Two Tabersonine 6,7-Epoxidases Initiate Lochnericine-Derived Alkaloid Biosynthesis in *Catharanthus roseus*1

Inês Carqueijeiro,a Stephanie Brown,b Khoa Chung,b Thu-Thuy Dang,b Manish Walia,a, Sébastien Besseau,a Thomas Dugé de Bernonville,a Audrey Oudin,a Arnaud Lanoue,a Kevin Billet,a Thibaut Munsch,a Konstantinos Koudounas,a Céline Melin,a Charlotte Godon,a,4 Bienvenue Razafimandimby,d Johan-Owen de Craene,a Gaëlle Glévarec,a Jillian Marc,a Nathalie Giglioli-Guivarc’h,b Marc Clastre,a Benoît St-Pierre,a Nicolas Papon,d Rodrigo B. Andrade,a Sarah E. O’Connor,b,2 and Vincent Courdavaulta,2

aUniversité de Tours, EA2106 Biomolécules et Biotechnologies Végétales, Tours, F-37200, France
bJohn Innes Centre, Department of Biological Chemistry, Norwich NR4 7UH, United Kingdom
cDepartment of Chemistry, Temple University, Philadelphia, Pennsylvania 19122, USA
dUniversité d’Angers, EA3142 Groupe d’Étude des Interactions Hôte-Pathogène, Angers, F-49933, France

Lochnericine is a major monoterpenoid indole alkaloid (MIA) in the roots of Madagascar periwinkle (*Catharanthus roseus*). Lochnericine is derived from the stereoselective C6,7-epoxidation of tabersonine and can be metabolized further to generate other complex MIAs. While the enzymes responsible for its downstream modifications have been characterized, those involved in lochnericine biosynthesis remain unknown. By combining gene correlation studies, functional assays, and transient gene expression analyses, we showed that the expression profiles of two 6,7-epoxidases (TEX1 and TEX2) are spatially separated in the plant. These results lay the groundwork for the metabolic engineering of tabersonine/lochnericine biosynthesis throughout the plant, as supported by the presence of lochnericine in flowers.

Biochemical characterization of TEX1/2 revealed their strict substrate specificity for tabersonine and their inability to epoxidize 16-hydroxylase, involved in vindoline biosynthesis in leaves. While the enzymes responsible for its downstream modifications have been characterized, those involved in lochnericine biosynthesis remain unknown. By combining gene correlation studies, functional assays, and transient gene expression analyses, we showed that the expression profiles of two 6,7-epoxidases (TEX1 and TEX2) are spatially separated in the plant. These results lay the groundwork for the metabolic engineering of tabersonine/lochnericine biosynthesis throughout the plant, as supported by the presence of lochnericine in flowers.

Monoterpenoid indole alkaloids (MIAs) are specialized plant metabolites characterized by potent biological activities, which place these compounds at the heart of defense systems that several Apocynaceae, Rubiaceae, Nyssaceae, and Loganiaceae species have evolved to combat bioaggressors (Luijendijk et al., 1996; Roepke et al., 2010; St-Pierre et al., 2013). For instance, *Catharanthus roseus* (Madagascar periwinkle) accumulates almost 130 distinct MIAs that collectively prevent organ consumption or result in herbivorous intoxication or death upon ingestion (Meisner et al., 1981; van der Heijden et al., 2004; Guirimand et al., 2010; Dugé de Bernonville et al., 2017). These poisonous properties also grant MIAs a prominent position in human pharmacopeia, such as the antimalarial quinine from *Cinchona officinalis*, the antiarrhythmic ajmaline synthesized in *Rauwolfia serpentina*, or the anticancer vinblastine and vincristine produced in *C. roseus*.

For decades, the high value of MIAs has stimulated the elucidation of their biosynthetic pathways, now opening new perspectives toward the engineered production of these compounds in heterologous organisms through gene transfers (Miettinen et al., 2014; Brown et al., 2015; Qu et al., 2015). Besides offering a new type of sourcing for MIAs, these studies resulted in a
better understanding of their metabolism in the aforementioned plants and especially in *C. roseus*, considered a model nonmodel system (Facchinelli and De Luca, 2008; Courdavault et al., 2014; Dugé de Bernonville et al., 2015a). Almost all MIAs derive from strictosidine, which undergoes many modifications to generate the different classes of MIAs, including corynanthe (e.g. ajmalicine), iboga (catharanthine), and aspidosperma (tabersonine; O’Connor and Maresh, 2006; Szabó, 2008). Initially, deglucosylation catalyzed by a dedicated glucosidase, strictosidine glucosidase, is required, which is then followed by distinct enzymes that determine the metabolic fluxes toward the synthesis of the different MIA classes (Geerlings et al., 2000). For instance, four medium-chain dehydrogenases/reductases including tetrahydroalstonine synthases and heteroyohimbine synthase have been associated with the formation of the corynanthe MIAs (Stavrínides et al., 2015, 2016). In addition, geissoschizine synthase, geissoschizine oxidase (CYP71D1V1), and four other enzymes have been shown to ensure the synthesis of iboga and aspidosperma MIAs through preakuanmimcine and stemmadenine formation (Tatsis et al., 2017; Qu et al., 2018).

More recently, the two missing enzymes involved in the biosynthesis of both catharanthine and tabersonine have been identified, hence completing the characterization of this highly studied biosynthetic pathway (Caputi et al., 2018).

