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Enantioselective Demethylation of Nicotine as a Mechanism for Variable Nornicotine Composition in Tobacco Leaf*[S]

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Background: Nornicotine composition in tobacco leaf has been a puzzle for more than half a century.
Results: Three recombinant nicotine demethylases selectively use (*R*)-nicotine *in vitro* and *in planta*.
Conclusion: Nornicotine composition in tobacco leaf can be reasonably explained by the combination of three nicotine demethylases.
Significance: This knowledge lays the base for the optimization of nornicotine composition in tobacco leaf in the future.

Nicotine and its *N*-demethylation product nornicotine are two important alkaloids in *Nicotiana tabacum* L. (tobacco). Both nicotine and nornicotine have two stereoisomers that differ from each other at 2′-C position on the pyrrolidine ring. (*S*)-Nicotine is the predominant form in the tobacco leaf, whereas the (*R*)-enantiomer only accounts for ~0.2% of the total nicotine pool. Despite considerable past efforts, a comprehensive understanding of the factors responsible for generating an elevated and variable enantiomer fraction of nornicotine (EF_{nnic} of 0.04 to 0.75) from the consistently low EF observed for nicotine has been lacking. The objective of this study was to determine potential roles of enantioselective demethylation in the formation of the nornicotine EF. Recombinant CYP82E4, CYP82E5v2, and CYP82E10, three known tobacco nicotine demethylases, were expressed in yeast and assayed for their enantioselectivities *in vitro*. Recombinant CYP82E4, CYP82E5v2, and CYP82E10 demethylated (*R*)-nicotine 3-, 10-, and 10-fold faster than (*S*)-nicotine, respectively. The combined enantioselective properties of the three nicotine demethylases can reasonably account for the nornicotine composition observed in tobacco leaves, which was confirmed *in planta*. Collectively, our studies suggest that an enantioselective mechanism facilitates the maintenance of a reduced (*R*)-nicotine pool and, depending on the relative abundances of the three nicotine demethylase enzymes, can confer a high (*R*)-enantiomer percentage within the nornicotine fraction of the leaf.

Nicotine, nornicotine, anabasine, and anatabine are the four major alkaloids found in tobacco (*Nicotiana tabacum* L.). In most tobacco plants, nicotine represents ~90% of the total alkaloid pool, with nornicotine, anabasine, and anatabine comprising the majority of the remaining ~10%. Nornicotine is the *N*-demethylation product of nicotine, a reaction that occurs primarily in tobacco leaves. In contrast, the biosynthetic reactions responsible for nicotine, anabasine, and anatabine synthesis are specifically localized in root tissue. There are three functional tobacco nicotine demethylases that have been reported in the literature, each being members of the cytochrome P 450 superfamily of monooxygenases. CYP82E4 is the major nicotine demethylase enzyme of tobacco (1–3). The gene encoding CYP82E4 is unique in that for most tobacco plants under commercial production, its expression is suppressed and nornicotine levels are relatively low (2–4% total alkaloid). Through an unknown genetic mechanism, however, *CYP82E4* gene expression can be activated, leading to tobacco plants that convert a substantial proportion of the nicotine pool into nornicotine during senescence and leaf cure (a process of controlled senescence and maturation). The amplitude of *CYP82E4* activation is highly variable, leading to nicotine to nornicotine conversion rates ranging from <5% to 98%. Although the mechanism underlying the spontaneous activation of a dormant *CYP82E4* locus is unknown, once activated, it is stably inherited and does not revert back to the suppressed state (4).

The other two characterized nicotine demethylase enzymes are CYP82E5v2 (5) and CYP82E10 (6). In contrast to the senescence-specific expression of *CYP82E4* in leaf tissue, *CYP82E5v2* and *CYP82E10* appear to be expressed constitutively in nonsenescent green leaves and/or root tissues. Although the expression levels of *CYP82E5v2* and *CYP82E10* are low compared with *CYP82E4* levels in plants possessing a strongly activated *CYP82E4* gene (5), in tobacco plants carrying a naturally suppressed or mutagenized *CYP82E4* gene, the *CYP82E5v2* and *CYP82E10* gene products are responsible for the majority of the 2–4% nornicotine levels typically observed in these plants (6).

