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# Functional Genomic Analysis of Alkaloid Biosynthesis in *Hyoscyamus niger* Reveals a Cytochrome P450 Involved in Littorine Rearrangement

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## Summary

**Tropane alkaloids are valuable pharmaceutical drugs derived from solanaceous plants such as *Hyoscyamus niger* (black henbane). The biosynthesis of these molecules, including the nature of the enigmatic rearrangement of (*R*)-littorine to (*S*)-hyoscyamine, is not completely understood. To test the hypothesis that a cytochrome P450 enzyme is involved in this rearrangement, we used virus-induced gene silencing to silence a cytochrome P450, CYP80F1, identified from *H. niger* roots by EST sequencing. Silencing CYP80F1 resulted in reduced hyoscyamine levels and the accumulation of littorine. Hyoscyamine was observed in CYP80F1-expressing tobacco hairy roots supplied with (*R*)-littorine. Expression in yeast confirmed that CYP80F1 catalyzes the oxidation of (*R*)-littorine with rearrangement to form hyoscyamine aldehyde, a putative precursor to hyoscyamine, and without rearrangement to form 3′-hydroxylittorine. Our data strongly support the involvement of CYP80F1 in the rearrangement of littorine to hyoscyamine.**

## Introduction

Tropane alkaloids such as scopolamine and hyoscyamine are found in various members of the Solanaceae family, including species of *Hyoscyamus*, *Atropa*, and *Datura* [1–4]. Such plants have a long history of use as medicines and hallucinogens, and tropane alkaloids remain an important class of plant-derived anticholinergic drugs. Atropine (racemic hyoscyamine), for example, is used as a premedication for anesthesia to reduce bronchial secretions and to block bradycardia associated with some anesthetic drugs.

Despite considerable efforts over many years and good progress toward elucidating the biosynthetic pathway leading to tropane alkaloids, knowledge of several key catalytic steps remains incomplete [3]. An intriguing mystery is the nature of the carbon skeleton rearrangement that occurs during the conversion of (*R*)-littorine to (*S*)-hyoscyamine (see Figure 1). In this step, the 3-phenyllactate moiety of littorine is rearranged to tropate. Isotope labeling studies have indicated that C1′ and C3′ form the phenylacetate moiety of hyoscyamine, and that C2′ forms the hydroxylmethyl group in a stereochemically controlled reaction [5–7]. In recent years, there has been some controversy regarding the types of enzymes involved in the conversion of littorine to hyoscyamine. An enzyme with a mechanism and properties similar to vitamin $B_{12}$-dependent isomerases has been suggested [1]. Retey and coworkers [8–10] have provided evidence for such a littorine isomerase that requires *S*-adenosylmethionine (SAM) as a cofactor. On the other hand, labeling and inhibitor studies and mechanistic considerations led Robins and coworkers to propose that a cytochrome P450 is involved, and that hyoscyamine aldehyde is formed as an intermediate [3, 11, 12]. Furthermore, Patterson and O'Hagan presented results that were inconsistent with a vicinal exchange mechanism expected for a SAM-dependent reaction [13].

In order to settle the debate regarding the last steps in hyoscyamine biosynthesis, an investigation of the hypothesis that a cytochrome P450 is involved was undertaken. Our functional genomics approach has included the generation of expressed sequence tags from an *H. niger* L. cDNA library and the testing of candidate cytochrome P450 gene function by virus-induced gene silencing (VIGS).

VIGS exploits the RNA silencing pathway directed against invading viruses to silence the expression of host genes [14] and has been used to silence genes involved in a variety of plant biosynthetic pathways [15–19]. Tobacco rattle virus (TRV), a tobravirus that silences genes in *Nicotiana benthamiana* [20, 21] and other Solanaceae [22], has been reported to infect *H. niger* [23]. In related work, proof-of-concept experiments showed that TRV-mediated gene silencing was effective in *H. muticus* (Egyptian henbane) for phytoene desaturase and known alkaloid biosynthetic enzymes (J.E.P. et al., unpublished data). In this paper, we report the use of VIGS to test the function of a candidate cytochrome P450 gene from *H. niger* for its involvement in littorine rearrangement. This has lead to the successful identification of CYP80F1, a multifunctional cytochrome P450 capable of littorine rearrangement.

## Results

### Expressed Sequence Tags from *Hyoscyamus niger*

Given the relatively limited knowledge of the genes involved in the biosynthesis, transport, and regulation of tropane alkaloids in the Solanaceae family, a collection of expressed sequence tags (ESTs) from *H. niger* was assembled as an aid to addressing this issue. Since alkaloid biosynthesis in *H. niger* occurs in the roots [1], a cDNA library was constructed so as to enhance the frequency of cDNAs corresponding to genes expressed root specifically. For this subtracted library, cDNA from *H. niger* root cultures was used as the tester, and leaf cDNA was used as the driver in a suppression PCR procedure. To generate ESTs from the library, bacterial colonies representing cDNA clones were picked randomly, and their plasmid inserts were sequenced. After sequence analysis, the 2286 EST sequences representing 214 clusters and 1299 singletons were subjected to similarity searches with BLAST.