In *C. roseus*, the metabolism of tabersonine is arguably one of the most interesting, due to the diversity of the resulting MIA end products and because of its organ-dependent organization. In leaves, tabersonine is mostly converted into vindoline, a highly abundant MIA and the precursor of vinblastine/vincristine (Westekemper et al., 1980). This conversion occurs via a seven-step biosynthetic pathway beginning with a C16-hydroxylation catalyzed by tabersonine 16-hydroxylase (T16H2; CYP71D351) and ending with acetylation (St-Pierre et al., 1998; Besseau et al., 2013; Qu et al., 2015). In roots, tabersonine mainly enters the biosynthetic pathway of hörhammericine and echitoveneine, two MIAs only differing by epoxidation or reduction at C6, C7 (Fig. 1). Encompassing only four distinct enzymes, the biosynthetic pathway of these MIAs is simpler than that of vindoline but also involves hydroxylation catalyzed by T19H followed by acetylation of the resulting hydroxyl (O’Connor and Maresh, 2006; Giddings et al., 2011). This last reaction was attributed initially to minovincine 19-O-acetyltransferase, but recent evidence suggested that acetylation is performed by the tabersonine derivative TAT (Laflamme et al., 2001; Carqueijeiro et al., 2018). Surprisingly, the two enzymes catalyzing modifications at C6, C7 still remain unknown, although 6,7-epoxidation activity has been associated with a cytochrome P450 (Rodriguez et al., 2003). While reduction of the 6,7-alkene gives rise to vincadifformine, epoxidation leads to the synthesis of lochnericine, named after a former name of *C. roseus*, *Lochnera rosea* (Gorman et al., 1959; Nair and Pillay, 1959). Interestingly, lochnericine and its hydroxylated form hörhammericine constitute some of the main MIAs accumulated in *C. roseus* roots and hairy roots, usually explaining why the engineering of vindoline synthesis in hairy roots so far failed due to competition for tabersonine supply (Rodriguez et al., 2003; Peebles et al., 2009; Sun et al., 2017; Carqueijeiro et al., 2018). Furthermore, this lack of characterization also impedes the ordering of the enzymatic steps in the pathway, and the requirement of a specific enzyme sequence still remains an open question.

In our continuing effort to fill the gaps in MIA biosynthetic pathways, we focused on the missing steps of root MIA biosynthesis in *C. roseus*. In this work, we describe the identification of two tabersonine 6,7-epoxidase isoforms displaying marked substrate specificity and a complementary gene expression profile. This gene discovery also allowed engineering of the synthesis of root MIA in yeast by expanding the enzymatic toolbox, which enables controlled modifications of the valuable tabersonine framework.

RESULTS

Identification of Tabersonine 6,7-Epoxidase Candidates through Gene Correlation Analysis

To initiate the identification of tabersonine 6,7-epoxidase candidate genes, we conducted a coexpression analysis of publicly available *C. roseus* RNA sequencing data, including the CDF97 consensus transcriptome and the transcriptomic resource generated through the interaction between *Manduca sexta* and *C. roseus* (Góngora-Castillo et al., 2012; Van Moerkercke et al., 2013; Xiao et al., 2013; Dugé de Bernonville et al., 2015b, 2017). Due to its closest position in the pathway, T19H was used as bait for a Spearman correlation study that allowed the retrieval of 1,526 genes at a 0.6 threshold (Supplemental Table S1). Based on previous experiments potentially associating tabersonine 6,7-epoxidase activity to P450s, we first focused on the 49 sequences encoding members of this large protein family (Rodriguez et al., 2003).

Since all the known P450s metabolizing tabersonine belong to the CYP71 clan, we hypothesized that tabersonine 6,7-epoxidase should be one of the 29 CYP71 members in our gene candidate list (Supplemental Table S1). Within this group, we next focused on the identification of paralogues of T16H2/T16H1 and T19H, two P450s known to modify tabersonine. Interestingly, the T16H paralogues included seven sequences, while only one paralogue of T19H was identified (Fig. 2A). All these candidates displayed an overall gene expression profile similar to T19H, notably characterized by a high expression in hairy roots and methyl jasmonate (MeJA) responsiveness (Fig. 2B), reflecting their high correlation with T19H (Fig. 2C). Sequence alignments also revealed that the seven T16H-like sequences correspond to four distinct genes in the *C. roseus* genome (CRO_T000497, CRO_T013902, CRO_T019212, and
Figure 1. Main routes of tabersonine metabolism in *C. roseus*. Solid and dashed arrows represent characterized and uncharacterized putative reactions, respectively. DAT, Deacetylvindoline 4-O-acetyltransferase; TAT, tabersonine derivative 19-O-acetyltransferase; T19H, tabersonine 19-hydroxylase. The positions of the carbon atoms subjected to modification are numbered. Note that the convention of carbon numbering shown here is not consistent with IUPAC nomenclature but reflects historical versions of numbering found in the enzymatic literature.
CRO_T027852; Supplemental Table S2), exhibiting an overall identity of around 65%, except for CRO_T019212 and CRO_T013902, which were 86% identical (Supplemental Table S3). The T19H-like sequence (CRO_T02923) was 73% identical to T19H. Finally, all five deduced protein sequences bear conserved P450 motifs (Supplemental Fig. S1) and were tested for tabersonine 6,7-epoxidase activity.