Recent interest in tobacco nornicotine content stems from its role as a precursor in the formation of a well characterized carcinogen. During the maturation, curing, and storage of tobacco leaves, nornicotine in the leaf may be *N*′-nitrosated to *N*′-nitrosonornicotine (NNN),[2] one of the major tobacco-specific *N*-nitrosamines whose carcinogenic properties have been documented in numerous studies (7, 8). In an effort to reduce the levels of NNN in tobacco products, for the tobacco varieties

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[S] This article contains supplemental Figs. S1–S8 and Results.
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[2] The abbreviations used are: NNN, *N*-nitrosonornicotine; EF, enantiomer fraction.

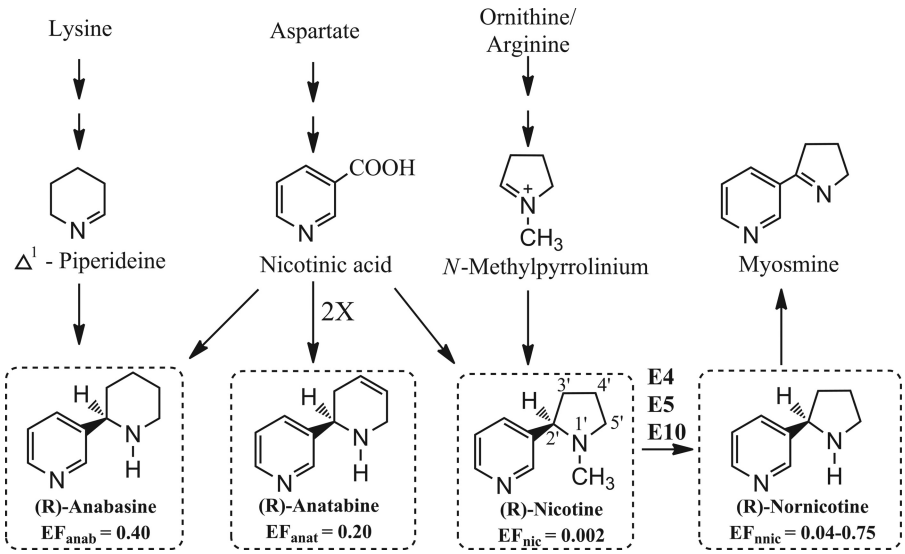

FIGURE 1. **Biosynthesis and enantiomeric composition of the four main alkaloids in *N. tabacum* L.** The four major alkaloids in tobacco are *boxed* with only the (*R*)-form structures depicted. The EF values shown were taken from leaf samples. *E4*, CYP82E4; *E5*, CYP82E5v2; *E10*, CYP82E10.

most prone to producing high nornicotine containing plants, a system has been implemented (termed the LC protocol) to identify and eliminate high nicotine-converting plants during the seed production process.

Different alkaloid enantiomers have different pharmacological activities. Both nicotine and nornicotine exist as either (*R*)- or (*S*)-enantiomeric isoforms that differ at the 2′-C position of the pyrrolidine ring. It has been reported that (*S*)-nicotine is more physiologically potent (9) and (*S*)-NNN (presumably produced from (*S*)-nornicotine) is more carcinogenic than the (*R*)-isoform (10). Due to the importance of enantiomer composition, the enantiomers of all four major tobacco alkaloids have been investigated. Of the four alkaloids, nornicotine is the only one that has a wide range of enantiomer fraction (EF) (Fig. 1). Despite extensive studies, the nornicotine enantiomeric composition in tobacco leaves cannot be explained by current data. In tobacco approximately 0.2% of the nicotine is the (*R*)-enantiomer (0.002 EF) (11), whereas nornicotine displays a higher and more variable EF_{nnic} (0.04 – 0.75) in the leaf (12, 13). Understanding the mechanisms behind the discrepancies of the enantiomeric composition between substrate and product will not only help to better understand the accumulation of enantiomers of nicotine and nornicotine in tobacco leaves, but could also provide a basis for future manipulation of the enantiomeric composition of nicotine, nornicotine, and their metabolites.

