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# A virus-induced gene silencing approach to understanding alkaloid metabolism in *Catharanthus roseus*

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ABSTRACT

The anticancer agents vinblastine and vincristine are bisindole alkaloids derived from coupling vindoline and catharanthine, monoterpenoid indole alkaloids produced exclusively by the Madagascar periwinkle (*Catharanthus roseus*). Industrial production of vinblastine and vincristine currently relies on isolation from *C. roseus* leaves, a process that affords these compounds in 0.0003–0.01% yields. Metabolic engineering efforts to either improve alkaloid content or provide alternative sources of the bisindole alkaloids ultimately rely on the isolation and characterization of the genes involved. Several vindoline biosynthetic genes have been isolated, and the cellular and subcellular organization of the corresponding enzymes has been well studied. However, due to the leaf-specific localization of vindoline biosynthesis, and the lack of production of this precursor in cell suspension and hairy root cultures of *C. roseus*, further elucidation of this pathway demands the development of reverse genetics approaches to assay gene function in planta. The bipartite pTRV vector system is a Tobacco Rattle Virus-based virus-induced gene silencing (VIGS) platform that has provided efficient and effective means to assay gene function in diverse plant systems. A VIGS method was developed herein to investigate gene function in *C. roseus* plants using the pTRV vector system. The utility of this approach in understanding gene function in *C. roseus* leaves is demonstrated by silencing known vindoline biosynthetic genes previously characterized in vitro.


## 1. Introduction

The bisindole alkaloids vinblastine **9** and vincristine **10** are produced exclusively by the Madagascar periwinkle (*Catharanthus roseus*) (Fig. 1). These compounds are potent inhibitors of microtubule assembly, and are used extensively to treat a variety of cancers. Although synthetic methods have been developed to produce these valuable natural products, which are derived in planta through the coupling of the terpenoid indole alkaloids (TIA) vindoline **8** and catharanthine **7** (Fig. 1), industrial production still relies on the low yields obtained through extraction and isolation from *C. roseus* leaves (Guéritte and Fahy, 2005). As such, an alternative production method that improves the yields of these costly molecules would be widely beneficial. Metabolic engineering efforts to develop alternative sources do, however, rely heavily on the identification and characterization of the genes and corresponding enzymes responsible for producing these compounds.

Most, if not all, plant TIAs are derived from the central precursor strictosidine, which is ultimately formed from indole-containing tryptamine and the monoterpenoid glycoside secologanin through the catalytic action of strictosidine synthase (O'Connor and Maresh, 2006). A few genes involved in strictosidine biosynthesis have been isolated characterized, but there are several steps that still remain to be elucidated (Facchini and De Luca, 2008). Strictosidine glucosidase hydrolyzes strictosidine to form a reactive aglycone, which can be transformed to form the iboga-type TIA catharanthine **7**, or the vindoline **8** precursor tabersonine **1** (aspidosperma-type; Fig. 1) (O'Connor and Maresh, 2006). Strictosidine glucosidase has been well characterized, but there is essentially nothing known about the downstream enzymes responsible for the formation of tabersonine **1** or catharanthine **7** (Facchini and De Luca, 2008; Scott and Qureshi, 1969). In contrast, genes responsible for all but one step in the conversion of tabersonine **1** to vindoline **8** have been isolated and characterized (Fig. 1) (Facchini and De Luca, 2008; Liscombe et al., 2010). The P450-dependent tabersonine-16-hydroxylase (T16H), installs a hydroxyl group at

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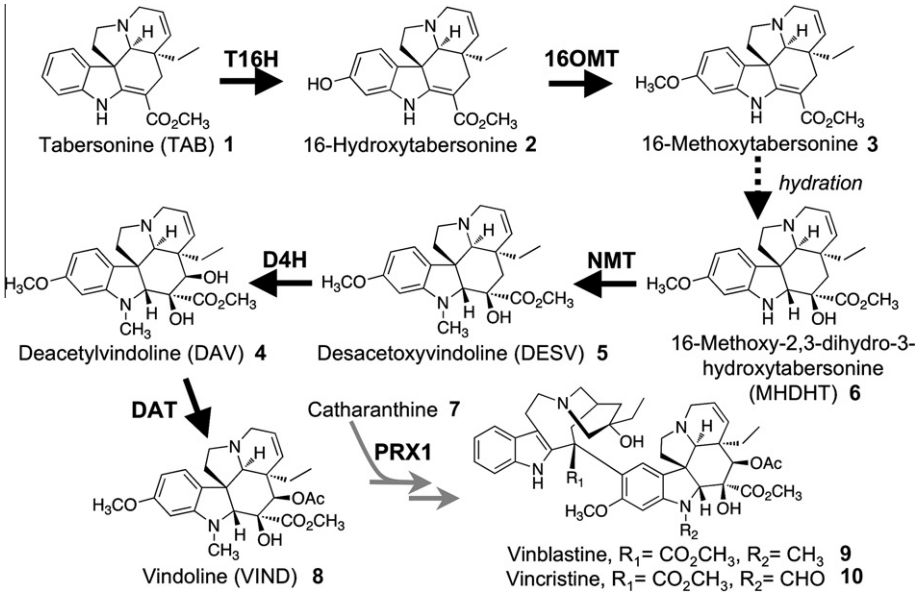


**Fig. 1.** Proposed biosynthesis of vindoline **8** from tabersonine **1** in *C. roseus*, and incorporation of vindoline **8** into the bisindole alkaloids, vinblastine **9** and vincristine **10**. Enzyme names are indicated in bold above solid black arrows. Cognate cDNAs have been isolated for all steps in the conversion of tabersonine **1** to vindoline **8**, except for the so-called hydration step (dotted arrow). Grey arrows indicate multiple enzymatic steps. Abbreviations: T16H, tabersonine 16-hydroxylase; 16OMT, 16-hydroxytabersonine 16-*O*-methyltransferase; NMT, 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline 4-*O*-acetyltransferase; PRX1, anhydrovinblastine synthase.