**CRO_T019212 and CRO_T013902 Encode Two Functional Tabersonine 6,7-Epoxidases**

To assay for tabersonine 6,7-epoxidase activity, the coding sequences of the four T16H-like genes (CRO_T000497, CRO_T013902, CRO_T019212, and CRO_T027852) and the T19H-like gene (CRO_T02923) were expressed in yeast by cloning their coding sequences into autoreplicative plasmids bearing inducible promoters. The transformed yeast strains were cultured and induced with Gal before feeding with tabersonine (125 µM) for 24 h, followed by analysis of the resulting products by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS; Fig. 3A).

While the two control yeast strains expressing T16H2 and T19H performed the expected site-specific hydroxylations of tabersonine, no conversion was observed when CRO_T027852, CRO_T000497, and CRO_T002923 were expressed, suggesting that the encoded proteins do not use tabersonine as a starting substrate. By contrast, a substantial consumption of tabersonine was observed when CRO_T019212 and CRO_T013902 were expressed, and this was accompanied by the formation of compounds with a similar mass-to-charge ratio (m/z) 353 but eluted at a higher retention time.
Interestingly, these compounds coeluted with lochnericine and displayed similar fragmentation patterns, suggesting that both enzymes synthesized lochnericine (Fig. 3A; Supplemental Fig. S2). To further identify the nature of the modification catalyzed by these proteins, the CRO_T019212 enzymatic product from a milligram-scale reaction was purified by reverse-phase HPLC and analyzed by NMR spectroscopy (Fig. 3B; Supplemental Fig. S3). The proton and carbon chemical shifts of the enzymatic product are very similar to those reported in the literature for tabersonine. However, the H-6 and H-7 protons in this case are strongly shielded relative to the corresponding protons in tabersonine and are found at 3.11 and 3.49 ppm, respectively. Similarly, C6 and C7 also are shielded and are found at 57.2 and 54 ppm, respectively. This suggests that the unsaturation across (C-6)-(C-7) [corresponding to C-15, C-14, respectively in Supplemental Fig. S3] has been lost, and the C-6 and C-7 atoms are likely bonded to oxygen. The m/z increase of 16 suggests that only one oxygen atom has been added, resulting in an epoxidation at the C-6,C-7 positions. Moreover, the H-6 proton shows nuclear Overhauser effect (NOE) correlations to both H-4 protons (corresponding to H-17 in Supplemental Fig. S3). This strongly suggests that the epoxide motif is directed upward, as the alternative configuration would place the H-6 proton upward, which would be expected to exhibit only one or possibly no NOE interaction with the H-17 protons. Collectively, these data indicate that this compound might be lochnericine. Comparison with previously reported NMR assignments for lochnericine shows good agreement. This result thus confirms that both CRO_T019212 and CRO_T013902 display tabersonine 6,7-epoxidase activity. As a consequence, CRO_T019212 and CRO_T013902 were named tabersonine 6,7-epoxidase isoform 1 (TEX1 [CYP71D521]; GenBank accession no. MG873080) and isoform 2 (TEX2 [CYP71D347]; GenBank accession no. MG873081), respectively.

**TEX1 and TEX2 Display Similar Kinetic Parameters**

To gain insight into TEX1 and TEX2 enzymatic specificities, their biochemical properties were analyzed subsequently following purification of the corresponding enriched yeast microsomes. TEX1 and TEX2 microsomes were first incubated with increasing amounts of tabersonine to compare the kinetics parameters of the two enzymes (Table 1; Supplemental Fig. S4). Both enzymes displayed very close apparent \( K_m \) and \( V_{max} \) values, suggesting that TEX1 and TEX2 exhibit similar catalytic characteristics toward tabersonine. We next analyzed their substrate specificities by assaying TEX1- or TEX2-enriched microsomes against other MIAs and non-MIA compounds such as flavonoids. Ajmalicine, vindoline, and catharanthine, as well as the flavonoids naringenin and kaempferol, were not converted by TEX1 or TEX2, suggesting that both enzymes were specific to the tabersonine backbone of MIAs (Table 1). Furthermore, vincadifformine was not epoxidized by either enzyme, confirming that the presence of unsaturation at the C6,C7 position was required strictly for the formation of an epoxide, as expected for these oxidases. Finally, while 19-hydroxytabersonine still contains the C6-C7 alkene, it was not metabolized by TEX1 or TEX2, thus suggesting that the addition of a hydroxyl close to the C6,C7 position prevents TEX activity. This hypothesis was confirmed in vivo by co-expressing T19H and TEX1 or TEX2 in yeast, which resulted in the formation of 19-hydroxytabersonine, while hörhammericine (19-hydroxylochnericine) was not observed (Supplemental Fig. S5). Altogether, these results suggested that TEX1 and TEX2 are highly specific to tabersonine and do not accept modifications of the C6,C7 functionality.
The presence of two TEX isoforms in *C. roseus* prompted us to investigate lochnerine metabolism at the whole-plant level by comparing the amount of lochnerine and derived MIAs with TEX1 and TEX2 gene expression profiles. Both transcript levels were analyzed in the major *C. roseus* organs, revealing opposite expression patterns. While TEX1 was expressed only in roots, TEX2 transcripts were detected in all organs except roots, including stem, young and mature leaves, and flowers, in agreement with our transcriptomic analysis (Figs. 2B and 4A). As a control, we showed that T19H exhibited an expression pattern restricted to roots and flowers, as reported previously (Carqueijeiro et al., 2018). As such, TEX1 expression is consistent with that of T19H for the high accumulation of lochnerine, hörhammericine, and 19-acetylhörhammericine in roots (Supplemental Table S4). By contrast, no lochnerine or derived MIAs were detected in young/mature leaves that could be linked to the high expression of TEX2 in these organs. However, the presence of TEX2 transcripts in flowers may explain the unexpected detection of lochnerine in this reproductive organ. We next compared the absolute expression level of these three genes in wild-type roots, hairy roots, and adventitious roots generated from freshly cut stems of *C. roseus* (Fig. 4B). Interestingly, the differential expression between TEX1 and TEX2 was confirmed in roots, with a substantially higher amount of TEX1 transcripts. By contrast, TEX2 was associated with high tabersonine 6,7-epoxidase activity in both hairy and adventitious roots, resulting in a marked accumulation of lochnerine (Supplemental Table S5). Again, T19H exhibited dramatically lower expression, in agreement with the low amounts of hörhammericine and 19-acetylhörhammericine (Supplemental Table S5). Finally, we confirmed the high MeJA responsiveness of both TEX1 and TEX2 (500- and 4,500-fold induction, respectively), as suggested by transcriptomics (Figs. 2B and 4C). In agreement with the presence of putative transmembrane helices at their N termini, the expression of TEX1 and TEX2 fused to yellow fluorescent protein (YFP) suggested that both enzymes are anchored to the endoplasmic reticulum, as observed previously for other P450s that metabolize tabersonine (Fig. 4, D–K; Supplemental Fig. S6; Besseau et al., 2013; Carqueijeiro et al., 2018).