The large differences between the enantiomeric composition of the precursor nicotine and the product nornicotine have puzzled researchers for more than half a century (14). Racemization of nornicotine was proposed to explain the high percentage of (*R*)-nornicotine accumulation (15), supported by the claim that (*R*)-nornicotine was observed in leaves when feeding (*S*)-nicotine (14). However, racemization was not confirmed when tested in cell culture assays (16, 17).

Besides racemization, the significant differences in (*R*/*S*)-nornicotine ratios could be caused by enantioselective demethylation of (*R*)-nicotine as suggested in excised leaf assays (14, 18) and tobacco cell cultures (17). Although Leete and Chedekel (19) claimed that the demethylation rates of (*R*)- and (*S*)-nicotine were the same in a whole plant feeding assays, there was a slightly higher amount of (*R*)-nornicotine recovered.

Currently, three tobacco nicotine demethylase enzymes have been biochemically characterized *in vitro* (1, 2, 5, 6), but their enantioselectivities are not known. In this study, we describe the enantioselectivity of these three enzymes by using recombinant CYP82E4, CYP82E5v2, and CYP82E10 *in vitro* assays. We also show through *in vitro* and subsequently *in planta* analysis that the cooperation of the three demethylases could generate the enantiomeric composition of nornicotine observed in tobacco leaves.

EXPERIMENTAL PROCEDURES

Expression of Nicotine Demethylases CYP82E4, CYP82E5v2, and CYP82E10 in Yeast—CYP82E4, *CYP82E5v2*, and *CYP82E10* cDNAs were cloned into the yeast expression vector pYeDP60 and transformed into yeast strain WAT11 (1, 2, 5, 6). WAT11 is a yeast line engineered to enhance the expression of plant P-450s through the coexpression of the *Arabidopsis* P-450 reductase gene (20). Transformed WAT11 yeast cells were spread on synthetic galactose induction plates, and a single colony was used to inoculate 10 ml of synthetic galactose induction medium followed by growth with shaking at 30 °C for 24 h. An aliquot of this culture was diluted 1:50 into 250 ml of YPGE medium (10 g $liter^{-1}$ yeast extract, 20 g $liter^{-1}$ bactopeptone, 5 g $liter^{-1}$ glucose and 30 g $liter^{-1}$ ethanol). The culture was grown until the glucose was completely consumed as indicated by the Diastix urinalysis reagent strip. DL-Galactose was added to a final concentration of 2% (w/v) to induce expression of the transgenes. Cells in culture were grown for an additional 20 h prior to the preparation of microsomal fractions.

Yeast Microsome Preparation—Induced yeast cells were collected and used for microsome preparation (2). The collected cells were washed twice with TES buffer and TES-M buffer, followed by resuspension in extraction buffer and lysis with glass beads. The cell extracts were centrifuged for 20 min at

TABLE 1

Comparison of CYP82E4 Michaelis-Menten constants as reported in the literature

In this study, 0.5 mg ml^{-1} of microsomes from yeast overexpressing CYP82E4, CYP82E5v2, or CYP82E10 were incubated with varying amounts of nicotine for 10 min, followed by extraction and quantification of (*R*)-nornicotine and (*S*)-nornicotine. Each data point is the average of three replicates ± S.D.

Tobacco nicotine demethylase	Constant	This study		Report
		R	*S*	*RS*
CYP82E4	K_m [a]	1.90 ± 0.36	2.76 ± 0.65	3.9[b] (2)
	V_{max} [c]	0.55 ± 0.02	0.17 ± 0.01	0.54[b] (2)
CYP82E5v2	K_m	3.02 ± 1.27	2.3 ± 2.37	5.6 ± 1.4 (5)
	V_{max}	0.04 ± 0.004	0.003 ± 0.0007	0.7 ± 0.02 (5)
CYP82E10	K_m	0.78 ± 0.24	3.70 ± 5.22	3.9 (6)
	V_{max}	0.12 ± 0.01	0.001 ± 0.002	

[a] Units for K_m: μM.
[b] Results are from leaf microsomes of plants with high *CYP82E4* expression. [c] Units for V_{max}: nmol min^{-1} mg^{-1} protein.

20,000 × *g*, and the supernatant was ultracentrifuged at 100,000 × *g* for 90 min. Pellets containing the microsomal protein were suspended in TEG-M buffer. Protein concentration was determined using the Bradford protein assay.