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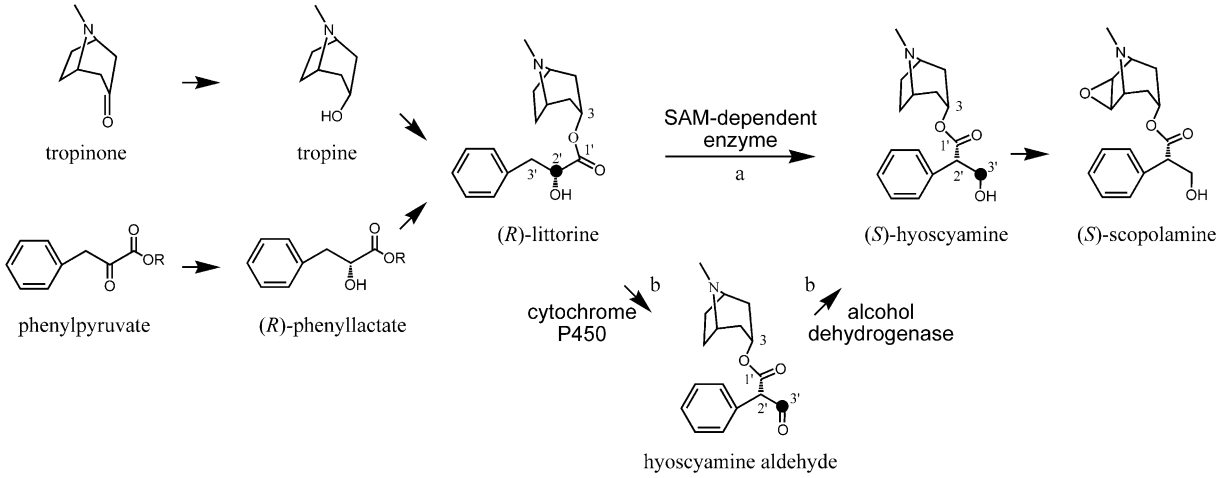


Figure 1. The Proposed Tropane Alkaloid Biosynthetic Pathway in Members of the Solanaceae

Two different routes for the conversion of littorine to hyoscyamine are shown. Route a involves the SAM-dependent conversion proposed by Retey et al. [8], while route b, which is supported by the results of this paper, is a two-step process catalyzed by a cytochrome P450 and an alcohol dehydrogenase. The 2′ carbon atom of littorine is labeled with a black dot to indicate its new position as the 3′ carbon atom in hyoscyamine after rearrangement.

As a starting point to investigate the possibility that a cytochrome P450 was involved in the conversion of littorine to hyoscyamine, the *H. niger* ESTs showing similarity to cytochrome P450s were examined. A total of 25 ESTs (4 clusters and 11 singletons) showed similarity to cytochrome P450 sequences. By virtue of the involvement of its homologs in plant secondary metabolism, a clone (pRL011) representing the CYP80B subfamily was one of the candidates chosen for functional analysis with VIGS.

## Suppression of a *Hyoscyamus* Cytochrome P450 by Virus-Induced Gene Silencing

A TRV-based VIGS system was found to be an effective tool for transient loss-of-function experiments in *Hyoscyamus*. Proof-of-concept experiments showed that TRV-mediated silencing of phytoene desaturase was more effective in *H. muticus* (Egyptian henbane) than in *H. niger*, and, consequently, the former species was used as the VIGS host plant. The two *Hyoscyamus* species have similar leaf alkaloid levels and profiles (data not shown). The silencing of several known genes in tropane alkaloid biosynthesis in *H. muticus*, as evidenced by reduced alkaloid levels in infected plants (unpublished data), demonstrated that recombinant TRV could successfully silence the tropane alkaloid pathway in roots, the site of alkaloid biosynthesis in this species [1].

For functional identification, the DNA insert of pRL011 was subcloned into the VIGS binary vector pTRV2 [21]. The resulting plasmid (pRL033) was used to transform *Agrobacterium tumefaciens*, and gene silencing was effected by infiltration of the transformants into the leaves of *H. muticus* plants (see Experimental Procedures for details).

Negative control experiments showed no change in alkaloid profiles in plants subjected to VIGS (see Figure 2A). Vector control plants appeared healthy, and plants undergoing silencing of the phytoene desaturase gene showed the expected bleached phenotype (data not shown). Significantly, silencing of the gene corresponding to pRL011 by using the VIGS construct pRL033 had a profound and reproducible effect on alkaloid quality and quantity, as shown in Figure 2A. In this case, the total tropane alkaloid content, and, notably, that of hyoscyamine, was decreased, yet littorine levels increased in the same plants. Since littorine is thought to be a precursor of hyoscyamine, this indicates a role for the cytochrome P450 gene corresponding to pRL011 in the conversion of littorine to hyoscyamine.