the 16-position (Schroder et al., 1999), which is subsequently methylated by 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT) (Levac et al., 2008). An as yet undiscovered "hydrating" step results in the hydroxylation of the 3-position, after which methylation of $N^1$ by 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase (NMT) can occur (Liscombe et al., 2010). Finally, a 4-hydroxy group is introduced by desacetoxyvindoline 4-hydroxylase (D4H; Vazquez-Flota et al., 1997), which is then acetylated by deacetylvindoline 4-*O*-acetyltransferase (DAT) to yield vindoline **8** (St.-Pierre et al., 1998). The spatial organization of the pathway has been a primary focus of study in recent years, and it is clear that the biosynthesis of the precursors to the bisindole alkaloids involves several different specialized cell types, and is developmentally-regulated (Facchini and De Luca, 2008; Guirimand et al., 2011; Murata et al., 2008). Although some transcripts, enzymes, and/or activities specific to vindoline **8** biosynthesis have been detected in experimental model systems like hairy root and cell suspension cultures, these tissues do not produce vindoline **8**. As such, seedlings and young leaves of mature plants are essentially the only model systems available to study vindoline **8** biosynthesis (Aerts et al., 1994). Recent cell- and tissue-specific EST sequencing efforts addressing the restricted localization of vindoline **8** biosynthesis in *C. roseus* leaves (Murata et al., 2006, 2008; Shukla et al., 2006) have provided EST collections enriched in alkaloid biosynthetic gene transcripts, thereby facilitating the discovery of novel genes (Levac et al., 2008; Liscombe et al., 2010; Murata et al., 2008). Unfortunately, current reverse genetics and functional genomics approaches that allow one to probe gene function in vivo have been most effective (and sometimes only successful) when applied to hairy root and cell suspension cultures of *C. roseus*. These include RNA interference in hairy root cultures (Runguphan et al., 2009) and cell suspension cultures (Courdavault et al., 2005; Papon et al., 2004) and T-DNA activation tagging in cell suspension cultures (van der Fits and Memelink, 2000). Though effective, these experiments are often cumbersome and can take months or years to complete, and most importantly, they cannot be used to probe vindoline **8** biosynthesis due to the limited metabolism exhibited by these experimental systems. With an abundance of new *C. roseus* sequence data coming available in the near future through large-scale, transcriptome sequencing initiatives (NIH-GO Medicinal Plant Consortium, http://www.medicinalplantgenomics.msu.edu; Genome Canada PhytoMetaSyn, http://www.phytometasyn.ca), there is an urgent need for more efficient methods to screen candidate genes and to validate gene function in planta.

Virus-induced gene silencing (VIGS) is an efficient and effective technique for probing gene function in diverse plant systems. This approach relies on natural plant defense mechanisms to direct degradation of cognate mRNA transcripts of a gene, or gene family, that have been targeted for silencing (Burch-Smith et al., 2004). VIGS has most often been utilized in studies of Solanaceous plants, such as *Nicotiana benthamiana*, which was the primary host for the development of this technique (Liu et al., 2002; Ratcliff et al., 2001; Ruiz et al., 1998). However, a growing number of medicinal plants have also proved amenable to this technique, for example: *Papaver somniferum* (Hileman et al., 2005), *Eschscholzia californica* (Wege et al., 2007), *Thalictrum spp.* (Di Stilio et al., 2010), *Aquilegia* spp. (Gould and Kramer, 2007); and the Solanaceous tropane alkaloid-producer *Hyoscyamus niger* (Li et al., 2006). Herein, the development of a method is reported to utilize the pTRV vector system for VIGS in *C. roseus*. The utility of this VIGS approach in functional analyses of *C. roseus* genes is demonstrated by silencing three known steps in vindoline **8** biosynthesis. The ability to use VIGS as a method to investigate gene function in *C. roseus* should greatly facilitate the discovery and characterization of novel genes that contribute to the rich metabolism of this important medicinal plant.

## 2. Results and discussion

### 2.1. C. roseus is susceptible to VIGS

A report that *C. roseus* was susceptible to experimental infection by Tobacco Rattle Virus (TRV) (ICTVdB, The Universal Virus Database, v.4, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/), prompted us to investigate whether *C. roseus* would be amenable to VIGS using the pTRV vector system (Liu et al., 2002). To develop a method for

VIGS in *C. roseus*, a marker gene that yields a readily visible phenotype upon silencing was required. *Protoporphyrin IX magnesium chelatase subunit H* (*ChlH*) was selected, as silencing of this gene impairs chlorophyll biosynthesis, thereby causing a photobleached phenotype (Hiriart et al., 2002). PCR primers were designed based on a partial open reading frame (ORF) encoding *C. roseus* ChlH [Fig. S1a; 93% amino acid identity to *Antirrhinum majus* ChlH (Hudson et al., 1993)] present in the PlantGDB EST assembly database (plantgdb.org). Two distinct fragments of the *C. roseus ChlH* ORF were amplified by PCR and cloned into pTRV2 to generate pTRV2-*ChlH*(F1R2) and pTRV2-*ChlH*(F3R1) (Fig. S1b). These constructs were used to transform *Agrobacterium tumfaciens* GV3101 by electroporation, and transformants were prepared for use in VIGS experiments as described in the Experimental. Several methods of *Agrobacterium*-mediated infection, were tested namely "Agrodrench" (Ryu et al., 2004), vacuum infiltration (Hileman et al., 2005), syringe infiltration (Ratcliff et al., 2001), and the pinch-wounding method normally used for introducing *A. rhizogenes* into *C. roseus* for generating hairy root cultures (Runguphan et al., 2009). The only method that was consistently successful in our hands was the pinch method, using plants possessing at least one pair of fully-expanded true leaves. Photobleaching occurred in leaves of pTRV2-*ChlH* treated plants as early as 1 week post-inoculation, and was visible in all affected plants between 2 and 3 weeks post-inoculation (Fig. 2a). Photobleaching was never observed in empty pTRV2-treated (EV) plants (Fig. 2b). The silencing effect is transient, in that it is typically restricted to the first two leaf pairs to emerge after inoculation (Fig. 2a), but highly effective (~100% silenced plants, Fig. S1c). In contrast to other methods of introducing *A. tumefaciens* habouring pTRV, which can have a substantial effect on the survival rate of the treated plants (Hileman et al., 2005), the pinch method results in essentially a 100% plant survival rate. Transcript levels of *ChlH* in photobleached leaves of pTRV2-*ChlH*(F3R1)-treated (*ChlH*-vigs) plants were significantly lower than those observed in EV plants ($P < 0.001$; Fig. 2c). Expression of *ChlH* was also significantly lower in EV plants, compared to mock-infiltrated (M) plants ($P < 0.05$). pTRV2-derived TRV coat protein transcripts were detected by qPCR in all photobleached *ChlH*-vigs leaves, and EV plants, but not in mock infiltrated plants (Fig. S2), demonstrating that the pTRV2-infiltrated plants examined were indeed successfully infiltrated. VIGS plants sometimes exhibited a subtle mosaic pattern typical of TRV infection (Ratcliff et al., 2001), but otherwise displayed no visible signs of viral infection.

