosynthesis (15, 17). Phylogenetic analysis places these candidates within a distinct clade that includes functionally characterized plant γ -TMT enzymes (Fig. 2) (15–17). The other analyzed candidates fall within a clade representing functionally diverse type-I and -II methyltransferases (Fig. 2) (19), including tabersonine 16-*O* methyltransferase, *O* methyltransferases of benzyliso-

quinoline and ipecac alkaloid biosynthesis, and an anthranilate *N* methyltransferase. The apparent full-length cDNAs of the latter candidate group do not encode putative chloroplast transit peptides, according to ChloroP (20), and, therefore, the corresponding polypeptides are not expected to be plastidic. The γ -TMT homologs Cr2270 and Cr7756 appeared to code for full-length proteins, lacking only the 5' region that would be expected to encode transit peptides (Fig. S1). However, Cr2270 was the only apparent full-length γ -TMT homolog that is expressed in leaves (Table S1), and we were unable to amplify the Cr7756 ORF from leaf or seedling cDNA. ORFs encoding Cr2270, and Cr1196 (both lacking putative transit peptide region), as well as Cr6996, and Cr7789, were amplified from elicited seedling cDNA and expressed in *E. coli*. Recombinant Cr2270, Cr1196, and Cr6996 proteins were purified to homogeneity and used for NMT assays (Fig. S2). As previously reported (21), we also observed that recombinant Cr7789 was insoluble and could not be purified.

Recombinant Cr2270 Catalyzes Aromatic Nitrogen Methylation of 2,3-Dihydrotabersonine. The known enzymes of vindoline **7a** biosynthesis are highly substrate specific (7, 22), which presumably ensures that the enzymes act in a strictly defined sequence. De Luca and coworkers previously reported that the NMT of vindoline **7a** biosynthesis, partially purified from *C. roseus* leaves, only accepted aspidosperma substrates in which the 2,3 alkene (see Fig. 1 for numbering) is reduced and the 6,7 alkene is intact (13, 18). The model substrate (2*R*,3*S*)-2,3-dihydrotabersonine (DHT) **10** was among the few substrates readily turned over by this enzyme (13, 18), and because this compound is synthetically accessible through sodium cyanoborohydride reduction of tabersonine **1** (Fig. 3*A*), we used this as a test substrate to screen for the desired NMT activity. The structure of the synthetic DHT **10** substrate was confirmed by ¹H-NMR (Fig. 3*B*; full spectrum in Fig. S3*A*), ¹³C-NMR, and COSY-NMR (Fig. S3*B* and *C*). Analysis of enzyme assays by liquid chromatography-mass spectrometry (LC-MS) revealed that in the presence of SAM, Cr2270 converted DHT **10** to a more hydrophobic compound