## Cloning and Sequence Analysis of Full-Length *H. niger* CYP80F1 cDNA

The cDNA clone pRL011 is one of five ESTs that were found to be similar to the plant CYP80B subfamily members. Based on sequence data in comparison with a full-length cDNA (see below), the five clones appeared to represent RsaI restriction fragments of cDNAs of the same gene.

A full-length clone, pRL037, corresponding to the partial ORF of pRL011 was obtained by 5′ and 3′ RACE and PCR amplification. The cDNA insert of 1745 nt has an ORF of 510 amino acids, predicting a polypeptide of molecular mass 58.6 kDa. The predicted amino acid sequence shares 41%, 39%, and 38% sequence identity with the *Coptis japonica*, *Papaver somniferum*, and *Eschscholzia californica* (*S*)-*N*-methylcoclaurine-3′-hydroxylases, respectively [24, 25]. It is notable that the *N*-methylcoclaurine-3′-hydroxylases of the CYP80B subfamily hydroxylate an aromatic position of an *N*-methylated intermediate in the benzylisoquinoline alkaloid pathway. Sequence alignment of the *H. niger* enzyme with the *C. japonica* *N*-methylcoclaurine-3′-hydroxylase, CYP80B2, shows that the sequences share conserved eukaryotic cytochrome P450 regions: a helix K region, an aromatic region, and a heme binding region at the C-terminal end (see Figure S3 in the Supplemental Data available with this article online). Based on sequence comparisons, the *H. niger* enzyme was

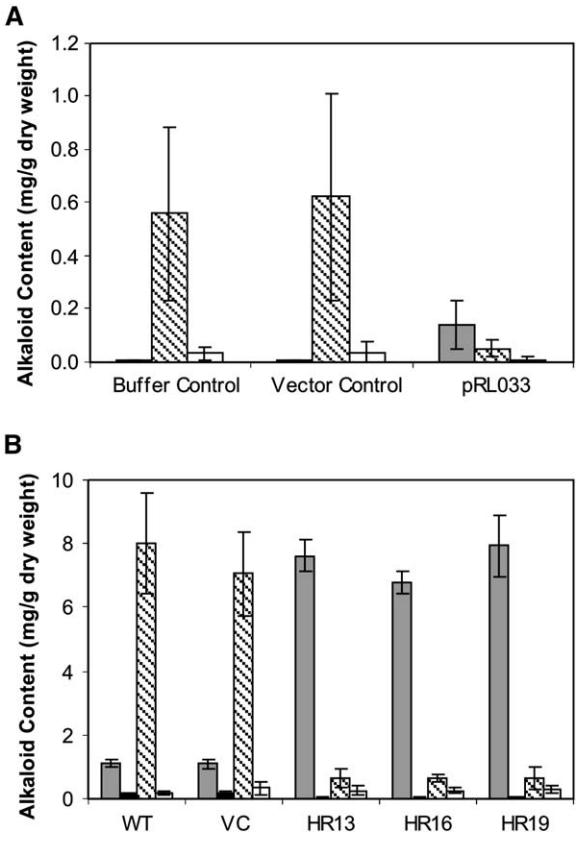


Figure 2. Effects of Suppression of CYP80F1 Gene Expression on Alkaloid Accumulation in *Hyoscyamus*

See Experimental Procedures for details.
(A) VIGS of CYP80F1 in *H. muticus*.
(B) RNAi of CYP80F1 in *H. niger*. *H. niger* hairy roots were generated from *A. rhizogenes* containing no vector (WT), the vector pH7GWIWG2(II) (VC), or pRL041 (containing a CYP80F1 gene fragment) in three separate hairy root lines (HR13, HR16, and HR19). Tissue levels are shown for littorine (gray), 3′-hydroxylittorine (black), hyoscyamine (striped), and scopolamine (white). Means and standard errors for 3–4 replicates are shown.

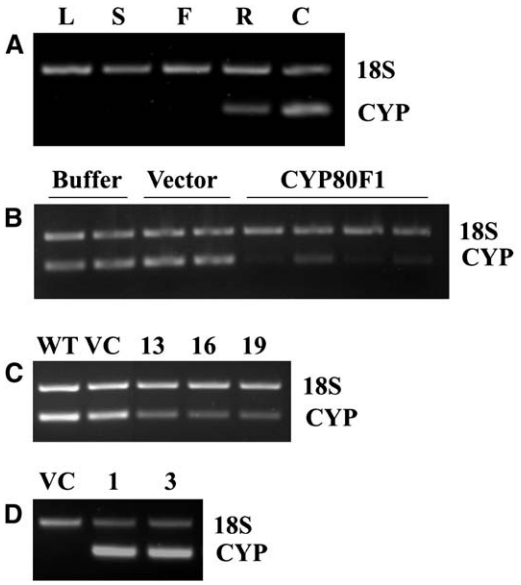


Figure 3. Expression of the CYP80F1 Gene in Wild-Type and Modified Plant Tissues

18S rRNA (18S) and CYP80F1 mRNA (CYP) levels were measured by RT-PCR (See Supplemental Data).
(A) Leaf (L), stem (S), flower (F), root (R), and cultured root (C) tissues of wild-type *H. niger*.
(B) Selected *H. muticus* control and CYP80F1 VIGS root.
(C) RNAi experiment showing selected *H. niger* hairy root lines transformed with wild-type *A. rhizogenes* (WT) and *A. rhizogenes* containing pH7GWIWG2(II) (VC) or PRL041 (containing a CYP80F1 gene fragment; labels 13, 16, and 19; see Supplemental Data).
(D) Tobacco hairy root lines transformed with pMDC32 (VC) or pRL042 (containing the CYP80F1 ORF; labels 1 and 3; see Supplemental Data).