### 2.2. Effect of the pTRV system and ChlH-silencing on alkaloid biosynthesis

To establish a baseline for the effects of the pTRV vector system, and to determine if it was plausible to co-infiltrate pTRV2-*ChlH* and a pTRV2 construct targeting genes involved in alkaloid biosynthesis, gene expression (Fig. 2c) and alkaloid profiles (Fig. 2d) of VIGS plants were examined. Expression of known alkaloid biosynthetic genes in leaves of EV plants was not significantly affected ($P > 0.05$ for all genes; Fig. 2c). In contrast, transcript levels of *T16H* and *D4H* were significantly reduced in photobleached leaves of *ChlH*-vigs plants ($P < 0.05$ and $P < 0.0001$, respectively Fig. 2c). Light is one of the factors regulating transcription of *T16H* and *D4H* (Schroder et al., 1999; Vazquez-Flota et al., 1997; Vazquez-Flota and De Luca, 1998), and though little is known about the signaling cascades or mechanism(s) governing the regulation of these genes, *ChlH*-silencing and/or the resulting photobleached phenotype might perturb the light-dependent transcriptional activation of these genes. This effect should be taken into account during experimental design, if one is considering co-infiltration of a target gene-silencing construct and pTRV-*ChlH* construct, as a marker to guide harvesting of silenced leaves. While alkaloid composition

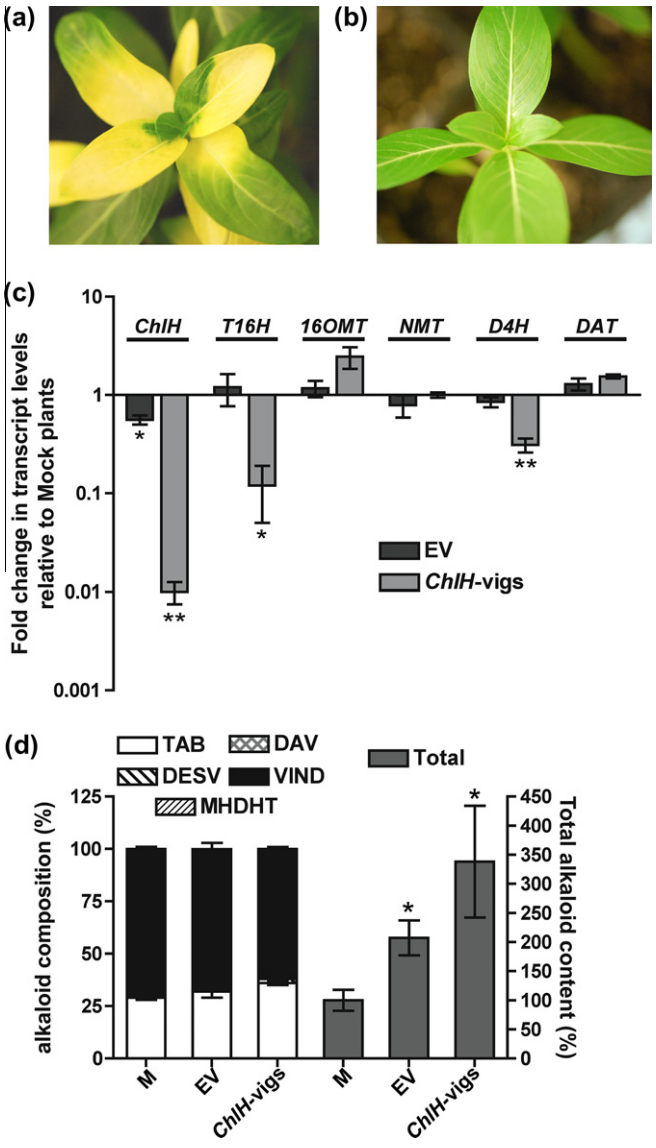


**Fig. 2.** Silencing of protoporphyrin IX magnesium chelatase gene (*ChlH*) as a visual marker for virus-induced gene silencing. (a) Representative *C. roseus* plant 2 weeks after infiltration with pTRV1/pTRV2-*ChlH*(F3R1) (1:1; *ChlH*-vigs) exhibiting photobleached phenotype. (b) Representative *C. roseus* plant 2 weeks after infiltration with pTRV1/empty pTRV2 (1:1; EV). (c) Fold change in *ChlH* and vindoline (VIND) biosynthetic gene transcripts levels in leaves of EV and *ChlH*-vigs plants relative to mock-infiltrated (M) plants, as determined by quantitative real-time PCR. Data represent mean ± SE of at least three technical replicates performed with cDNA prepared from 8 (EV) and 3 (*ChlH*-vigs) individual plants. Asterisks indicate significant differences between EV and mock, or EV and *ChlH*-vigs plants using Student's t-test (*$P < 0.05$; **$P < 0.0001$). (d) Alkaloid composition and total alkaloids in leaves of M, EV, and *ChlH*-vigs plants. Data represent mean ± SE alkaloid composition (as % of total) or total alkaloid content (normalized to M) from 5 (M), 8 (EV) and 3 (*ChlH*-vigs) individual plants. Asterisks indicate significant differences between EV and M, or *ChlH*-vigs and M plants using Student's *t*-test (*$P < 0.05$). Total leaf alkaloid content of from *ChlH*-vigs plants compared to EV plants was not significantly different ($P > 0.05$). Abbreviated alkaloid and biosynthetic gene (enzyme) names are defined in Fig. 1.

was unaffected by the pTRV system in EV plants, a significant accumulation of desacetoxyvindoline **5** (1–3% of total, $P < 0.05$) was observed in photobleached leaves of *ChlH*-vigs plants (Fig. 2d). The accumulation of desacetoxyvindolines **5**, the substrate of D4H, is probably a result of the decreased expression of *D4H* (Fig. 2c). Despite the reduced expression of *T16H* and *D4H*, the total alkaloid content was significantly higher (~3-fold) in photobleached leaves of *ChlH*-vigs plants compared to M plants, but was not significantly

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different from the total alkaloid content observed for EV plants ($P > 0.05$, Fig. 2d). The increased alkaloid content in EV and *ChlH*-vigs plants could perhaps be a response to the *Agrobacterium* infection. Nonetheless, the empty pTRV vector system does not appear to either inactivate alkaloid biosynthesis, or exert aberrant effects on the expression of known alkaloid biosynthetic genes, indicating that VIGS is a suitable methodology to study alkaloid biosynthetic gene function in vivo.