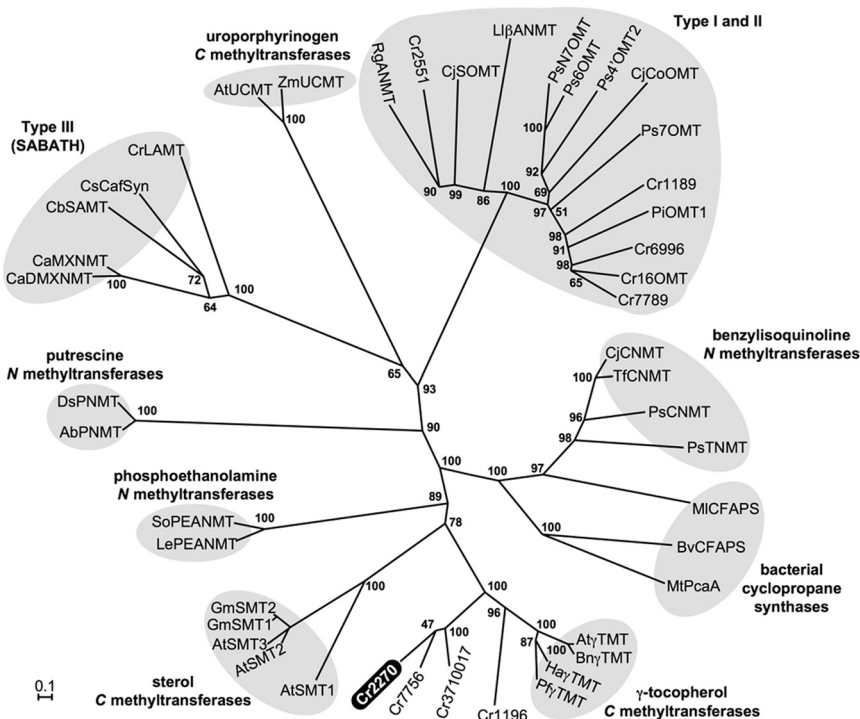


Fig. 2. Unrooted, amino acid similarity tree of plant and bacterial *S*-adenosyl-L-methionine dependent methyltransferases constructed using the Neighbor-Joining method. Bootstrap frequencies (as percentage of 1,000 iterations) are shown for each clade. Protein names and accession numbers are provided in Table S3.

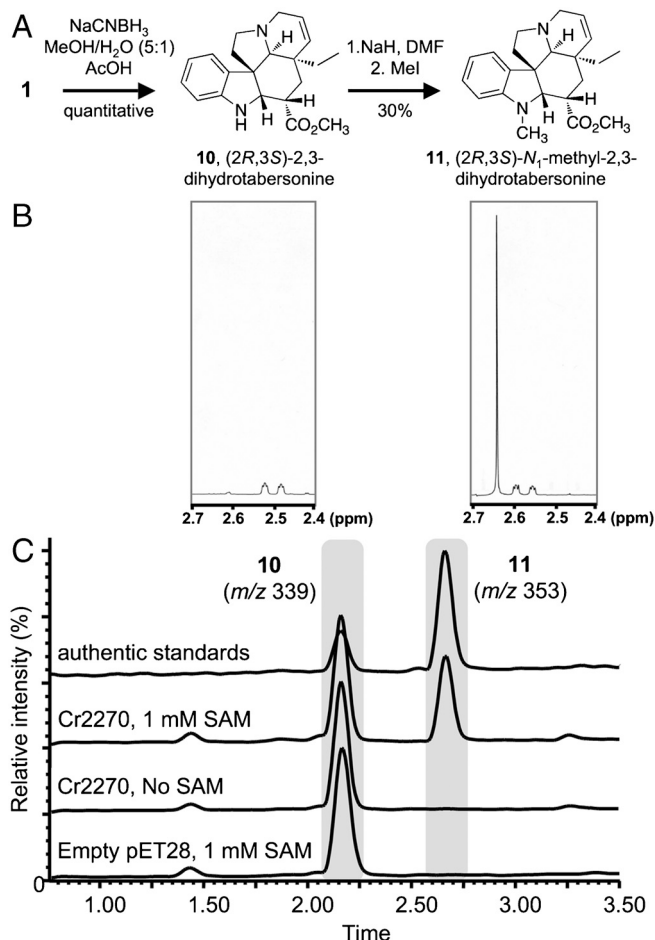


Fig. 3. Characterization of recombinant Cr2270. (A) Synthetic scheme for model *N*-methyltransferase substrate **10** [(2R,3S)-2,3-dihydrotabersonine] and product standard **11** [(2R,3S)-*N*¹-methyl-2,3-dihydrotabersonine]. (B) ¹H-NMR analysis of synthetic substrate and product standard confirming methylation of aromatic nitrogen. (C) Selected ion chromatograms (*m/z* 339 and 353) from LC-MS analysis of enzyme assays with purified, recombinant Cr2270.

that displayed the expected molecular weight of *N*-methyl-DHT **11** (Fig. 3C). Furthermore, an authentic standard of *N*-methyl-DHT **11** that was synthesized by treatment of DHT **10** with sodium hydride and iodomethane (Fig. 3A and B and Fig. S3 D–E) coeluted with the enzymatic product (Fig. 3C). In contrast, recombinant Cr1196 or Cr6996 was not capable of methylating DHT **10** when SAM was included in the assays. Steady-state kinetic analyses for Cr2270 showed that DHT **10** evidently serves as an excellent model substrate for Cr2270, exhibiting a K_m of 8.8 μ M and a k_{cat} of 2.4 s⁻¹ (Table 1 and Fig. S4 A and B). As expected, Cr2270 is sensitive to inhibition by *S*-adenosylhomocysteine (SAH) ($K_i = 7.3 \mu$ M) (Fig. S4).