classified as CYP80F1, a new CYP80 subfamily, by the Cytochrome P450 Nomenclature Commission. Based on enzyme activity (see below), the name littorine mutase/monooxygenase is proposed. CYP80F1 also shares 30%–35% identity with various members of the CYP75, CYP76, and CYP80 families in the CYP71 clan of cytochrome P450s. The relationship among known sequences similar to CYP80F1 was determined by phylogenetic analysis (see Supplemental Data). In terms of known enzymes, these include flavonoid hydroxylases, geraniol hydroxylase, 7-ethoxycoumarin *O*-deethylase, and berbamunine synthase. The latter enzyme couples (*S*)-*N*-methylcoclaurine with a second *N*-methylcoclaurine enantiomer and thus also utilizes *N*-methylated alkaloid substrates [26].

### Tissue-Specific Expression of CYP80F1

Figure 3A shows the results of competitive RT-PCR indicating the root-specific expression of CYP80F1. This pattern is consistent with its involvement in tropane alkaloid biosynthesis. Further studies are required to test if expression shows a cell-specific pattern similar to that of other tropane alkaloid biosynthetic genes [27, 28]. Similar RT-PCR experiments demonstrate the resulting reduction in root expression of CYP80F1 upon VIGS by using pRL033 in four different plants, confirming the desired gene silencing effect (Figure 3B).

### Suppression by RNA Interference and Overexpression of CYP80F1 in *H. niger*

To corroborate the VIGS results and obtain propagable plant tissue in which CYP80F1 is suppressed, RNA interference experiments in hairy roots of *H. niger* were carried out. In this case, the CYP80F1 gene fragment used for suppression differed from that used for VIGS (see Supplemental Data for the details). Suppression of gene expression was confirmed by RT-PCR (Figure 3C). As shown in Figure 2B, suppression of CYP80F1 by RNAi gave an alkaloid phenotype similar to that observed for VIGS experiments with CYP80F1. This is similar to the observed effects of cytochrome P450 inhibitors on alkaloid accumulation in *Datura stramonium* hairy roots, which showed a selective decrease in hyoscyamine accumulation [29]. In addition, a compound identified as 3′-hydroxylittorine (see below), which was found to be present in control roots, showed reduced levels in roots undergoing CYP80F1 gene silencing (Figure 2B).

In similar experiments, *H. niger* hairy root cultures were generated in which CYP80F1 was overexpressed. While competitive RT-PCR indicated modest overexpression of CYP80F1 of up to ~3-fold relative to wild-type (data not shown), no significant change in alkaloid content or profile was observed. Consequently, it appears that CYP80F1 does not limit the accumulation of alkaloids under the conditions tested.

### Expression of CYP80F1 in Tobacco Hairy Roots

The function of CYP80F1 was tested by expression in *N. tabacum* hairy roots, which are normally devoid of tropane alkaloids. The CYP80F1 ORF was cloned into the vector pMDC32 and was used to generate *N. tabacum* hairy root cultures. Two transgenic lines (1 and 3) were chosen and compared with the vector control line. Expression of CYP80F1 in lines 1 and 3 was confirmed by RT-PCR (Figure 3D). Alkaloid analysis for *N. tabacum* hairy roots expressing CYP80F1 showed a compound that eluted slightly earlier than littorine in (*R*)-littorine-supplemented cultures expressing CYP80F1 (see Supplemental Data). This was identified by GC/MS as hyoscyamine based on comparison with a standard (data not shown). This same compound was undetectable in vector control lines (see Supplemental Data). This confirms the VIGS and RNAi results indicating that CYP80F1 is involved in littorine-to-hyoscyamine conversion. In addition to hyoscyamine, traces of 3′-hydroxylittorine were also detected by GC/MS in lines 1 and 3 after they were fed with (*R*)-littorine (data not shown). When the CYP80F1-expressing hairy roots were supplied with the unnatural isomer, (*S*)-littorine, hyoscyamine accumulation was not observed, and only traces of 3′-hydroxylittorine were detected (data not shown). This indicates that the CYP80F1-dependent conversion of littorine to hyoscyamine is stereospecific. When hyoscyamine was supplied, no other alkaloids could be detected in the hairy roots expressing CYP80F1 (data not shown).