### 2.3. VIGS can be used to investigate vindoline biosynthetic gene function

The isolation and in vitro characterization of a *C. roseus* 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase (NMT; Fig. 1) was recently reported (Liscombe et al., 2010). This gene was selected to test the utility of the pTRV system in probing alkaloid biosynthesis and to confirm the function of NMT in vivo. A 377 bp fragment of the *NMT* ORF was cloned into pTRV2, and the construct (pTRV2-*NMT*) was introduced to *C. roseus* plants in the same manner as described above. *ChlH*-vigs plants were prepared in parallel as an indicator of when leaves should be harvested to examine silencing effects. The two youngest leaf pairs were harvested from *NMT*-vigs plants approximately 2 weeks post-infiltration, once *ChlH*-vigs plants were exhibiting the photobleached phenotype in the equivalent leaf pairs. Alkaloids and RNA were extracted in parallel from the same tissue sample and methanolic alkaloid extracts were analyzed by LC–MS to identify plants exhibiting a perturbed alkaloid profile.

Extracts from M and EV plants showed consistently large peaks corresponding to vindoline 8, while several *NMT*-vigs plants had diminished vindoline 8 peaks, and instead contained a new compound (*m/z* 385) not detected in M or EV plants (Fig. 3a). Two representative chromatograms that highlight the range of chemical phenotypes observed in the set of *NMT*-vigs plants are presented in Fig. 3a. The exact mass of the accumulating compound was determined by high-resolution mass spectrometry analysis, and was consistent with 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** ($C_{22}H_{28}N_2O_4$, calculated *m/z* 385.2122, observed *m/z* 385.2134), the substrate of NMT (Figs. 1, 3a). Quantitative real-time PCR analysis (qPCR) was employed to measure *NMT* expression in VIGS plants relative to M plants (Fig. 3b). In addition, successful infiltration of the *NMT*-vigs plants examined was confirmed by detection of pTRV2-derived TRV coat protein transcript using qPCR (Fig. S2). Transcript levels of *NMT* were found to be significantly lower (21 ± 5%, $P < 0.05$) in *NMT*-vigs plants compared to EV plants (79 ± 20%, Fig. 3b). These results strongly suggest that the accumulation of the NMT substrate 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** is a direct result of the VIGS-mediated reduction in *NMT* transcripts. While the total alkaloid content of *NMT*-vigs leaves (227% of

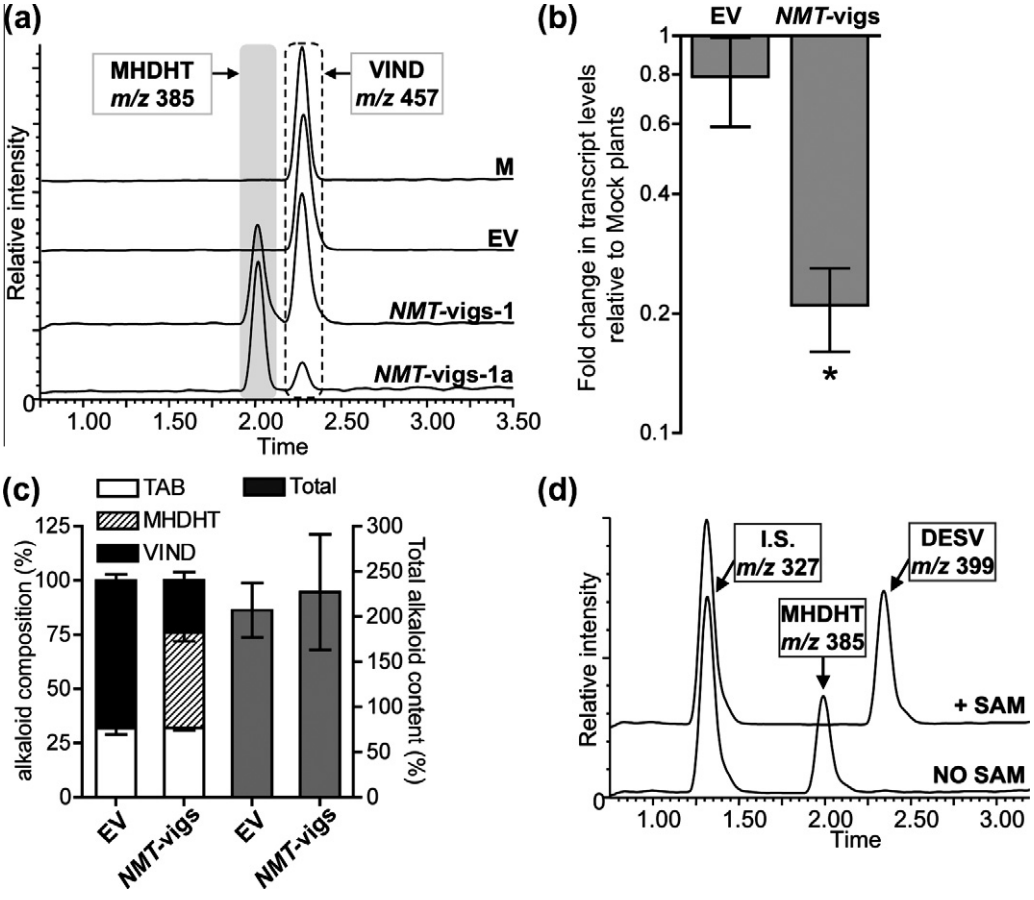


**Fig. 3.** Virus-induced silencing of 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase (*NMT*) transcripts in *C. roseus*. (a) Selected ion chromatograms (*m/z* 385 + 457) showing relative vindoline **8** (VIND, *m/z* 457) and 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** (MHDHT, *m/z* 385) in mock infected (M), empty vector infected (EV), and least (*NMT*-vigs-1) and most (*NMT*-vigs-1a) extreme phenotypes observed for pTRV2-*NMT* infected plants. (b) Relative expression of *NMT* in EV and *NMT*-vigs plants compared to M plants, measured by quantitative real-time PCR. Data represent mean ± SE of at least three technical replicates performed with cDNA prepared from 8 (EV) and 7 (*NMT*-vigs) individual plants. Asterisk indicates significant difference between *NMT*-vigs and EV plants using Student's *t*-test ($P < 0.05$). (c) Mean alkaloid composition and total alkaloid content (relative to M) in EV and *NMT*-vigs plants. Data represent mean ± SE alkaloid composition (as % of total) or total alkaloid content (normalized to M) from 8 (EV) and 7 (*NMT*-vigs) individual plants. (d) Selected ion chromatogram (*m/z* 327 + 385 + 399) of in vitro rescue assay demonstrating the *S*-adenosyl-L-methionine (SAM)-dependent conversion of MHDHT (*m/z* 385) accumulating in *NMT*-vigs plants to desacetoxyvindoline (DESV, *m/z* 399) by recombinant NMT. Abbreviated alkaloid names are defined in Fig. 1; I.S., ajmaline internal standard (*m/z* 327).