Consistent with the previously reported substrate specificity for the NMT of vindoline **7a** biosynthesis, Cr2270 does not methy-

Table 1. Apparent kinetic parameters for recombinant Cr2270

	Substrates	
	DHT	SAM
K_m (μ M)	8.8 \pm 1.0	22.0 \pm 1.8
V_{max} (μ mol s ⁻¹ mg ⁻¹ protein)	67.0 \pm 1.8	151.6 \pm 3.9
k_{cat} (s ⁻¹)	2.4 \pm 0.1	5.4 \pm 0.1
k_{cat}/K_m (s ⁻¹ M ⁻¹)	267,744	243,053

late tabersonine **1** (13, 18). A variety of other alkaloids were assayed with recombinant Cr2270, but none were converted to any product as evidenced by monitoring the disappearance of starting material and formation of any product by LC-MS (Fig. S5). The failure of Cr2270 to turn over tabersonine **1** indicates that the 2,3-single bond of the aspidosperma skeleton is a critical recognition element for the enzyme. Without this functionality, the substrate is not recognized by the enzyme in vitro, presumably due to conformational differences between the substrates, or because the nitrogen of DHT **10** is more electron rich than that of tabersonine **1**. The known alkaloid products of *C. roseus*—specifically the lack of *N*-methylated tabersonine—suggest that the substrate specificity is the same in vivo. In contrast, the 16-methoxy substitution on the indole ring does not appear to qualitatively affect the action of this NMT.

The substrate specificity of this NMT undoubtedly plays a role in determining the order of enzymatic transformations and defining the complement of alkaloids produced by *C. roseus* plants. The substrate specificity of subsequent steps is also consistent with the order of vindoline **7a** biosynthesis (Fig. 1): D4H activity relies on the presence of the *N*-methyl group and reduced 2,3-bond (22), and deacetylvindoline acetyltransferase (DAT) clearly requires the 4-hydroxy group for acetylation. Although *N* deformylvincristine (*N* desmethylvinblastine) has been isolated, it is likely that this compound arises from deformylation of vincristine **9b** in planta or during the isolation process (23), or via demethylation of vinblastine. The inherent substrate specificity of the biosynthetic enzymes has obvious metabolic engineering implications. Currently, all known *N*-methylated aspidosperma alkaloids in *C. roseus* lack the 2,3-double bond; understanding the molecular basis of substrate recognition by this enzyme may allow *N* methylation of a wider range of alkaloid intermediates for production of many previously undescribed alkaloid structures.

Recombinant Cr2270 Methylates the Proposed Native Substrates in Seedling Extracts. The precursors to vindoline **7a** and vindorosine **7b** proposed to serve as native substrates for the NMT (Fig. 1) are difficult to produce synthetically or isolate in sufficient quantities for kinetic studies. However, compounds with exact mass and formulas corresponding to these biosynthetic intermediates could be detected in crude extracts of *C. roseus* seedlings (Fig. 4). These extracts were therefore incubated with recombinant Cr2270 in the absence or presence of SAM to determine whether these intermediates are productive substrates for the enzyme. Notably, the compound with the exact mass matching 16-methoxy-2,3-dihydro-3-hydroxytabersonine **4a** (Fig. 1) was consumed after addition of the enzyme, and a compound (desacetoxyvindoline **5a**) with a mass corresponding to the addition of one methyl group accumulated (Fig. 4 A and C). Taken together with the results of the model substrate, this strongly suggests that Cr2270 also acts upon its predicted native substrate. A compound in crude extracts with an exact mass matching *N* desmethylvindolidine **4b** also appeared to be methylated by this NMT to yield the corresponding intermediate vindolidine **5b**, a presumed precursor in vindorosine **7b** biosynthesis (Fig. 4 B and C). Methylation of other putative vindoline **7a** or vindorosine **7b** intermediates in the extract was not detected. This targeted activity-based metabolic profiling approach (24) appears to be an excellent strategy to functionally characterize biosynthetic enzymes with native substrates that are challenging to isolate or synthesize.