### Silencing of TEX1 Negatively Impacts Lochnericine Accumulation in Roots

To investigate the role of TEXs in lochnerine biosynthesis in planta, we applied a transient silencing approach through virus-induced gene silencing. Although roots are less prone to silencing, we targeted TEX1 given the high amount of lochnerine accumulated in this organ type, thus enabling efficient analysis of the metabolic consequences of gene silencing. To conduct this study, young plantlets were transformed with a TEX1 silencing construct (viTEX1) or an empty vector (EV) as a control. These transformed plantlets exhibited strongly altered root phenotypes such as reduced growth, which might result from a virus or *Agrobacterium tumefaciens* propagation (Fig. 5A), as reported in a previous study (Weaver et al., 2014). However, we measured a substantial decrease of TEX1 expression in viTEX1 plants as compared with EV (Fig. 5B). Such gene silencing was accompanied by a decrease of lochnerine production and, conversely, by an increase of tabersonine accumulation in viTEX1 plants (Fig. 5C). This result thus confirms that TEX1 is involved in the biosynthesis of lochnerine in *C. roseus* plants.

### Metabolic Engineering of the Biosynthesis of 6,7-Oxygenated Tabersonine Derivatives in Yeast

Due to the highly valuable properties of some tabersonine derivatives exhibiting a C6,C7-epoxide, we next evaluated the possibility of reconstituting a tabersonine bioconverting system by coexpressing several tabersonine-metabolizing enzymes together in yeast (Kojima and Umezawa, 2006; Lim et al., 2008). We first chose to reconstitute the 19-acetylhörhammericine biosynthetic pathway by coexpressing TEX1, T19H, and TAT.

Due to the high substrate specificity of TEX1, which is not capable of epoxidizing 19-hydroxytabersonine, TEX1 is required to act before the subsequent reactions catalyzed by T19H and TAT (Table 1; Supplemental Fig. S5). Such sequential conversions were achieved by cloning the TEX1 coding sequence under the control of a constitutive promoter (phosphoglycerate kinase1 in pXP218), while T19H and TAT were controlled by

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TEX1 $K_m$ [µM]</th>
<th>$V_{max}$ [pmol s$^{-1}$]</th>
<th>TEX2 $K_m$ [µM]</th>
<th>$V_{max}$ [pmol s$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabersonine</td>
<td>2.08 ± 0.22</td>
<td>0.254 ± 0.006</td>
<td>6.45 ± 0.37</td>
<td>0.108 ± 0.002</td>
</tr>
<tr>
<td>Vincadiformine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19-Hydroxytabersonine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ajmalicine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vindoline</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catharanthine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naringenin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Gal-inducible promoters (GAL1/10 in pYEDP60 and pESC-Trp, respectively). Using a yeast strain transformed with these three plasmids, a first 24 h of growth in glycerol-enriched medium allowed an almost complete conversion of tabersonine into lochnericine (Fig. 6). The subsequent induction of T19H and TAT expression through Gal addition resulted in the hydroxylation and acetylation of lochnericine, leading to the synthesis of 19-acetylhörhammericine (Fig. 6). Interestingly, almost all synthesized MIAs were excreted to the yeast culture medium. The production of the final compound reached between 12% and 25% of the tabersonine substrate, similar to previous observations for such bioconversion (Carqueijeiro et al., 2018). This three-enzyme coexpressed system thus allows a complete reconstitution of the 19-acetylhörhammericine biosynthetic pathway from tabersonine and opens new opportunities toward the tailor-made synthesis of tabersonine-derived MIAs.