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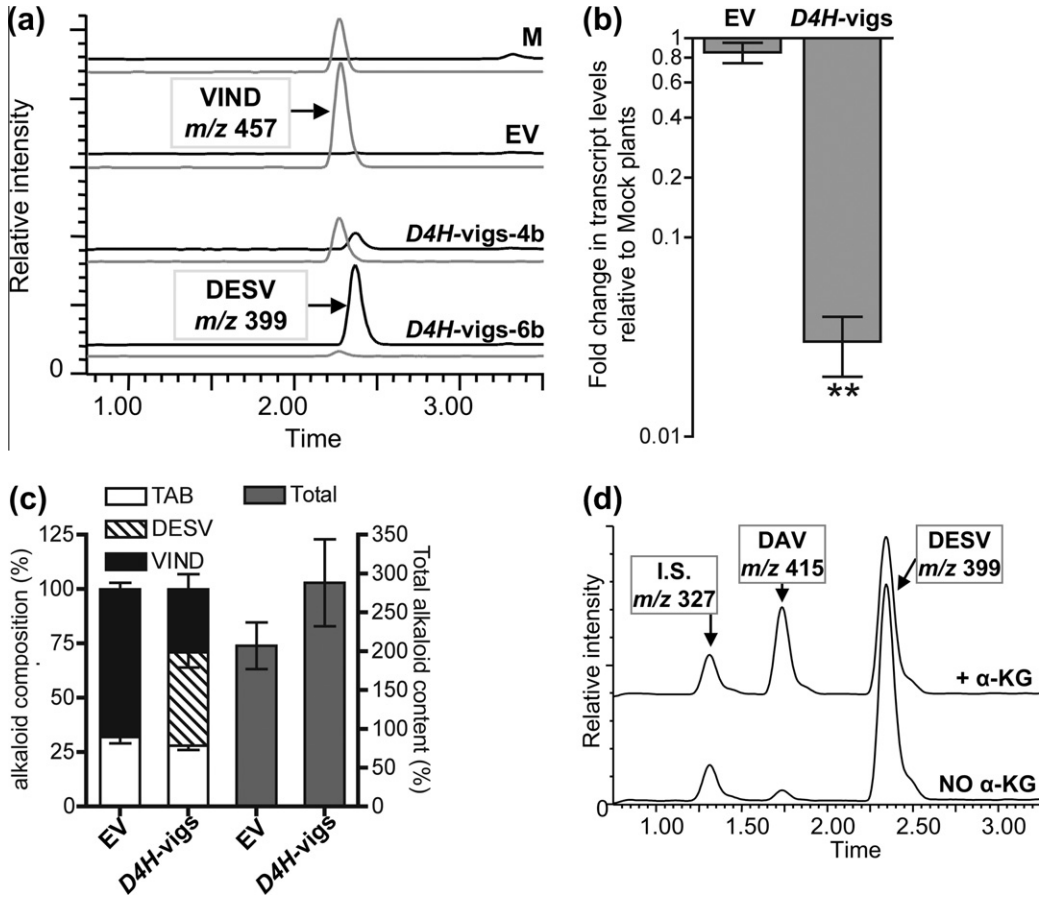


**Fig. 4.** Virus-induced silencing of desacetoxyvindoline 4-hydroxylase (*D4H*) transcripts in *C. roseus*. (a) Selected ion chromatograms (*m/z* 399 and *m/z* 457) showing relative vindoline **8** (VIND, *m/z* 457, grey trace) and desacetoxyvindoline **5** (DESV *m/z* 399, black trace) in mock infected (M), empty vector infected (EV), and least (*D4H*-vigs-4b) and most (*D4H*-vigs-6b) extreme phenotypes observed for pTRV2-*D4H* infected plants. (b) Relative expression of *D4H* in EV and *D4H*-vigs plants compared to M plants, measured by quantitative real-time PCR. Data represent mean ± SE of at least three technical replicates performed with cDNA prepared from 8 (EV) and 7 (*D4H*-vigs) individual plants. Asterisks indicate significant difference between *D4H*-vigs and EV plants using Student's t-test ($P < 0.0001$). (c) Mean alkaloid composition and total alkaloid content (relative to M) in EV and *D4H*-vigs plants. Data represent mean ± SE alkaloid composition (as % of total) or total alkaloid content (normalized to M) from 8 (EV) and 7 (*D4H*-vigs) individual plants. (d) Selected ion chromatogram (*m/z* 327 + 399 + 415) of in vitro D4H rescue assay demonstrating the α-ketoglutarate (α-KG)-dependent conversion of DESV (*m/z* 399) accumulating in *D4H*-vigs plants to deacetylvindoline **4** (DAV, *m/z* 415) using crude, soluble protein extract from young leaves of wild type *C. roseus* plants. Abbreviated alkaloid names are defined in Fig. 1; I.S., ajmaline internal standard (*m/z* 327).