### Characterization of CYP80F1 in Yeast

The activity of CYP80F1 was further characterized by expression in *Saccharomyces cerevisiae* strain WAT11, which has been engineered to express a plant cytochrome P450 reductase [30]. Microsomal fractions were prepared from recombinant yeast cells, and its enzyme activity was determined by using GC or GC/MS. Figure 4A shows a GC/MS trace of the trimethylsilylated alkaloid products of CYP80F1-containing yeast microsomes incubated with (*R*)-littorine and NADPH. When compared to products of control microsomes (Figure 4B), four new peaks (2, 2*, 3, and 3*) are evident. Production of the corresponding compounds was found to be dependent on NADPH, but not NADH (data not shown). Clotrimazole, a CYP inhibitor that has been found to selectively reduce hyoscyamine accumulation in *Datura stramonium* roots [29], inhibited the reaction in yeast microsomes by 50% at a concentration of 0.1 mM (data not shown). The reaction was not stimulated by SAM at 0.2 mM (data not shown).

Peaks 2 and 2* were identified as the geometric isomers of the trimethylsilyl derivatives of the enol form of hyoscyamine aldehyde (see Supplemental Data). Peaks 3 and 3* were identified as the trimethylsilyl derivative of

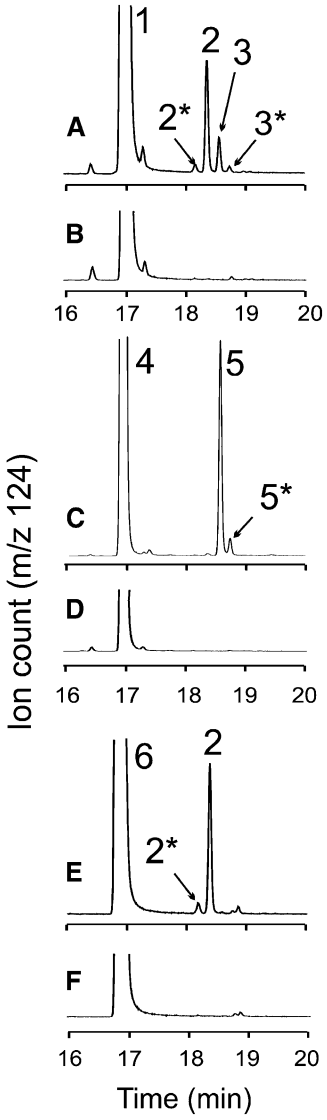


Figure 4. Conversion of Tropane Alkaloids by CYP80F1 in Yeast Microsomes

(A–F) Single ion monitoring GC/MS traces of trimethylsilylated alkaloids extracted from microsomes obtained from the yeast strains (A, C, and E) WAT11/pRL039 (expressing CYP80F1) and (B, D, and F) WAT11/pYES-DEST52 (empty vector) after incubation with (A and B) (*R*)-littorine, (C and D) (*S*)-littorine, or (E and F) hyoscyamine. Peaks were assigned to underivatized parent compounds as follows: 1, (*R*)-littorine; 2 and 2*, hyoscyamine aldehyde (*trans* and *cis* isomers, respectively, of the enol form); 3 and 3*, 3′-hydroxylittorine (*2′R,3′R* and *2′R,3′S* isomers, respectively); 4, (*S*)-littorine; 5 and 5*, 3′-hydroxylittorine (*2′S,3′S* and *2′S,3′R* isomers, respectively); 6, hyoscyamine. All chromatograms are normalized to the area of the scopolamine (internal standard) m/z = 138 peak, and the signals for (C) and (D) are attenuated 5-fold relative to those in (A), (B), (E), and (F). See Experimental Procedures for assay details.

(*2′R,3′R*)-3′-hydroxylittorine and (*2′R,3′S*)-3′-hydroxylittorine, respectively. The GC retention time and high- and low-resolution mass spectra of peak 3 matched the derivatized 3′-hydroxylittorine isolated and characterized from *H. niger* roots (see Supplemental Data). Also, methyl (*2′R,3′R*)-dihydroxy-3-phenylpropanoate and smaller amounts of methyl (*2′R,3′S*)-dihydroxy-3-phenylpropanoate were detected by chiral column

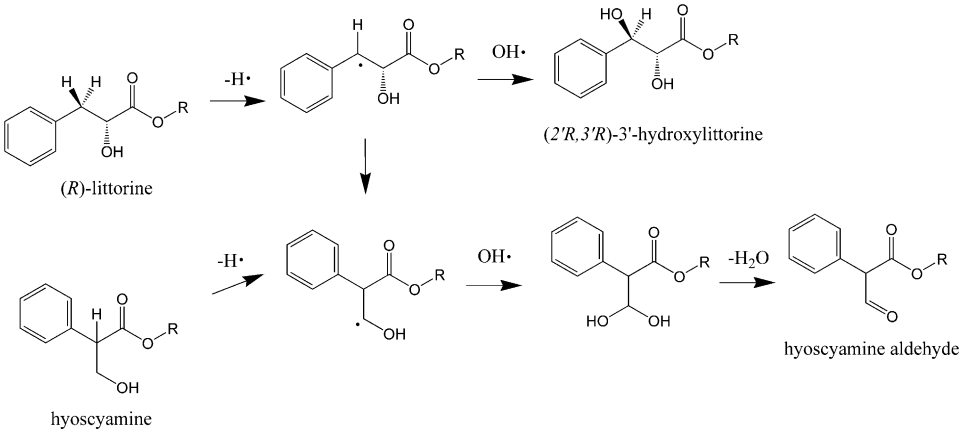


Figure 5. Tropane Alkaloid Reactions Catalyzed by CYP80F1-Containing Yeast Microsomes

Mechanistically, hydrogen abstraction at vicinal positions and oxygen rebound (shown as the formal addition of OH·) with optional rearrangement could explain the two products from littorine and hyoscyamine. Carbocation-based mechanisms are also possible [12].