In Vitro Enzyme Assays—Nicotine demethylase activity was assayed in a reaction mixture (20 μl) containing 0.5 mg ml^{-1} microsomal protein, 2.5 mM NADPH, 25 mM Tris buffer (pH 7.5) and varying amounts of (*R*)-, (*S*)-, or racemic [2′-^{14}C]nicotine. Five μM nicotine substrate was used in validation assays. Recovery of nicotine and nornicotine for all three assays ranged from 92 to 108%. (*R*)- and (*S*)- [2′-^{14}C]nicotine were separated from racemic [2′-^{14}C]nicotine (Moravek Biochemicals and Radiochemicals). (*R*)- and (*S*)-nicotine were base line separated by chiral high performance liquid chromatography (HPLC) (supplemental Fig. S1). The details of the chiral HPLC are described below. (*R*)-Nicotine comprised 49.8% of the original racemic [2′-^{14}C]nicotine analyzed. Nornicotine contamination in the [2′-^{14}C]nicotine was <0.5%. Reaction mixtures were incubated at room temperature for 10 min, and the reactions were stopped by addition of 20 μl of methanol containing 50 mM nicotine and nornicotine.

Nicotine demethylation was measured by resolving the nicotine and nornicotine in 10 μl of reaction mixture by thin layer chromatography (TLC) using Silica Gel 60 F254 plates (EMD Chemicals Inc.). The developing solvent for TLC was chloroform:methanol:ammonia hydroxide (85:15:2, v/v/v). [^{14}C]Nicotine and [^{14}C]nornicotine were scraped from the plates and quantified using a liquid scintillation counter (1900 TR, Packard Instrument Company). To determine EF_{nnic}, the remaining reaction mixture of each sample was used to separate the nicotine and nornicotine by TLC, and the nornicotine was methylated to nicotine by incubating for 30 min with 50 μl of formic acid and 100 μl of formaldehyde at 110 °C. The nicotine was base extracted by MTBE and collected after further separation into (*R*)- and (*S*)-enantiomers by chiral HPLC (17). A PerkinElmer Life Sciences series 200 HPLC was used with a Chiralcel OD-H column (0.46 cm (D) × 25 cm) (Chiral Technologies Inc.) and eluted with hexane:methanol (98:2, v/v) at 1.0 ml min^{-1}, with detection at 252 nm. (*R*) and (*S*) collections were quantified by liquid scintillation counting and data were analyzed using Sigmaplot 12 (Systat Software, San Jose, CA). Enantiomer fraction (EF) was calculated as: EF = *R* enantiomer/(*R* enantiomer + S enantiomer) (21).

To test the specificity of the three demethylases, methylanabasine was incubated with CYP82E4, CYP82E5v2 and CYP82E10 *in vitro*. After a 10 h incubation time, the reactions were stopped by base extraction with MTBE containing quinoline as an internal standard. Extracts were analyzed by GC-MS with a Varian CP-3800 GC coupled to a Varian Saturn 2200 MS/MS (Varian Medical Systems) using a Supelco SLB-5ms fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Supelco). Initial oven temperature was set at 150 °C for 0.5 min, increased 15 °C min^{-1} to 170 °C, then 1.5 °C min^{-1} to 195 °C, held for 2 min, then 20 °C min^{-1} to a final temperature 300 °C, and held for a final 20 min.

Plant Materials—TN 90LC is a standard commercial burley tobacco cultivar with a low tendency to demethylate nicotine to form nornicotine. Burley tobacco breeding line DH98-325-6 (with a very high propensity to demethylate nicotine to form nornicotine) was used as parent in a mutation breeding experiment to develop genetic stocks possessing different combinations of knock-out mutations in *CYP82E4*, *CYP82E5*, and *CYP82E10.* Development of these mutant lines has been described previously (6).

The original DH98-325-6 line along with seven derived mutant lines were evaluated at the Upper Coastal Plain Research Station (Rocky Mount, NC) during 2010, and the evaluation was repeated at Spindletop Farm (Lexington, KY) in 2011, both using standard production and curing practices. TN 90LC was added to the 2011 experiment. After curing, the fourth leaf from the top of each stalk was collected and used to quantify alkaloid contents and their enantiomer profiles. All the samples were oven-dried (55 °C) and ground to pass through a 1-mm sieve.