M) was not significantly different from EV (207% of M; $P > 0.05$; Fig. 3c), the vindoline **8** content of *NMT*-vigs plants was significantly lower (24% of total alkaloid; $P < 0.001$) than that observed for EV plants (68% of total; Fig. 3c). Moreover, tabersonine **1** content was identical (32% of total) for leaves from both EV and *NMT*-vigs plants (Fig. 3c). The accumulation of 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** (44% of total) at the expense of vindoline **8** in *NMT*-vigs plants confirms the role of NMT as an upstream enzyme in vindoline **8** biosynthesis, with 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** as the substrate, which is consistent with the current model of the pathway (Facchini and De Luca, 2008; Liscombe et al., 2010). To validate that it was indeed a reduction in NMT activity that led to 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** accumulation in *NMT*-vigs plants, we performed in vitro rescue assays were performed with recombinant NMT and alkaloid extract from *NMT*-vigs plants as substrate (Fig. 3d). In the absence of SAM, no turnover of 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** to desacetoxyvindoline **5**, the expected product of the NMT catalyzed reaction (Fig. 1), was observed. However, when SAM was included in the assay, 16-methoxy-2,3-dihydro-3-hydroxytabersonine **6** was completely converted to a product exhibiting a retention time and *m/z* 399 consistent with desacetoxyvindoline **5** (Fig. 3d) (Liscombe et al., 2010).

Two other biosynthetic genes previously characterized in vitro, *D4H* and *DAT* (St-Pierre et al., 1998; Vazquez-Flota et al., 1997), were then targeted to determine if additional genes of vindoline **8** biosynthesis could also be silenced using VIGS. A 400 bp fragment of the D4H ORF and a 423 bp fragment of the *DAT* ORF were cloned into pTRV2 to generate pTRV2-*D4H* and pTRV-*DAT*, respectively. *D4H* and *DAT* VIGS experiments were set up as described above for *NMT* silencing, using *A. tumefaciens* GV3101 harboring the pTRV2-*D4H* or pTRV-*DAT* construct. The two youngest leaf pairs were harvested from VIGS plants approximately 2 weeks post-inoculation. Harvest time was guided by the appearance of photobleaching of the corresponding leaves in *ChlH*-vigs positive control plants. Gene expression and alkaloid profiling was performed and compared to those data for M and EV plants (Fig. 2). Successful infiltration of the VIGS plants examined was confirmed by detection of pTRV2-derived TRV coat protein transcript using qPCR (Fig. S2).

As observed for *NMT*-vigs samples, leaves from several *D4H*-vigs plants had diminished vindoline **8** peaks, but in this case accumulated a unique compound (*m/z* 399) not detected in M or EV plants (Fig. 4a). The extremes of *D4H*-vigs alkaloid phenotypes observed in this study are also presented in Fig. 4a. The exact mass of the accumulating compound was determined by high-resolution mass spectrometry analysis, and was consistent with

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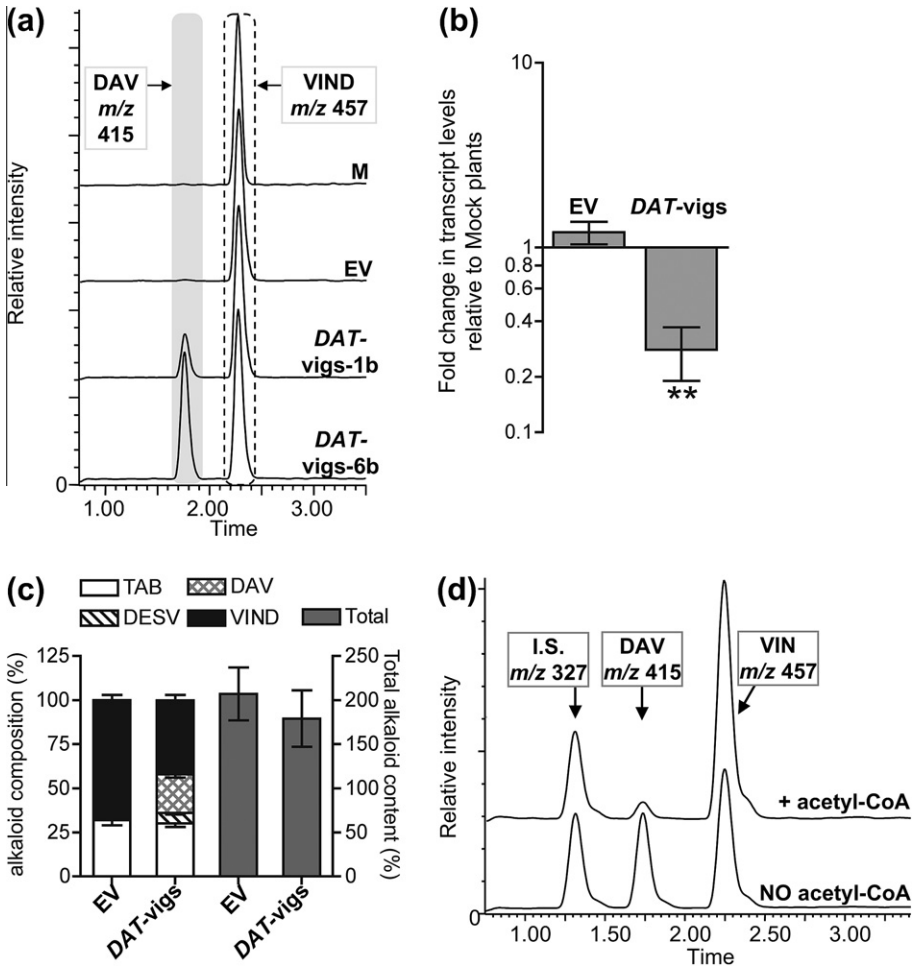


**Fig. 5.** Virus-induced silencing of deacetylvindoline 4-*O*-acetyltransferase (*DAT*) transcripts in *C. roseus*. (a) Selected ion chromatograms (*m/z* 415 + 457) showing relative vindoline **8** (VIND, *m/z* 457) and deacetylvindoline **4** (DAV, *m/z* 415) in mock infected (M), empty vector infected (EV), and least (*DAT*-vigs-1b) and most (*DAT*-vigs-6b) extreme phenotypes observed for *DAT*-silenced plants. (b) Relative expression of *DAT* in EV and *DAT*-vigs plants compared to M plants, measured by quantitative real-time PCR. Data represent mean ± SE of at least three technical replicates performed with cDNA prepared from 8 (EV) and 7 (*DAT*-vigs) individual plants. Asterisks indicate significant difference between *DAT*-vigs and EV plants using Student's t-test ($P < 0.001$). (c) Mean alkaloid composition and total alkaloid content (relative to M) in empty vector and *DAT*-vigs plants. Data represent mean ± SE alkaloid composition (as % of total) or total alkaloid content (normalized to M) from 8 (EV) and 7 (*DAT*-vigs) individual plants. (d) Selected ion chromatogram (*m/z* 327 + 415 + 457) of in vitro DAT rescue assays demonstrating the acetyl-CoA-dependent conversion of DAV (*m/z* 415) accumulating in *DAT*-vigs plants to VIND (*m/z* 415) using crude, soluble protein extract from methyl jasmonate-elicited *C. roseus* seedlings. Abbreviated alkaloid names are defined in Fig. 1; I.S., ajmaline internal standard (*m/z* 327).