GC/MS after hydrolysis and methylation of extracts of the microsomal assay (see Supplemental Data).

Thus, the major products of CYP80F1 from (*R*)-littorine are hyoscyamine aldehyde and (*2′R,3′R*)-3′-hydroxylittorine. Neither compound has been previously reported as a natural product. However, the *para*-hydroxy derivative of littorine ("4′-hydroxylittorine") reported to occur in a number of *Hyoscyamus* species [31, 32] may actually be 3′-hydroxylittorine.

In order to determine the substrate specificity of CYP80F1, other compounds were tested for activity in the yeast microsome system. The unnatural isomer, (*S*)-littorine (Figures 4C and 4D, peak 4), was shown to act as a substrate, giving the nonrearranged hydroxylation product, (*2′S,3′S*)-3′-hydroxylittorine, and a minor amount of (*2′S,3′R*)-3′-hydroxylittorine (peaks 5 and 5*, Figure 4C; Supplemental Data) when compared to products of control microsomes (Figure 4D). Interestingly, CYP80F1 was also found to oxidize hyoscyamine to hyoscyamine aldehyde (peaks 2 and 2*, Figure 4E), while control microsomes showed no new peaks (Figure 4F).

Unlike hyoscyamine, no products of CYP80F1 were detected from 3′-hydroxylittorine (data not shown). In order to test this, a source of 3′-hydroxylittorine was required. Analysis of *H. niger* roots indicated that a compound with the expected properties of 3′-hydroxylittorine was present. A total of 125 μg was isolated and characterized (see Supplemental Data). This compound represents a novel plant natural product whose occurrence tends to confirm the validity of the yeast microsome results implicating 3′-hydroxylittorine as a product of CYP80F1.

## Discussion

As late as 2005, evidence for the role of a SAM-dependent enzyme with similarities to vitamin $B_{12}$-dependent enzymes was put forth for the biotransformation of littorine to hyoscyamine [8–10]. On the other hand, Robins, O'Hagan, and coworkers have provided data and a rationale favoring the involvement of a cytochrome P450 in the first step of a two-step conversion [3, 11, 13]. The data presented here are consistent with a mechanism involving cytochrome P450. In vitro, CYP80F1 converts (*R*)-littorine mainly to hyoscyamine aldehyde and (*2′R,3′R*)-3′-hydroxylittorine. In vivo, suppression of the CYP80F1 gene by using both VIGS and RNAi with different gene fragments results in the accumulation of littorine and reduction of hyoscyamine levels and, in the case of RNAi in root cultures, a decrease in 3′-hydroxylittorine levels. In addition, expression of CYP80F1 in tobacco roots supplied with (*R*)-littorine results in the accumulation of hyoscyamine. While, individually, each of these results is subject to interpretation, taken together the results clearly support the role of a cytochrome P450, CYP80F1, in the conversion of littorine to hyoscyamine.

The precise route from littorine to hyoscyamine remains to be confirmed. Formally, 3′-hydroxylittorine could be rearranged to hyoscyamine in a pinacol rearrangement-like reaction. On the other hand, we consider it more likely that hyoscyamine is derived from the CYP80F1-formed aldehyde (route b in Figure 1). Further work is required to clarify this aspect of hyoscyamine formation. Also, the relevance of the observed oxidation of hyoscyamine by CYP80F1 requires further investigation.

The yeast expression data provide important insights into the nature of littorine mutase/monooxygenase. As summarized in Figure 5, at least three reactions can be attributed to the enzyme: (a) littorine rearrangement/dehydrogenation, (b) littorine hydroxylation, and (c) hyoscyamine dehydrogenation. In terms of the cytochrome P450 enzyme mechanism, two of these three reactions can be rationalized to involve hydrogen abstraction of the benzylic position of littorine [3, 12]. Littorine rearrangement has been discussed at length by O'Hagan and Robins [12]. Figure 5 illustrates the proposed cytochrome P450-catalyzed free radical mechanism for littorine rearrangement. An alternative carbocation-based mechanism is also possible [12]. As for typical cytochrome P450 hydroxylation reactions [33], the free radical mechanism includes hydrogen abstraction and rebound steps, but it also includes an unusual rearrangement. The resulting *gem*-diol then dehydrates to the aldehyde. The oxygen rebound and dehydration

steps resemble the mechanism proposed for cytochrome P450-mediated alcohol dehydrogenation [34].