Expression of Cr2270 Transcripts Is Coordinately Regulated with Known Alkaloid Biosynthetic Genes. *C. roseus* seedlings produce alkaloids as part of their normal developmental program, and this accumulation can be enhanced by methyl jasmonate (25). As previously demonstrated, we noted the amount of vindoline **7a** found in seedlings increased over the course of a 10-d methyl jasmonate

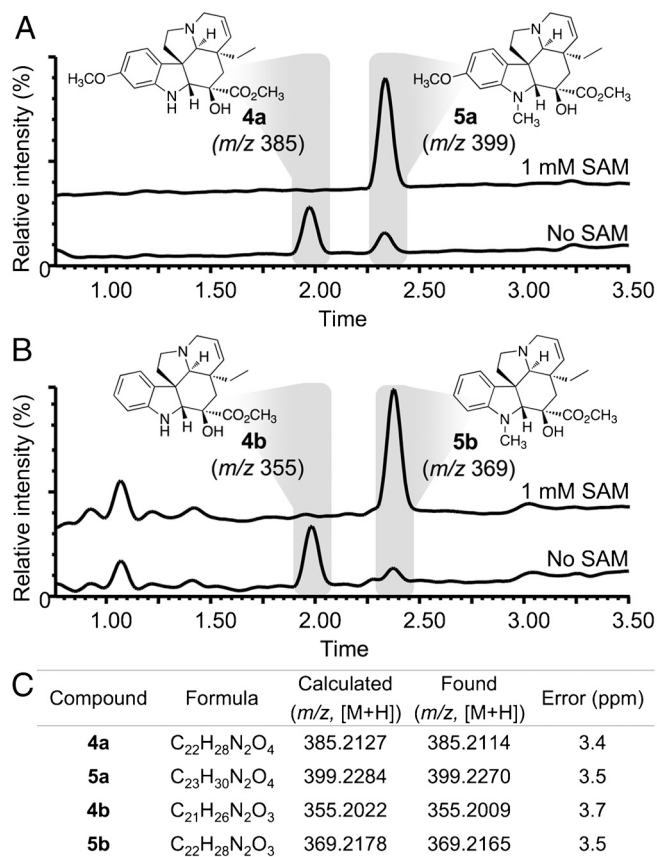


Fig. 4. Targeted activity-based metabolite profiling to examine native substrate specificity of purified, recombinant Cr2270. Crude methanolic alkaloid extract from methyl jasmonate-elicited *C. roseus* seedlings was incubated with Cr2270, and with or without *S*-adenosyl-L-methionine. Assays were analyzed by LC-MS to examine Cr2270-dependent methylation of (A) 16-methoxy-2,3-dihydro-3-hydroxytabersonine (selected ion chromatograms for *m/z* 385 and 399 are displayed) and (B) *N*-desmethylvindolidine (selected ion chromatograms for *m/z* 355 and 369 are displayed). (C) Exact mass data for extract components.

elicitation when compared to nonelicited controls (Fig. 5A). We therefore used qPCR to track the expression levels of *Cr2270*, in addition to the four previously characterized vindoline **7a** biosynthetic genes. We noted that all known vindoline **7a** biosynthetic genes and the NMT *Cr2270* gene were up-regulated relative to the nonelicited seedlings (Fig. S6). The expression profile for *Cr2270* exhibits the same pattern as *16OMT*, suggesting that expression of these genes is coordinately regulated (Fig. 5B). In contrast, expression of the putative methyltransferase gene *Cr1196*, which appears to have no role in alkaloid biosynthesis, was not up-regulated by methyl jasmonate (Fig. 5B). The coordinate regulation of *Cr2270* expression with other vindoline **7a** biosynthetic genes in response to methyl jasmonate, along with the lack of transcriptional induction of a highly similar methyltransferase that is not involved in alkaloid biosynthesis (*Cr1196*; Fig. 2), provides further support for the role of *Cr2270* as the 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N* methyltransferase in vindoline **7a** biosynthesis.