desacetoxyvindoline **5** ($C_{23}H_{30}N_2O_4$, calculated *m/z* 399.2278, observed *m/z* 399.2273), the substrate of D4H (Figs. 1, 4a). Quantification of *D4H* transcripts indicated that *D4H* expression was nearly abolished in *D4H*-vigs leaves (3 ± 1%, $P < 0.0001$) compared to EV plants (91 ± 10%, Fig. 4b). These data strongly support a VIGS-mediated accumulation of the D4H substrate, desacetoxyvindoline **5**, which represented 43% of the total alkaloid content (Fig. 4c). Again, while total alkaloid content was unaffected ($P > 0.05$), vindoline **8** content was significantly lower in *D4H*-vigs plants (29% of total; $P < 0.005$) compared to EV plants (Fig. 4c). Moreover, in vitro rescue assays showed that it was indeed a VIGS-mediated reduction in D4H activity that caused an accumulation of desacetoxyvindoline **5** (Fig. 4d). When the α-ketoglutarate (α-KG) cofactor was included in an enzyme assay using protein extract from young leaves of wild-type *C. roseus* plants and alkaloid extract from *D4H*-vigs plants, a large increase in a product exhibiting a retention time and *m/z* 415 consistent with deacetylvindoline **4** is observed (Fig. 4d). In contrast, this large accumulation of deacetylvindoline **4** was not observed in the absence of α-KG.

LC–MS analysis of alkaloid extracts from leaves of *DAT*-vigs plants still contained a substantial amount of vindoline **8**, but consistently accumulated a less hydrophobic compound of *m/z* 415 (Fig. 5a). High-resolution mass spectrometry analysis showed that the exact mass of this compound was consistent with deacetylvindoline **4** ($C_{23}H_{30}N_2O_5$, calculated *m/z* 415.2227, observed *m/z* 415.2236), the substrate of DAT (Figs. 1, 5a). Concomitantly, transcript levels of *DAT* were significantly lower in leaves from *DAT*-vigs plants (28 ± 9%, $P < 0.001$) compared to leaves from EV plants (121 ± 17%), indicating that a VIGS-mediated reduction in *DAT* expression ultimately caused an accumulation of its substrate. Quantification of leaf alkaloids present in *DAT*-vigs plants showed that, as with *NMT*- and *D4H*-vigs plants, total alkaloid content (179 ± 32% of M) did not differ significantly from that observed in EV plants (207 ± 30% of M, $P > 0.05$; Fig. 5c). However, vindoline **8** content was significantly reduced as a result of *DAT* silencing (42 ± 3% of total, $P < 0.0001$), compared to EV plants (68 ± 3% of total; Fig. 5c). The lost vindoline **8** content was replaced by the biosynthetic precursors deacetylvindoline **4** (22% of total) and desacetoxyvindoline **5** (6% of total). The deacetylvindoline **4**-accumulating phenotype of *DAT*-vigs leaves could be rescued in vitro using protein extract from methyl jasmonate-elicited *C. roseus* seedlings as a source of DAT (Fig. 5d). Deacetylvindoline **4** in

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alkaloid extracts from *DAT*-vigs plants was converted to vindoline **8** when the cofactor acetyl-CoA was included in the assays, whereas no conversion was observed in the absence of acetyl-CoA (Fig. 5d). Notably, a substantial amount of deacetylvindoline **4** appeared to remain in DAT rescue assays with elicited seedling protein extract and acetyl-CoA (Fig. 5d). While this could represent incomplete turnover of deacetylvindoline **4** by DAT in the assay, a prominent deacetylvindoline **4** peak was also present in LC–MS chromatograms when alkaloid extracts of *D4H*-vigs leaves were incubated with leaf protein extract in the absence of α-KG (Fig. 4d). In consideration of these data, it is suggested that these unexpected deacetylvindoline **4** peaks are artifacts of hydrolysis of the 4-*O*-acetyl group of vindoline **8** by a nonspecific acetylesterase previously detected in *C. roseus* plants (Fahn et al., 1985).

Notably, vindoline **8** accumulated to some degree in all plants when *NMT*, *D4H* or *DAT* vindoline **8** biosynthetic genes were effectively knocked-down (Figs. 3–5). This could be due to the presence of redundant genes, but at least *D4H* and *DAT* are known to be single copy genes (St-Pierre et al., 1998; Vazquez-Flota et al., 1997). Most likely however, vindoline **8** arises in these plants from areas of the examined leaves not affected by VIGS, and therefore maintaining full biosynthetic capacity. Another possible explanation could be that vindoline **8** observed in these plants accumulated before silencing effects took over, as vindoline **8** biosynthetic genes are known to be highly expressed early in leaf development for the rapid synthesis of vindoline **8** (Levac et al., 2008; St-Pierre et al., 1998; Vazquez-Flota et al., 1997). Nonetheless, gene expression analysis showed that the levels of transcripts targeted for VIGS were indeed reduced (Figs. 3b, 4b, 5b), and in each case, gene silencing was accompanied by marked perturbations of alkaloid profiles (Figs. 3c, 4c, 5c).

### 2.4. Concluding remarks

A VIGS method was developed to assay gene function in *C. roseus*, and it was demonstrated how this approach can be used to acces components of the vindoline **8** biosynthetic pathway. In the present study, VIGS was used to confirm the in vivo function of vindoline **8** biosynthetic genes previously characterized in vitro. However, it is expected that VIGS will also be an efficient way to probe the function of uncharacterized genes, thereby facilitating screening of candidate genes from *C. roseus* sequencing efforts. VIGS should be a particularly useful approach for gene discovery because, in contrast to characterization in vitro, one can also assay the function of candidate transcripts where only a part of the coding sequence is known. While our primary goal in undertaking this work was to develop a functional assay of vindoline **8** biosynthesis in leaves, it has yet to be determined whether VIGS will allow functional analysis of genes located elsewhere, such as root tissues. Moreover, biosynthetic genes involved in the production of metabolites that are readily transported throughout the plant might also be difficult targets. Metabolic perturbations in areas affected by VIGS could be masked by the accumulation of compounds transported from non-silenced areas that still maintain their biosynthetic capacity. It is anticipated that the ability to use the VIGS technique in *C. roseus* will advance our understanding of the rich metabolism in the leaves of this important medicinal plant.