If oxygen rebound were to occur without rearrangement, then 3′-hydroxylittorine would be formed, as observed. It is notable that the stereochemistry of the 3′-hydroxylittorine product is as expected if it shares an intermediate with the rearrangement reaction resulting from removal of the 3′-*pro-R* hydrogen [12] (see Figure 5).

The branching in the reaction mechanism for littorine shown in Figure 5 also provides a possible explanation for the observed in vivo conversion rates of fluoro-substituted phenyllactates [35]. The expected higher stabilization of the benzylic radical (or cation) in the para-substituted littorine versus the ortho-substituted isomer would be expected to favor 3′-hydroxlittorine formation at the expense of hyoscyamine aldehyde. Indeed, the conversion of 3-(4′-fluorophenyl)lactic acid to hyoscyamine in *Datura stramonium* root cultures appears to be lower than that of 3-(3′-fluorophenyl)lactic acid [35]. It is also notable that since (*S*)-littorine gives primarily a single nonrearranged product, the CYP80F1-catalyzed rearrangement appears to be highly stereospecific.

Attack at C3′ of hyoscyamine would give the aldehyde in a reaction that may share an intermediate with littorine rearrangement (see Figure 5). Thus, the products of CYP80F1 from two substrates can be rationalized by the involvement of two hydrogen abstraction steps, an optional rearrangement, followed by oxygen rebound.

## Significance

**_Hyoscyamus niger_ (black henbane) and other plants containing tropane alkaloids have long been used as medicines and hallucinogens, and tropane alkaloids remain widely prescribed pharmaceutical drugs. Here, we address a longstanding question about the nature of the enzyme(s) responsible for the conversion of (*R*)-littorine to (*S*)-hyoscyamine, a key step in tropane alkaloid biosynthesis. We demonstrate that a cytochrome P450, CYP80F1, is a littorine mutase/monooxygenase that oxidizes and rearranges littorine to form hyoscyamine aldehyde. This suggests the possibility that the (*R*)-littorine to (*S*)-hyoscyamine conversion is a two-step process and that a second enzyme, presumably an alcohol dehydrogenase, is needed to form hyoscyamine. CYP80F1 was identified through a novel functional genomics approach involving virus-induced gene silencing (VIGS) of a candidate cytochrome P450. Clear evidence for the involvement of CYP80F1 in the tropane alkaloid pathway was demonstrated by littorine accumulation and substantial reduction in pathway endproduct alkaloids in CYP80F1-suppressed plants. In addition to its littorine mutase activity, assays with recombinant CYP80F1 show that this multifunctional enzyme acts as a hyoscyamine dehydrogenase, and that it is capable of hydroxylating littorine to form 3′-hydroxylittorine. The identification of CYP80F1 and its characterization as a littorine mutase/monooxygenase may allow for the engineering of plants with increased or modified tropane alkaloid levels, and it will further the goal of reconstituting tropane alkaloid biosynthesis in microorganisms.**

## Experimental Procedures

### Chemical Synthesis

(*R*)- and (*S*)-littorine enantiomers were synthesized essentially as described previously [36] from tropine (Fluka, Oakville, ON) and 3-phenyllactic acids (Aldrich, Oakville, ON) by heating over dry HCl(g). For both enantiomers, GC (see Supplemental Data) indicated a purity of 94%–95% with an enantiomeric excess >96%.

Methyl (*2R,3R*)-2,3-dihydroxy-3-phenylpropionate was derived from ethyl (*2R,3S*)-3-phenylglycidate (a kind gift of B. Sharpless). Similarly, a mixture of diastereomers of methyl 2,3-dihydroxy-3-phenylpropionate was derived from ethyl 3-phenylglycidate (Aldrich; see Supplemental Data).

### Plant Sample Preparation

Plant samples for alkaloid analysis were lyophilized and weighed. Each sample was homogenized in 4 ml 30% (w/v) aqueous $NH_4OH$:ethanol (1:19) and then sonicated in a water bath for 20 min. The sample was centrifuged, and the supernatant was removed. The pellet was extracted twice with 2 ml aliquots of ethanol. The pooled extracts were dried under $N_2$, taken up in 1 ml 0.1 N HCl, and centrifuged. The supernatant was neutralized with 100 μl 1 M sodium carbonate (pH 10) and applied to an Extrelut QE column (1 ml capacity, EM Science, Gibbstown, NJ). After 15 min, 10 ml dichloromethane was applied and the eluate was collected. Eicosane (Aldrich) was then added to the sample as an internal standard, and the solvent was removed under a nitrogen stream. For GC or GC/MS analysis (see Supplemental Data), the residue was dissolved in 250 μl *N,O-bis*(trimethylsilyl)acetamide (BSA; Aldrich)/pyridine (1:1).