On the Evolution of *N*-Methyltransferase Function. Sequence analysis of *Cr2270* and related proteins (Fig. 2 and Fig. S1) suggest that *Cr2270* represents a previously undescribed class of NMT that likely arose from γ -TMTs. *Arabidopsis thaliana* possesses a single gene that encodes a functional γ -TMT (17), whereas *Brassica napus* (26) and *C. roseus* both have at least four γ -TMT-like genes. In the latter case, we now know that members of this gene family

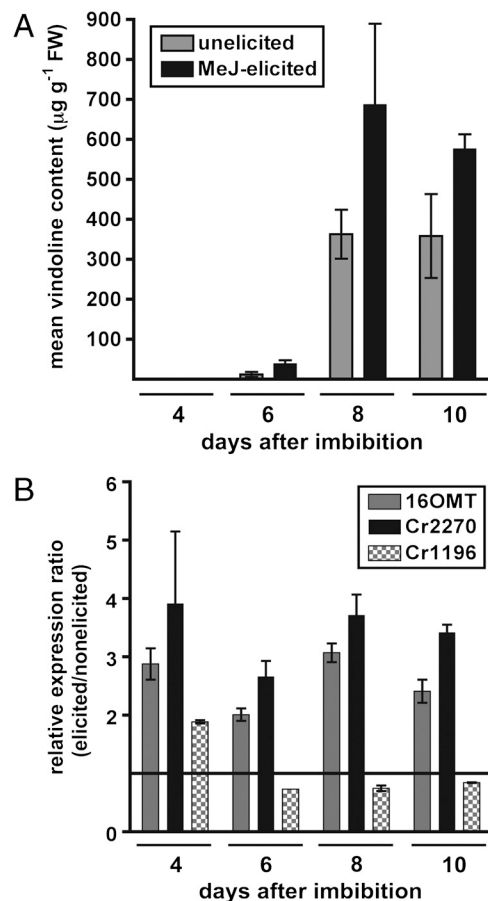


Fig. 5. Targeted metabolite and transcript profiling of *C. roseus* seedlings elicited with methyl jasmonate. (A) Mean vindoline content of elicited seedlings after receiving methyl jasmonate treatment 2 d after imbibition compared to unelicited seedlings. (B) Quantitative real-time PCR analysis of *16OMT*, *Cr2270*, and *Cr1196* gene expression in *C. roseus* seedlings treated in the same manner as A. Data are displayed as a relative expression ratio compared to untreated seedlings. Error bars represent SEM from at least three experiments.

encode enzymes that are functionally distinct. We speculate that *Cr2270* arose from γ -TMT gene duplication events, which would explain why these gene products reside in the same subcellular compartment. Moreover, although DHT methylation by *Cr2270* is inhibited by γ -tocopherol (Fig. S7A), γ -tocopherol is not turned over by this enzyme (Fig. S7B). Perhaps the evolution of substrate specificity was facilitated by the presence of nitrogen-containing γ -tocopherol derivatives, such as 5-nitro- γ -tocopherol, which is generated in diverse plant systems (27). Rubisco large subunit methyltransferase (*RubLSMT*) is known to modulate γ -TMT activity through methylation of a C-terminal lysine residue (28). Interestingly, the *RubLSMT* consensus motif (28) is conserved in γ -TMTs, and *Cr1196*, but has been lost in *Cr2270*, *Cr3710017*, and *Cr7756* (Fig. S1).