Nicotine and nornicotine were quantitatively analyzed by gas chromatography (GC) (PerkinElmer Life Sciences Autosystem XL with Prevent^{TM}) according to the LC protocol. The absolute amounts of nicotine and nornicotine isomers were calculated based on total amount and R/S ratio. Data were analyzed by Sigmaplot 12 (Systat Software, San Jose, CA).

RESULTS

Enantioselectivity of CYP82E4, CYP82E5v2, and CYP82E10—To test the enantioselectivity of CYP82E4 for nicotine, different amounts of (*R*)- or (*S*)- [2′-^{14}C]nicotine were incubated with the enzyme preparations (supplemental Results and Fig. S8). The V_{max} for (*R*)-nicotine (0.55 nmol min^{-1} mg^{-1} protein) was 3-fold higher than the V_{max} for (*S*)-nicotine (0.17 nmol min^{-1} mg^{-1} protein), and there was no significant difference between the K_m values of (*R*)-nicotine *versus* (*S*)-nicotine (Table 1). The

V_{max} of (*R*)-nicotine was close to the previously reported V_{max} using racemic nicotine as substrate (0.54 nmol min^{-1} mg^{-1} protein) (2). Results from inhibition assays illustrate the competitive inhibition between the two nicotine enantiomers (supplemental Fig. S2).

Although CYP82E5v2 and CYP82E10 account for <5% of the nicotine demethylation observed in plants that accumulate high levels of nornicotine, they are responsible for the majority of the nornicotine found in plants with a nonactivated or mutant *CYP82E4* gene (6). In addition to CYP82E4, the enantioselectivities of CYP82E5v2 and CYP82E10 were also determined (Table 1). Enzyme kinetic analyses showed that CYP82E5v2 and CYP82E10 almost exclusively used (*R*)-nicotine over (*S*)-nicotine, and in both cases the V_{max},*R* was >10-fold higher than V_{max},*S*. Compared with the Michaelis-Menten constants found in the literature for these enzymes, the K_m,*R* values for CYP82E4 and CYP82E5v2 in this study were about half of those derived in the previous studies using racemic nicotine and about one fifth of the reported value for CYP82E10 (Table 1). After demethylation of either (*R*)- or (*S*)-nicotine, only the corresponding form of the nornicotine enantiomer was detected in these assays (supplemental Fig. S3*A*), consistent with the results from leaf disc feeding assays (supplemental Fig. S3*B*). Therefore, no racemization was observed during the CYP82E4-, CYP82E5v2-, and CYP82E10-catalyzed demethylation reactions.

Combination of the Three Demethylases Can Generate Leaf-like Nornicotine Compositions in Vitro—After demonstrating the enantioselectivity of the three nicotine demethylases, the next question asked was whether they could convert a low *R* percentage of nicotine into the high *R* percentages of nornicotine reported in the literature. Plants with all three demethylases silenced by knock-out mutation accumulated approximately 3% of the total nicotine pool as (*R*)-nicotine (Fig. 4*B*). Therefore, 3% (*R*)-nicotine was assumed to be the nicotine composition at the time of synthesis and was used in the subsequent assays.

In tobacco plants, nicotine is primarily stored within cellular vacuoles, whereas P-450 enzymes of the class that includes CYP82E4, CYP82E5v2, and CYP8210 are anchored on the endoplasmic reticulum membrane via a noncleaved N-terminal signal sequence with the rest of the protein facing the cytosol (2). Although the concentration of endogenous nicotine is 60 mM in the vacuoles of the leaf tip (22), which is a level much higher than that needed to drive the maximal rate of nicotine demethylation, the actual concentration of nicotine in the cytosol that is accessible for the demethylase enzymes is not known. Therefore, we assayed large range of nicotine concentrations *in vitro*. Regardless of the initial nicotine concentration, however, no differences were observed in the resulting nornicotine enantiomer ratios (supplemental Fig. S4*A*). Nicotine substrates with different (*R*/*S*) ratios were also tested in *in vitro* CYP82E4 assays to determine the relationship between (*R*)-nicotine substrate and (*R*)-nornicotine produced in the presence of (*S*)-nicotine (supplemental Figs. S4*B*). Because there was no concentration effect *per se* on the product profile, (*R*)-nicotine mixtures with variable concentrations were used to cover a wide range of (*R*/*S*)-nicotine ratios as substrate. Based on the results presented in supplemental Fig. S4*B*, it would require a EF_{nic} from 0.008 to 0.27 to obtain the 0.04–0.75 EF_{nnic} reported in the literature. These values of (*R*)-nicotine, particularly the upper end, are much higher than found in tobacco plants and are also greater than the nicotine composition in the plants with the three demethylase knock-out mutations, which implies the involvement of demethylases other than CYP82E4.