To illustrate the potential of such an approach, we next added a supplemental decoration capacity by introducing tabersonine C16 modifications through the simultaneous expression of T16H2 and 16-hydroxytabersonine 16-O-methyltransferase (16OMT), two enzymes involved in vindoline biosynthesis (Levac et al., 2008; Besseau et al., 2013) that are not naturally involved in these root-specific pathways. Both coding sequences were cloned under the control of GAL1/10-inducible promoters in pESC-Leu, and the resulting plasmid was cotransformed with the TEX1-expressing plasmid to generate a yeast strain coexpressing TEX1, T16H2, and 16OMT. Using the sequential culture process described above, we noted that lochnericine resulting from TEX1 constitutive expression was converted further into a compound exhibiting an m/z 383 consistent with...
lochnericine (16-methoxylochnericine) after T16H2- and 16OMT-induced expression (Supplemental Fig. S7). This result suggested that these three enzymes can successively metabolize tabersonine into this rare MIA in yeast. As a consequence, we finally coexpressed TEX1, T19H, TAT, T16H2, and 16OMT in the same yeast cells. After feeding with tabersonine and sequential gene expression, we observed that this yeast strain produced a compound with an m/z 411 consistent with 16-methoxy-19-acetylhörhammericine as a result of the five-step enzymatic sequence (Supplemental Fig. S7). While we succeeded previously in the synthesis of this compound through the bioconversion of hörhammericine (Carqueijeiro et al., 2018), the achievement of this production from tabersonine constitutes a marked improvement, since tabersonine can be obtained in a higher yield in plants relative to hörhammericine (Koroch et al., 2009). Furthermore, the development of such a yeast strain further enriches the toolbox dedicated to the metabolic engineering of MIAs and the capacity to achieve a tailor-made synthesis of this compound type.

DISCUSSION

While lochnericine constitutes one of the main MIAs from C. roseus roots, the key enzyme responsible for the epoxide structure has remained unknown for several decades. In this work, we describe the identification of two isoforms of tabersonine 6,7-epoxidase named TEX1 and TEX2 and, thus, completed the main biosynthetic route of tabersonine derivatives in C. roseus roots.

By combining gene expression correlation analyses and yeast assays, we identified two enzymes (TEX1 and TEX2) catalyzing the synthesis of a similar product. NMR characterization revealed the compound to be lochnericine (Figs. 2 and 3). Additional evidence of their function in planta was obtained through TEX1 gene inactivation, resulting in a decrease of lochnericine accumulation in C. roseus roots (Fig. 5, B and C). While TEX1 (CYP71D521) and TEX2 (CYP71D347) both belong to the CYP71 clan, we noted that their degree of identity was higher to T16H compared with T19H, even though T16H acts on the indole moiety of tabersonine. On the other hand, TEX1/2 and T19H both act on the terpenoid backbone of tabersonine (Supplemental Fig. S1; Supplemental Table S3). Surprisingly, identity was even lower (46%) to the only other MIA biosynthetic enzyme known so far to catalyze epoxidation, tabersonine 3-oxidase (T3O; Kellner et al., 2015; Qu et al., 2015), suggesting that TEX1 and TEX2 may originate from an ancestor closer to T16H or that selection pressure was lower on T3O. This finding thus reinforces the importance of diversification of the CYP71 clan for the biosynthesis of poststrictosidine MIAs, as observed in C. roseus (Schröder et al., 1999; Besseau et al., 2013; Kellner et al., 2015; Tatsis et al., 2017). Interestingly, as suggested by locus tagging, TEX1 (CRO_T019212) and TEX2 (CRO_T013902) occupy distant positions in the C. roseus genome, implying that a duplication event was followed by gene translocation along with recruitment of the two gene copies into distinct lochnericine synthesis-responsive mechanisms.

At the biochemical level, both isoforms display similar kinetic parameters and substrate specificities (Table 1; Supplemental Fig. S4). Besides being specific to the tabersonine backbone, TEX1 and TEX2 require the presence of the C6,C7-alkene to perform epoxidation, as revealed by the lack of vincadifformine conversion. This requirement is based on their catalytic mechanism, as described for other epoxidases such as zeaxanthin epoxidase (Marin et al., 1996) and squalene epoxidase (Rasbery et al., 2007). Surprisingly, neither TEX1 nor TEX2 accepts 19-hydroxytabersonine as a substrate, in contrast with T19H, which can hydroxylate both tabersonine and lochnericine (Table 1; Giddings et al., 2011). This substrate specificity thus suggests that TEX1 and TEX2 are highly sensitive to the environment proximal to the C6,C7 double bond, and we can hypothesize that the addition of a hydroxyl at C19 increases steric hindrance and/or local polarity, altering substrate recognition. As a physiological consequence, this specificity directs the reaction order in the lochnericine/hörhammericine biosynthetic pathway that strictly requires that 6,7-epoxidation proceeds before 19-hydroxylation, to yield lochnericine and then hörhammericine. This hypothesis is further supported by the high amount of lochnericine measured in roots versus the very low accumulation of 19-hydroxytabersonine (Supplemental
Interestingly, T19H displays a high affinity and catalytic efficiency for tabersonine ($K_m$ of 300 nm; Giddings et al., 2011). While comparing catalytic properties remains quite difficult between different studies, especially for P450s that are not all quantifiable by carbon monoxide spectrum (e.g. TEX1 and TEX2), this noticeable difference, as well as our observations in yeast (Supplemental Fig. S5), argue for a higher catalytic activity of T19H versus TEX1 or TEX2. However, the higher expression levels of TEX1/TEX2 than T19H may have a stronger impact than the higher affinity of T19H toward tabersonine, leading to a preferential flux of tabersonine toward the biosynthesis of lochnericine (Fig. 4B). One can also hypothesize that a multitissue organization may orchestrate this preferential synthesis, with TEX and T19H expressed in distinct root tissues, as observed in leaf for vindoline synthesis (Coudavault et al., 2014).