Conclusion

The discovery of this NMT further closes the gap in the tabersonine to vindoline branch of monoterpene indole alkaloid biosynthesis, with only one uncharacterized step remaining in this pathway. This discovery will facilitate metabolic engineering efforts for both enhanced production of bisindole alkaloids in plants or in heterologous expression systems. Moreover, these data will allow a detailed study of the substrate specificity of this enzyme, which controls the production of the distinct alkaloid structures produced naturally. Furthermore, the amino acid sequence of this enzyme provides a suggestion as to how one

piece of this specialized metabolic pathway evolved from an entirely distinct metabolic pathway.

Materials and Methods

Plant Growth. *Catharanthus roseus* cv. 'Little Bright Eyes' seeds were purchased from B & T World Seeds (<http://b-and-t-world-seeds.com>). Sterile seedlings were cultured and treated with methyl jasmonate according to Aerts et al. (25).

Chemicals. Tabersonine was generously provided by Viresh Rawal (University of Chicago, Chicago). Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich.

Synthesis of (2R,3S)-2,3-Dihydrotabersonine. Excess sodium cyanoborohydride (0.027 g, 0.430 mmol) was added to a solution of tabersonine (0.024 g, 0.071 mmol) in 2.4 mL of methanol-water (5:1) acidified with acetic acid (pH 4). This mixture was stirred under argon for 16 h at room temperature. Additional sodium cyanoborohydride (0.008 mg, 0.127 mmol) was then added, and the reaction was allowed to stir at room temperature for another 4 h. The mixture was then evaporated to dryness, resuspended in 500 μ L of water, and then extracted 4 times with ethyl acetate (500 μ L). The organic fractions were combined, evaporated to dryness, and purified by flash silica chromatography (100% MeCl₂) in quantitative yield to give (2R,3S)-2,3-dihydrotabersonine (0.024 g, 0.071 mmol). ¹H-NMR (CD₃OD, 400 MHz): δ 7.02 (d, 1H, *J* = 7.5 Hz), 6.95 (ddd, 1H, *J* = 8.0, 7.5, 1.0 Hz), 6.60 (ddd, 1H, *J* = 7.5, 7.5, 1.0 Hz), 6.51 (d, 1H, 8.0 Hz), 5.79–5.75 (m, 1H), 5.37–5.35 (m, 1H), 4.02 (d, 1H, *J* = 2.5 Hz), 3.77 (s, 3H), 3.64–3.60 (m, 1H), 3.34 (d, 1H, *J* = 5.5 Hz), 3.22 (dt, 1H, *J* = 9.0, 3.0 Hz), 2.62 (d, 1H, *J* = 15.5 Hz), 2.40–2.34 (m, 1H), 2.30 (s, 1H), 2.24 (dt, 1H, *J* = 13.0, 3.0 Hz), 2.01 (dt, 1H, *J* = 13.0, 8.5 Hz), 1.66–1.63 (m, 2H), 1.05–0.97 (m, 2H), 0.56 (t, 3H, *J* = 7.5 Hz). ¹³C-NMR (CD₃OD, 400 MHz): δ 177.34, 152.56, 136.46, 135.69, 129.07, 124.62, 124.25, 119.15, 109.92, 70.82, 68.11, 54.61, 53.59, 52.66, 52.29, 44.40, 40.82, 38.76, 35.15, 32.95, 8.72. High resolution mass spectrometry (HRMS): calculated for C₂₁H₂₆N₂O₂ [M + H⁺]: *m/z* 339.2067; found: *m/z* 339.2061.