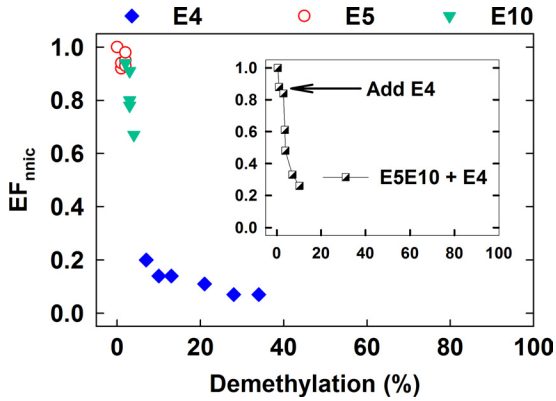

FIGURE 2. **Generation of leaf-like nornicotine enantiomeric compositions using CYP82E4 (*E4*), CYP82E5v2 (*E5*), and CYP82E10 (*E10*) *in vitro* assays.** Nicotine solutions (0.03 EF_{nic}) were incubated with each of three demethylases separately or collectively (*inset*), and the nornicotine enantiomeric composition was analyzed at varying incubation times. For collective incubation assays, equivalent amounts of CYP82E5v2 and CYP82E10 were mixed and incubated with the substrate, followed by the addition of an equal amount of CYP82E4 after 30 min. Total protein for all single and collective enzyme experiments was the same. Each data point represents the average of two independent replications. An alternative representation of these data can be found in supplemental Fig. S5.

Each of the three tobacco nicotine demethylases was incubated with 3% (*R*)-nicotine in a time course study, and product compositions at different reaction times were observed for each demethylase (Fig. 2 and supplemental Fig. S5). During a 3-h reaction, <5% of the total nicotine substrate was demethylated by CYP82E5v2 and CYP82E10, and the nornicotine formed consisted of >70% of the (*R*) form, which reaches the upper limit of EF_{nnic} found in tobacco plants. In contrast to CYP82E5v2 and CYP82E10, CYP82E4 demethylated >30% of the nicotine in a 3-h reaction, and the nornicotine product consisted of 5–20% (*R*) form, which is close to the lower limit of EF_{nnic} found in tobacco plants. It was thereby logical to speculate that a mixture of the three nicotine demethylases could potentially produce nornicotine with the full range 0.04–0.75 EF_{nnic} from an initial nicotine pool of 0.03 EF_{nic}. To test the combined effects of the three demethylases on nornicotine composition, a mixture of equal amounts of CYP82E5v2 and CYP82E10 protein was first incubated with 0.03 EF_{nic} nicotine for 30 min, followed the addition of an equivalent amount of CYP82E4 protein for another 2.5 h (Fig. 2 *inset*). The EF of the nornicotine product decreased continuously as the duration of the incubation time increased, resulting in a wide range of EF_{nnic}.

Substrate Specificity of CYP82E4, CYP82E5v2, and CYP82E10—A series of nicotine analogues have been shown to be metabolized in tobacco through *N*-dealkylation processes (18, 23–26) (Fig. 3). The tobacco used in these reports all had a high propensity for demethylating nicotine. What was unknown, however, was whether nicotine and the nicotine ana-