As already depicted for other MIA biosynthetic genes in *C. roseus* (Dugé de Bernonville et al., 2015b; Stavrinides et al., 2015, 2016), more than one gene copy governs lochnericine synthesis in planta. Interestingly, while tabersonine 6,7-epoxidase activity has been detected only in roots (Rodriguez et al., 2003), we showed that TEX1 and TEX2 display complementary gene expression profiles, with TEX1 in roots and TEX2 in aerial organs and hairy/adventitious roots (Fig. 4, A–C). In this study, the observed discrepancies between RNA sequencing and qPCR expression profiles may originate from the high degree of identity between both genes, resulting in chimeric transcript reconstitution or spurious read attribution that caused a mix of TEX1 and TEX2 expression patterns in the transcriptomic analysis (Figs. 2B and 4A). Based on the qPCR results, we suggest that the expression of TEX1 is mainly responsible for lochnericine synthesis in roots, while TEX2 is responsible for lochnericine

Table S4; Carqueijeiro et al., 2018). Interestingly, T19H displays a high affinity and catalytic efficiency for tabersonine ($K_m$ of 300 nm; Giddings et al., 2011). While comparing catalytic properties remains quite difficult between different studies, especially for P450s that are not all quantifiable by carbon monoxide spectrum (e.g. TEX1 and TEX2), this noticeable difference, as well as our observations in yeast (Supplemental Fig. S5), argue for a higher catalytic activity of T19H versus TEX1 or TEX2 that should favor the formation of 19-hydroxytabersonine in vivo at the expense of lochnericine. However, the higher expression levels of TEX1/TEX2 than T19H may have a stronger impact than the higher affinity of T19H toward tabersonine, leading to a preferential flux of tabersonine toward the biosynthesis of lochnericine (Fig. 4B). One can also hypothesize that a multitissue organization may orchestrate this preferential synthesis, with TEX and T19H expressed in distinct root tissues, as observed in leaf for vindoline synthesis (Coudavault et al., 2014).

As already depicted for other MIA biosynthetic genes in *C. roseus* (Dugé de Bernonville et al., 2015b; Stavrinides et al., 2015, 2016), more than one gene copy governs lochnericine synthesis in planta. Interestingly, while tabersonine 6,7-epoxidase activity has been detected only in roots (Rodriguez et al., 2003), we showed that TEX1 and TEX2 display complementary gene expression profiles, with TEX1 in roots and TEX2 in aerial organs and hairy/adventitious roots (Fig. 4, A–C). This contrasting distribution pattern resembles that of T16H1 and T16H2 isoforms in flowers and leaves of *C. roseus* (Besseau et al., 2013). In this study, the observed discrepancies between RNA sequencing and qPCR expression profiles may originate from the high degree of identity between both genes, resulting in chimeric transcript reconstitution or spurious read attribution that caused a mix of TEX1 and TEX2 expression patterns in the transcriptomic analysis (Figs. 2B and 4A). Based on the qPCR results, we suggest that the expression of TEX1 is mainly responsible for lochnericine synthesis in roots, while TEX2 is responsible for lochnericine

Figure 6. Reconstitution of the tabersonine-to-19-acetylhörhammericine biosynthetic pathway in yeast. Yeast expressing TEX1 and T19H/TAT under the control of constitutive and Gal-inducible promoters, respectively, were fed with tabersonine (1) and grown during 24 h in the absence of Gal to specifically allow TEX1 activity, leading to lochnericine (2) production. Induction of T19H and TAT expression via Gal addition then resulted in the conversion of lochnericine into hörhammericine (3) and 19-acetylhörhammericine (4) during the subsequent 24-h growth period. The three chromatograms successively highlight the MIA composition (expressed as relative peak areas) in the yeast culture medium at 0, 24, and 48 h after tabersonine feeding, std, Standards.
production in aerial organs. This hypothesis is supported by the accumulation of lochnericine in *C. roseus* flowers and by the coexpression of *T19H* in this organ (Fig. 4A; Supplemental Table S4). However, we never observed lochnericine or its root-type derivatives in leaves, despite the high TEX2 expression. This may be explained by the presence of other biosynthetic enzymes in these organs that also can metabolize MIAs with a tabersonine backbone, such as T16H2 or other yet unknown enzymes, resulting in the formation of uncharacterized MIAs. For instance, although we did not measure it in this work, lochnericine (16-methoxylochnericine) and hörhammerine (16-methoxyhörhammericine) already have been detected in *C. roseus* and *Catharanthus lanceus* leaves, respectively (Maloney et al., 1965; Farnsworth et al., 1968). Interestingly, TEX2 promoter sequence analysis revealed the presence of a CTCGAG motif that is known to bind phytochrome-interacting factors (Supplemental Fig. S8). By interacting with phytochromes, these transcription factors participate in many light responses that may explain the expression of TEX2 in *C. roseus* aerial organs (Leivar and Quail, 2011). Besides expression in these organs, TEX2 also displays high expression in adventitious and hairy roots in our growth conditions (Fig. 4B). This high expression, in turn, may cause the high accumulation of lochnericine/hörhammerine in hairy roots, making TEX2 a preferential target in inactivation strategies aimed at producing hairy roots with reoriented MIA metabolism (Supplemental Table S5; Sun et al., 2017). Also, the MeJA inducibility of both TEX1 and TEX2 is supported by the increased synthesis of lochnericine upon MeJA treatment and may reflect TEX involvement in plant defense mechanisms (Peebles et al., 2009).