Synthesis of (2R,3S)-N¹-Methyl-2,3-Dihydrotabersonine. Sodium hydride (0.003 g, 0.066 mmol, 60% in mineral oil) was added to dihydrotabersonine (0.015 g, 0.044 mmol) in dimethylformamide (1 mL) under argon. The solution was warmed to 50 °C for 1 h at which time it was cooled and iodomethane (0.004 g, 0.066 mmol) was added. The solution was then stirred for 16 h at room temperature and then warmed to 50 °C for an additional 6 h. Methanol was added, and the solution was evaporated and purified by flash silica chromatography to give *N* methyl-dihydrotabersonine (0.005 g, 0.014 mmol) in 30% yield. ¹H-NMR (CD₃OD, 400 MHz): δ 7.05 (ddd, 1H, *J* = 8.4, 7.2, 1.2 Hz), 7.04 (d, 1H, *J* = 7.6 Hz), 6.66 (ddd, 1H, *J* = 7.6, 7.2, 0.8 Hz), 6.45 (d, 1H, 7.6 Hz), 5.74–5.70 (m, 1H), 5.37–5.34 (m, 1H), 3.78–3.73 (m, 1H), 3.73 (s, 3H), 3.61–3.60 (m, 1H), 3.35–3.32 (m, 1H), 3.26–3.21 (m, 1H), 2.64 (s, 3H), 2.60–2.55 (m, 1H), 2.35–2.25 (m, 2H), 2.21 (s, 1H), 2.02–1.97 (m, 1H), 1.74–1.61 (m, 2H), 1.05–0.98 (m, 2H), 0.50 (t, 3H, *J* = 7.6 Hz). ¹³C-NMR (CD₃OD, 400 MHz): δ 177.57, 154.46, 136.78, 136.37, 129.30, 124.06, 123.90, 119.56, 108.81, 77.48, 71.61, 54.27, 54.21, 52.79, 52.24, 44.48, 39.15, 38.42, 36.88, 35.12, 32.63, 8.80. HRMS: calculated for C₂₂H₂₈N₂O₂ [M + H⁺]: *m/z* 353.2224; found: *m/z* 353.2238.

Alkaloid Extraction. Aseptically grown *C. roseus* seedlings (~0.1 g) were ground to a fine powder under liquid N₂ and extracted in 1 mL of methanol, containing ajmaline (10 μ M) as an internal standard. The solution was centrifuged, filtered through a 2- μ m disposable filter, and then subjected to analysis by LC-MS.

RACE to Obtain 3' Ends of Cr1196 and Cr2551. Total RNA was isolated from elicited seedlings using a Plant RNeasy kit (Qiagen), according to the manufacturer's protocol. First strand cDNA synthesis was performed using M-MuLV Reverse Transcriptase (New England Biolabs), and cDNA cloning primer (Table S2) according to manufacturer's instructions. The missing regions were amplified by PCR using primers listed (Table S2). Amplicons were cloned into pGEM-T Easy and subjected to DNA sequencing.

Construction of Expression Plasmids. cDNA was obtained from seedlings as described above, except that an oligo d(T) primer was used in place of the cDNA cloning primer. Open reading frames of candidate cDNAs were amplified by PCR using a sense and antisense primer pairs (Table S2), and High Fidelity Platinum Taq Polymerase (Invitrogen). PCR products were cloned into pGEM-T Easy (Promega). The ORFs were then subcloned into multiple cloning

sites of pET28a (EMD) or pRSETA (Invitrogen) using restriction sites engineered into the primers (Table S2).

Production and Purification of Recombinant Protein. *Escherichia coli* Rosetta 2 (DE3) pLysS cells (EMD) harboring the pET28-Cr2270, pET28-Cr6996, or pRSET-Cr1196 expression constructs were grown in 1 L of Luria-Bertani medium supplemented with 50 μ g mL⁻¹ kanamycin (pET28) or 100 μ g mL⁻¹ ampicillin (pRSET), 34 μ g mL⁻¹ chloramphenicol [and 3% (vol/vol) ethanol for Cr2270] at 37 °C with shaking (220 rpm) to an OD₆₀₀ of 0.6, and then induced with 1 mM IPTG. After 3 h of growth at 37 °C, cells were collected by centrifugation at 5,000 \times *g*, flash-frozen in liquid N₂, and stored at -80 °C until further use. Pelleted cells were resuspended in buffer A [100 mM Tris-HCl (pH 7.5), 100 mM KCl, 10% glycerol, 20 mM β -mercaptoethanol] (14) and lysed by sonication. For Cr2270 and Cr1196, Tergitol NP-10 (0.1%, vol/vol) was added to the supernatant, which was subsequently incubated for 2 h at 4 °C with gentle agitation. Cell debris was removed by centrifugation (10,000 \times *g*) for 30 min at 4 °C. The supernatant was bound to Talon cobalt affinity resin (Clontech), which was then washed twice with 4 mL of buffer A and eluted in a stepwise manner with 750 μ L of buffer A containing increasing concentrations (10, 50, 100, and 200 mM) of imidazole. To remove imidazole, these fractions were exchanged into buffer B [100 mM NaPO₄ (pH 7.4), 25% glycerol, 20 mM β -mercaptoethanol] using a Zeba desalt spin column (Pierce).