## 3. Experimental

### 3.1. Plant growth

*C. roseus* cv. 'Little Bright Eyes' seeds were obtained from B and T World Seeds (http://www.b-and-t-world-seeds.com). Seeds were germinated and plants were cultivated in Miracle-Gro Potting Mix (Scotts; https://www.scotts.com) on shelves in our laboratory, under fluorescent lights on a 12 h photoperiod.

### 3.2. Chemicals

Tabersonine **1** was generously provided by Viresh Rawal (University of Chicago, Chicago). Unless otherwise noted, all other chemicals were purchased from Sigma–Aldrich.

### 3.3. Cloning and construction of VIGS constructs

VIGS vectors, pTRV1 and pTRV2-MCS, were obtained from The Arabidopsis Biological Resource Center (https://www.arabidopsis.org). The *ChlH* sequence was identified in the *C. roseus* EST assemblies at plantgdb.org (CrPUT-9364). All oligonucleotide primers used for cloning are listed in Table S1. Fragments of open reading frames encoding *C. roseus ChlH* (F1R2, 564 bp; F3R1, 499 bp), NMT (377 bp), D4H (400 bp), and DAT (423 bp) were amplified with High Fidelity Taq polymerase (Invitrogen) from methyl jasmonate-elicited seedling cDNA prepared as described by Liscombe et al. (2010). PCR amplicons were cloned into the multiple cloning site of pTRV2-MCS using the restriction sites engineered into the primers. The resulting pTRV2 constructs were sequenced using the pTRV2-seq1 primer (Table S1).

### 3.4. Preparation of agrobacterium

*Agrobacterium tumefaciens* GV3101 for VIGS was prepared according to Hileman et al. (2005) with a few modifications. Electrocompetent *Agrobacterium* was transformed with pTRV1, empty pTRV2 vector (EV), or pTRV2 containing a fragment of the target gene for silencing. Single colonies were used to inoculate 1 ml Luria Broth (LB) overnight cultures supplemented with 50 μg $ml^{-1}$ kanamycin. Transformants were screened by PCR with the gene-specific primers originally used to amplify the gene fragments (Table S1). Positive transformants were used to inoculate 5 ml LB [containing 10 mM MES (2-[*N*-morpholino]ethanesulfonic acid) and 20 μM acetosyringone], which were grown overnight at 28 °C. *These* cultures were pelleted at 5000*g* and resuspended in inoculation solution (10 mm MES, 200 μM acetosyringone, 10 mm $MgCl_2$) to an absorbance at 600 nm ($OD_{600}$) of 1.5. *Agrobacterium* resuspended in inoculation solution was incubated at 28 °C with shaking for 2.5 h. Strains harboring pTRV2 constructs were then mixed 1:1 with pTRV1 culture, and used to inoculate the plants.

### 3.5. Agrobacterium-mediated introduction of VIGS vectors into C. roseus

*C. roseus* seedlings or young plants were inoculated in the same manner as described previously by Runguphan et al. (2009), where modified fine forceps possessing loops at the tips are dipped in *Agrobacterium* cultures and used to pinch the stem, just below the apical meristem and the youngest leaf pair. Plants used for VIGS had at least one pair of fully-expanded true leaves.

### 3.6. Tissue harvest

Single leaves or paired leaves from the first two leaf pairs to emerge following inoculation were harvested separately, weighed, packaged in aluminum foil, flash-frozen in liquid nitrogen, then stored at −80 °C until extraction.

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### 3.7. Parallel RNA and alkaloid extraction

To obtain gene expression and alkaloid profiles from the same sample, a single leaf or leaf pair (20–150 mg) was ground to a fine powder under liq. $N_2$ with sand (40 mg) and the ground tissue split equally. RNA was extracted from half of the ground tissue using an RNeasy Plant Mini Kit following the manufacturer's instructions, while alkaloids were extracted from the remaining powdered tissue with HPLC-grade MeOH (1 ml) containing 25 μM ajmaline (internal standard). Alkaloid extracts were then vortexed, centrifuged at 17,000*g* for 10 min, filtered through a 0.2 μm syringe filter, and diluted at least 1:500 in HPLC-grade MeOH, before analysis of 2–7 μl by LC–MS.

### 3.8. Liquid chromatography–mass spectrometry (LC–MS)

Alkaloid profiles were analyzed as described previously (Liscombe et al., 2010). Total alkaloid content was defined as the sum of tabersonine **1** and vindoline **8** measurements in each sample, as these are typically the most abundant alkaloids in the leaves of *C. roseus* plants used in this study, unless another intermediate had accumulated due to VIGS-mediated alkaloid biosynthetic gene silencing. vindoline **8** and tabersonine **1** peaks were identified by co-elution with authentic standards. Relative alkaloid quantitation was achieved by integrating chromatogram peaks, dividing by internal standard (ajmaline, *m/z* 327) peak area, and normalizing per gram fresh weight extracted.

### 3.9. Quantitative real-time PCR (qPCR)

Gene expression analysis by qPCR was performed according to Liscombe et al. (2010), using the primers described in Table S1. TRV coat protein transcript levels were measured according to Rotenberg et al. (2006).

### 3.10. Rescue assays

NMT assays were performed with methanolic alkaloid extract (5 μl) from plant 'NMT2270#1' as a substrate and recombinant NMT as previously described (Liscombe et al., 2010). Rescue assays for *D4H*-silenced plants were performed using crude soluble protein extract from young leaves from wild-type *C. roseus* plants (1 g FW) as a source of D4H, and methanolic alkaloid extract (5 μl) from plant 'D4H_sept30_2b' as substrate, according to (Vazquez-Flota et al., 1997). DAT assays were carried out essentially as described by St-Pierre et al. (1998), using crude soluble protein extract from methyl jasmonate-elicited *C. roseus* seedlings (Liscombe et al., 2010) as a source of DAT, and methanolic alkaloid extract (5 μl) from plant 'DAT_sept15_2b' as a substrate. All enzyme assays were analyzed by LC–MS.

### 3.11. Accession numbers

Sequence data from this article can be found in the GenBank/EMBL/DDBJ data libraries under the Accession Number HQ60 8936 (*C. roseus ChlH*).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.07.001.

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