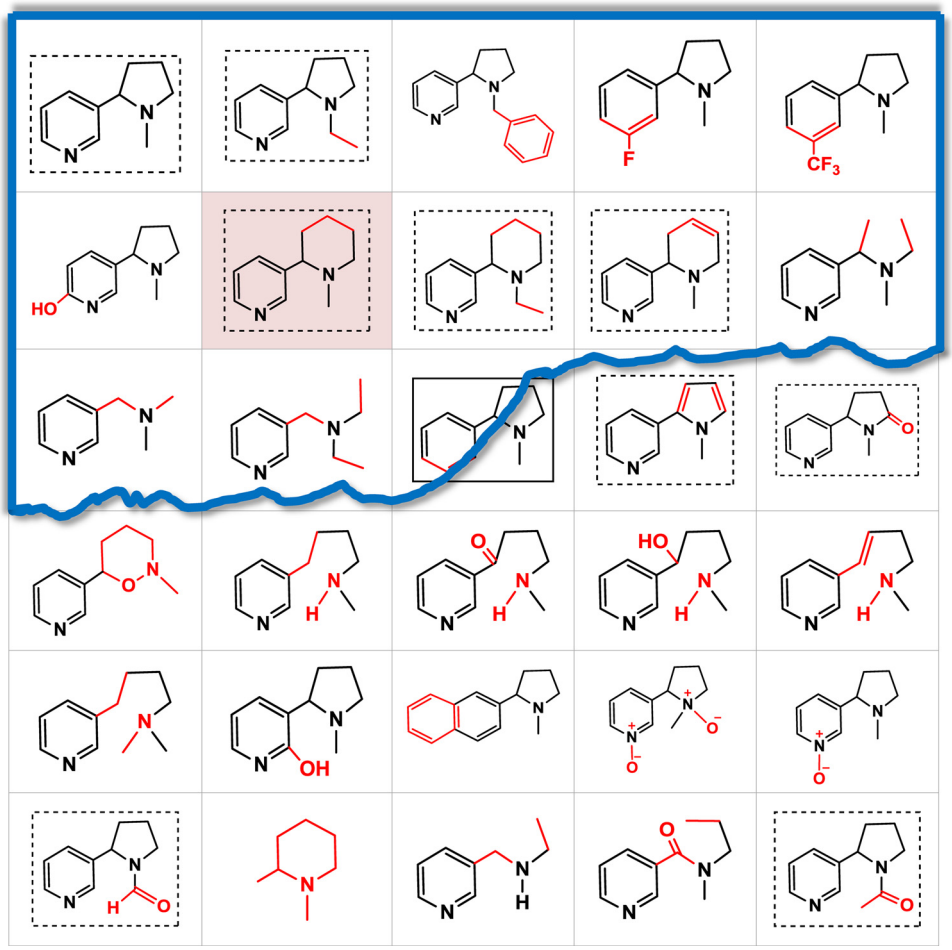

FIGURE 3. **Nicotine and nicotine analogues of tobacco.** The compounds depicted represent those that have been previously fed to tobacco leaves and cells, followed by analysis for potential *N*-dealkylation products (18, 23–26). Compounds shown to be dealkylated in tobacco are encompassed by the *bold blue line*. Reports regarding (*R*,*S*)-1-methyl-2-phenylpyrrolidine (*solid border*; cut in half by the *blue line* on the figure) have been inconsistent. Nicotine is depicted in the *top left corner*, and all structural deviations from nicotine are shown in *red*. The methylanabasine substrate tested in the current study is *background-shaded* in *red*. Compounds surrounded with a *dashed border* are found naturally in tobacco.

logues were metabolized by the same enzyme. One of these compounds, methylanabasine (highlighted by a *red background* in Fig. 4), was chosen to test the substrate specificities of the three nicotine demethylases. Very low concentrations of methylanabasine can be found naturally in tobacco leaves (27), and this compound can also be formed in tobacco and *Nicotiana glauca* by aberrant biosynthesis through the feeding of *N*′-methyl-Δ^1-piperideinium choride (28). Anabasine was formed from methylanabasine by microsome preparations of yeast expressing CYP82E4, CYP82E5v2, and CYP82E10; product identity was confirmed by GC-MS (supplemental Fig. S6). These results suggest that the three nicotine demethylases can potentially act on a broad range of substrates.

Effects of Nicotine Demethylase Enzymes on Enantiomeric Composition of Nicotine and Nornicotine Level in Air-cured Leaf Laminas—To determine the effects of nicotine demethylase enzymes *in vivo*, three genetic stocks with different combinations of mutations in the three nicotine demethylase genes (6) were grown in the field during 2010, and samples of the air-cured leaf were analyzed for alkaloid levels and nicotine and nornicotine enantiomeric composition. The experiment was repeated in 2011, adding the tobacco line TN 90LC (Fig. 4) as a control.