Enzyme Assays. NMT assays contained 1 μ g of purified protein, 0.78–200 μ M 2,3-dihydrotabersonine, 1.13–1000 μ M *S*-adenosyl-L-methionine, and 10 μ M ajmaline (internal standard), in buffer C [100 mM NaPO₄, pH 7.4; 20 mM β -mercaptoethanol, 1 mM EDTA], in a total volume of 50 μ L. Assays were incubated at 30 °C for 30 or 60 min and quenched with the addition of 500 μ L methanol. Reactions were then centrifuged at 17,000 \times *g* for 10 min to pellet precipitated protein. Assays were further diluted 1:150, and 7 μ L were analyzed by LC-MS.

Kinetic Analysis. Kinetic assays were performed under optimal conditions (30 °C, pH 7.4). For DHT and SAH kinetics, SAM was fixed at 200 μ M, and for SAM kinetics DHT was fixed at 100 μ M. Kinetic data were fitted by non-linear regression and analyzed using GraphPad Prism version 4 for Macintosh (GraphPad Software).

Liquid Chromatography-Mass Spectrometry. Ultra performance liquid chromatography analysis was performed using an Acquity Ultra Performance BEH C18 column with a 1.7- μ m particle size, 2.1 \times 100 mm dimension, and a flow rate of 0.5 mL min⁻¹. The column elution was coupled to MS analysis carried out using a Micromass LCT Premier TOF Mass Spectrometer with an electrospray ionization source. Both modules are from Waters Corporation. The capillary and sample cone voltages were 3,000 V and 30 V, respectively. The desolvation and source temperatures were 300 and 100 °C, respectively. The cone and desolvation gas flow rates were 60 and 800 L h⁻¹. Alkaloid methyltransferase assays were analyzed in ES⁺ mode using the following gradient: 10:90 to 50:50 acetonitrile-formic acid water over 5 min. Tocopherol methylation was analyzed in ES⁻ mode using the following gradient: 10:90–50:50 acetonitrile-formic acid water over 5 min, 95:5 acetonitrile-formic acid water for 1 min, and 100% acetonitrile for 6 min. Analysis was performed with MassLynx 4.1, and integrations were carried out using the QuantLynx tool.

Quantitative Real-Time PCR (qPCR). Gene expression analysis was performed as described by Runguphan et al. (29) using the qPCR primers listed in Table S2. In addition to *C. roseus rps9*, *EF1 α* was used as a second endogenous reference transcript (30). The 2^{- $\Delta\Delta$ Ct} method was used for relative gene expression analysis (31).

Phylogenetic Analysis. Amino acid alignments were performed using ClustalX (32). The neighbor-joining phylogeny was generated, and bootstrap analysis was performed with TREECON (33). GenBank accession numbers for the sequences used are provided in Table S3.

ACKNOWLEDGMENTS. The authors are grateful to Dr. Viresh Rawal (University of Chicago, Chicago) for the gift of tabersonine, and Dr. Li Li of the DCIF (Massachusetts Institute of Technology, Cambridge, MA) for obtaining exact mass data. We gratefully acknowledge support from GM074820. We also acknowledge additional support from National Science Foundation and the American Cancer Society. D.K.L. is the recipient of a Natural Sciences and Engineering Research Council of Canada postdoctoral fellowship. A.R.U. is the recipient of a National Institutes of Health postdoctoral fellowship.

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