Finally, the identification of TEX1/TEX2 expands the toolbox dedicated to the metabolic engineering of MIAs in yeast that we developed recently (Carqueijeiro et al., 2018). First, by ensuring an efficient epoxidation of tabersonine (Fig. 6), it allows a controlled and easy synthesis of lochnericine, given the abundant sourcing of tabersonine (Koroch et al., 2009). Lochnericine displays cytotoxic properties against human leukemia cells in combination with other MIAs. Therefore, yeast bioproduction constitutes an alternative sourcing for this compound that may facilitate future uses (Fernández-Pérez et al., 2013). Second, it enables the full reconstitution of the 19-acetylhörhammerine pathway up to the synthesis of uncommon MIAs, such as 16-methoxy-19-acetylhörhammerine, by mixing enzymatic modules from roots (*T19H* and *TAT*) and leaves (*T16H2* and 16OMT; Fig. 6; Supplemental Fig. S7). While such an association also has been reported in *C. roseus* hairy roots through *T16H1* and 16OMT overexpression, it did not enable full control of the synthesis of the final MIAs and was limited to the production of some intermediates such as lochnericine (Sun et al., 2018). By contrast, in our yeast system, the sequential expression of genes enabled a rationalized enzyme activity, resulting in a streamlined reorientation of the metabolic flux up to the expected MIA. Since the yield of these conversions remained low, further optimizations will be required to control the relative yields. However, this study offers concrete opportunities toward the production of tabersonine analogs. Tabersonine is a lead anti-Alzheimer compound, and structural variants may have improved properties for amyloid β-fibril disruption (Kai et al., 2015).

In conclusion, we identified two P450s, TEX1 and TEX2, catalyzing the epoxidation of tabersonine to produce lochnericine in an organ-dependent manner, thus completing one MIA biosynthetic branch of *C. roseus* roots. Their high substrate specificity sheds light on the biosynthetic order governing the synthesis of 19-acetylhörhammerine, placing TEX1/TEX2 as obligatory starting enzymes of this pathway. Such identification also broadens our capacity to achieve MIA production in heterologous organisms such as yeast, allowing for a tailor-made synthesis of MIA resulting from combining distinct enzymatic modules. Expanding the combinatorial synthesis of MIAs and improving the metabolic engineering of tabersonine derivatives is important, given the pharmaceutical potential of highly oxidized congeners of tabersonine, including jerantinine, pachyphine, taberhanine, melodinine, or conophyline, whose production and access could be facilitated (Kam et al., 2003; Kojima and Umezawa, 2006; Lim et al., 2008; Feng et al., 2010).

### MATERIALS AND METHODS

#### Chemicals

Tabersonine was purchased from ChromaDex, while lochnericine and hörhammerine were purified from *Catharanthus roseus* hairy roots according to Giddings et al. (2011). Vincadifformine was prepared by the hydrogenation of tabersonine as described by Zhao and Andrade (2013).

#### Transcriptomic Analysis and Identification of Tabersonine 6,7-Epoxidase Candidate Genes

The CDF97 reference transcriptome (Dugé de Bernonville et al., 2015b) was used to obtain expression levels from a wide range of experimental conditions (Supplemental Table S1). Raw reads were obtained from the National Center for Biotechnology Information Short Read Archive with the SRA Toolkit version 2.6.2 and quasimapped onto reference transcripts with Salmon version 0.8.2 to quantify transcript abundance (Patro et al., 2017). Spearman nonparametric correlations were calculated with R version 3.4.1 (R Development Core Team, 2013). Among transcripts that correlated best with *T19H*, P450s were identified according to reference transcript annotations performed by BlastX (Blast+ version 2.2.29; Camacho et al., 2009) against Uridiprot and by hmmscan version 3.1 (Eddy, 2011) against PfamA. Candidates were clustered according to either their sequence similarities with a neighbor-joining tree in ClustalO version 1.2.2 (Sievers et al., 2011) or their expression levels with a Euclidean distance together with a Ward clustering algorithm in R.

#### Heterologous Expression of Tabersonine 6,7-Epoxidase Candidate Genes in Yeast

Full-length open-reading frames of CRO_T000497, CRO_T013902, CRO_T027852, CRO_T002923, T16H2, and *T19H* were amplified using dedicated primers (Supplemental Table S6) that have been designed to introduce BamHI or SseI restriction sites at their extremities, allowing cloning into the pESC-His plasmid. Recombinant plasmids were independently used to transform the
Tabersonine 6,7-Epoxidase Functional Assay and Large-Scale Product Synthesis

Functional assays of tabersonine 6,7-epoxidase activity were conducted by feeding 175 µL of Gal-induced recombinant yeast strains with tabersonine (125 µM) during 16 h at 30°C under continuous shaking. Reactions were centrifuged (15 min at 25,000 g) before subsequent UPLC-MS analysis. For large-scale product synthesis of CRO_T019212 (TEX1), 100 mL of the TEX1-expressing yeast strain was grown, induced, and fed with 3.4 mg of tabersonine for 24 h at 30°C. A purifications was conducted according to Carqueijeiro et al. (2018) before a final purification performed by preparative HPLC on a Thermo Dionex Ultimate 3000 chromatography apparatus using a Phenomenex Luna C18 (5 µm, 30 × 250 mm) column. The column was used with 0.1% acetic acid (solvent A) and acetonitrile (solvent B). A linear gradient from 5% B to 50% B over 25 min was used to separate the alkaloids. Chromatography was performed at a flow rate of 30 mL min⁻¹ and monitored with a UV detector at 254 nm. Fractions (30 mL) were collected and pooled and then characterized by NMR.