### Isolation of (*2′R,3′R*)-3′-Hydroxylittorine and Hyoscyamine Aldehyde

A total of 125 μg of a compound identified as (*2′R,3′R*)-3′-hydroxylittorine was purified from 200 g lyophilized *H. niger* roots (see Supplemental Data). Hyoscyamine aldehyde was isolated from multiple yeast microsomal CYP80F1 reactions (see below) and was identified by GC/MS and analysis of the product of $NaBH_4$ reduction (see Supplemental Data).

### Plant Materials

*H. niger* seeds were provided by Plant Gene Resource of Canada (Saskatoon, Canada). *H. niger* and *H. muticus* plants were grown in soil in a controlled environment chamber with 16 hr/23°C–24°C days and 8 hr/20°C nights under ~100 μmol/$m^2$/s light intensity. Cultured roots of *H. niger* were obtained as reported previously [37] and were grown at 25°C in the dark on a rotary shaker (100 rpm) in Gamborg B5 medium containing 3% (w/v) sucrose and 1 μM indole-3-butyric acid. Sterile *Nicotiana tabacum* cv. Xanthi shoots were maintained in vitro (hormone-free MS medium, 3% sucrose [pH 5.8], 0.8% agar).

### *H. niger* cDNA Library Construction and Expressed Sequence Tags

Total RNA was prepared from leaves or cultured roots of *H. niger* as described by Carpenter and Simon [38]. The polyA$^+$ RNA fraction was isolated with the PolyATract mRNA Isolation System (Promega, Madison, WI). Double-stranded cDNAs were synthesized, and suppression PCR was conducted by using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA) according to the protocol provided by the manufacturer. Double-stranded cDNA from cultured roots and leaves were used as "tester" and "driver," respectively. The resulting PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The resulting plasmids were used to transform ElectroMax DH10B *E. coli* cells (Invitrogen) by electroporation. The resulting $1.2 \times 10^5$ colonies were pooled and stored as a glycerol stock. DNA sequences of the inserts of randomly picked clones were determined and analyzed as described in Supplemental Data.

### VIGS in *H. muticus*

For gene silencing experiments, the plasmids pTRV1 and pTRV2 [21] were used. pTRV1 encodes tobacco rattle virus RNA1, and pTRV2 is a VIGS vector based on TRV RNA2. The cDNA clone, pRL011 (corresponding to the nucleotides 948–1270 in the CYP80F1 full-length cDNA), was selected from the *H. niger* cDNA library, and its insert was ligated into the vector pTRV2 by using appropriate restriction enzymes. pTRV1 and pTRV2 constructs were introduced separately into *A. tumefaciens* strain C58 by electroporation. The selected transformants were grown overnight at 28ºC in LB broth supplemented with kanamycin (50 mg/l) and rifampicin (50 mg/l). After centrifugation, bacteria were resuspended in buffer containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES [pH 5.6]), 10 mM $MgCl_2$, and 100 μM acetosyringone to $OD_{600}$ = 1 and were allowed to stand at room temperature for 2–4 hr. *Agrobacterium* cultures containing pTRV1 and pTRV2 constructs were mixed in 1:1 ratio and infiltrated into the underside of two or three leaves of 8-week-old *H. muticus* plants with a 1 ml syringe. Mock-infected control plants were infiltrated with *Agrobacterium* resuspension buffer. Vector control plants were infiltrated with a mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2 lacking a cDNA insert. After 5 weeks, leaf or root material was harvested for alkaloid analysis.

### Isolation of Full-Length CYP80F1 cDNA

In order to isolate the full-length CYP80F1 cDNA, 5′ and 3′ RACE reactions were performed on polyA$^+$ RNA of *H. niger*-cultured roots by using the Marathon cDNA Amplification Kit (Clontech). The full-length cDNA was cloned into the pCR2.1-TOPO vector (Invitrogen), and DNA sequences were determined for eight independent clones. One of these clones was designated pRL037. Phylogenetic analysis of the corresponding predicted amino acid sequence was performed as described in Supplemental Data.

### RNAi Suppression and Overexpression of CYP80F1 in *H. niger* and *N. tabacum*

For CYP80F1 gene suppression and overexpression in *H. niger* hairy roots and expression in tobacco hairy roots, the vectors pH7GWIW-G2(II) [39] and pMDC32 [40] were used in combination with *A. rhizogenes* (see Supplemental Data).

### Characterization of CYP80F1 Expressed in Yeast

CYP80F1 was characterized by yeast expression by using the vector pYES-DEST52 (Invitrogen) and the yeast strain WAT11 [30]. For enzyme assays, the complete reaction mixture (200 μl) contained 300 μg microsomal protein, 100 mM potassium phosphate buffer (pH 7.4), 3 mM NADPH, and 865 μM alkaloid substrate (see Supplemental Data for the details of yeast microsome preparation and enzyme assays).

### Supplemental Data

Supplemental Data including the results of GC and MS analyses, DNA sequence comparisons, RT-PCR experiments, and characterization of CYP80F1 expressed in yeast are available at http://www.chembiol.com/cgi/content/full/13/5/513/DC1/.

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## Accession Numbers

The sequence of CYP80F1 has been deposited in GenBank with accession code DQ387048.