Microsome Purification and Steady-State Enzyme Kinetics

Microsomes enriched with TEX1 or TEX2 were isolated using the high-density procedure as described by Heitz et al. (2012). After total microsomal protein quantification, TEX1 and TEX2 activity assays were conducted in a final volume of 50 µL containing 100 mM Tris-HCl, pH 8, 4 mM DTT, 500 µM NADPH, various amounts of substrates, and 37 µg of microsomal proteins. Reactions were initiated by adding NADPH to the reaction mixtures at 30°C and stopped by the addition of methanol (50 µL) before UPLC-MS analysis. Additional assays performed without NADPH or with microsomes from yeast harboring the empty expression vector were used as controls. For kinetic parameter determination, tabersonine was used at concentrations ranging from 0.5 to 50 µM. Nonlinear regression (SigmaPlot 12.0) was used to determine Km and Vmax using the Michaelis-Menten equation. For substrate specificity evaluation, reactions were carried out at 30°C during 30 min with vincadifformine, 19-hydroxytabersonine, ajmalicine, vindoline, catharanthine, naringenin, or kaempferol at 100 µM. For UPLC-MS analyses, reactions were stopped by adding 1 volume of methanol. Solutions were vortexed and centrifuged for 10 min at 16,000g before collecting 5 µL of the supernatant for injection.

UPLC-MS Analyses

UPLC-MS analyses were conducted using equipment and conditions described by Parage et al. (2016). Data collection was carried in selected ion monitoring mode for the following compounds: tabersonine, m/z 337, retention time (RT) = 10.9 min in Figure 3 and 11.52 min in Figure 6 and Supplemental Figures S5 and S7; catharanthine, m/z 337, RT = 9.71 min; 16-hydroxytabersonine, m/z 353, RT = 8 min; 19-hydroxytabersonine, m/z 353, RT = 5.65 min in Figure 3 and 7.8 min in Supplemental Figure S5; ajmalicine, m/z 353, RT = 9.76; lochnericine, m/z 353, RT = 10.03 min in Figure 3 and 10.4 min in Figure 6 and Supplemental Figures S5 and S7; vinsamide, m/z 339, RT = 11.4 min; 16-methoxytabersonine, m/z 367, RT = 12.3 min; 16-hydroxylochnericine, m/z 369, RT = 6.4 min; hörhammericine, m/z 369, RT = 6.5 min; 16-methoxylochnericine, m/z 383, RT = 11.2 min; 16-hydroxyhörhammericine, m/z 385, RT = 4.11 min; 16-methoxyhörhammericine, m/z 399, RT = 10.7 min; 19-acetylhörhammericine, m/z 411, RT = 12.46 min; 16-methoxy 19-acetylhörhammericine, m/z 441, RT = 11.25 min; vindoline, m/z 457, RT = 12.62 min; 16-methoxyvindoline, m/z 385, RT = 11.2 min; 16-methoxy-19-acetylhörhammericine, m/z 441, RT = 11.2 min; naringenin, m/z 275 (negative electrospray charge [m-]), RT = 12.28 min; and kaempferol, m/z 285 (m+), RT = 13.12 min. Discrepancies in RT values resulted from conducting analyses on distinct UPLC devices.

NMR Spectroscopy

NMR spectra (1D and 2D NMR) were acquired using a Bruker Avance III 400 NMR spectrometer equipped with a BBFO plus 5-mm probe. TopSpin version 3.2 was used for analysis. The residual 1H- and 13C-NMR signals of CDC1 (δ 7.26 and 77.16, respectively) were used for calibration. Proton spectrum used 64 scans; COSY, eight scans; NOESY, eight scans; TOCSY, eight scans; HSQC, 16 scans; HMBC, 64 scans.

Gene Expression Measurement (Reverse Transcription-PCR)

The expression levels of TEX1, TEX2, and T19H were analyzed by quantitative reverse transcription-PCR according to Carqueijeiro et al. (2018) and Liscombe and O’Connor (2011) using primers compiled in Supplemental Table S6, KPS9 and EFla as endogenous reference genes, and retrotranscribed RNAs from different C. roseus organs (including roots, stems, young and mature leaves, flowers [cv Apricot Sunstorm], adventitious roots, and hairy roots).

Subcellular Localization Studies

Subcellular localizations of TEX1 and TEX2 were studied by creating fluorescent fusion proteins using the p sca-cassette YFPi plasmid (Guirimand et al., 2009). The full-length open reading frames of enzymes were amplified using specific primer couples (Supplemental Table S6), which were designed to introduce the SpeI restriction site at both cDNA extremities. PCR products were cloned at the 5’ end of the YFP coding sequence to generate the TEX1-YFP and TEX2-YFP fusion proteins and to maintain the functionality of the predicted N-terminal transmembrane helices (Supplemental Fig. S6). The resulting plasmids were used for transient transformations in combination with plasmids expressing the endoplasmic reticulum-CFP marker (Nelson et al., 2007). Transient transformation of C. roseus cells by particle bombardment and fluorescence imaging were performed following the procedures described previously (Fourreaux et al., 2016). Briefly, C. roseus plated cells were bombard-
in maintaining cell cultures/plants and for valuable technical assistance. We also acknowledge the Fédération CaSciModOT for access to the computing grid. We thank Dr. David Nelson (University of Tennessee, Memphis) for CYP annotation.

Received May 8, 2018; accepted June 13, 2018; published June 22, 2018.

LITERATURE CITED


Carqueijeiro et al.