With all three nicotine demethylases silenced (genotype = *e4e4e5e5e10e10*), 3% of nicotine was accumulated as the (*R*)-enantiomer (EF_{nic} = 0.03) (Fig. 4*B*). Any of the three demethylase enzymes can demethylate (*R*)-nicotine and reduce EF_{nic} to under 0.002 (Fig. 4*B*). Only CYP82E4 can significantly convert (*S*)-nicotine to (*S*)-nornicotine. All of these results were consistent with the selectivity of the three demethylases *in vitro*. With different combination of mutations in nicotine demethylase genes, a broad range of EF_{nnic} is observed, consistent with *in vitro* assays.

DISCUSSION

The hypothesis of enantioselective demethylation was proposed to explain how the wide range of EF_{nnic} in tobacco leaves could result from an initial nicotine pool with a low EF. *In vitro*, all three nicotine demethylases, CYP82E4, CYP82E5v2, and CYP82E10, demethylated (*R*)-nicotine faster than (*S*)-nicotine, but they exhibited very different product accumulation patterns. Although considered as minor nicotine demethylases in tobacco, CYP82E5v2 and CYP82E10 have very strong, if not exclusive, selectivity for (*R*)-nicotine and can produce 0.75 EF_{nnic} from 0.03 EF_{nic}. CYP82E4 can demethylate both (*R*)- and (*S*)-nicotine, and the highest EF_{nnic} produced by CYP82E4 *in vitro*

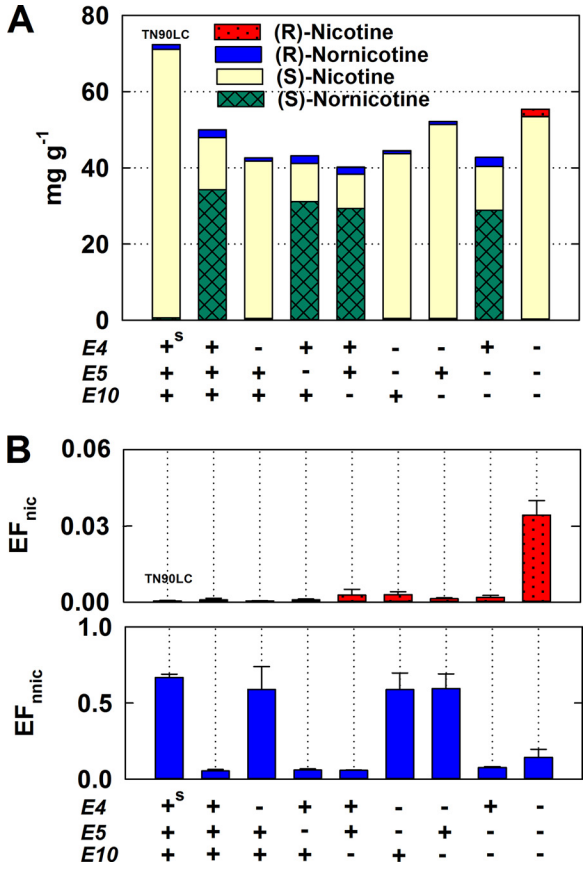

FIGURE 4. **Effects of nicotine demethylase enzymes on nicotine, nornicotine enantiomers in air-cured leaf lamina in 2010 and 2011.** *A*, changes of enantiomer levels of nicotine and nornicotine in nicotine demethylase mutants. *B*, effects of nicotine demethylases on nicotine and nornicotine composition. Each *bar* is the average of 2 years of results. TN 90LC data are from 2011 only. The *error bars* represent S.D. + and − below the *bars* indicate the presence/absence of a functional demethylase gene. TN 90LC has functional *CYP82E4* gene, but the gene expression is suppressed ($+^{s}$). *E4*, *CYP82E4*; *E5*, *CYP82E5v2*; *E10*, *CYP82E10*.

from 0.03 EF_{nic} of nicotine substrate was 0.20. The selectivity of three nicotine demethylase enzymes shown *in vitro* results was consistent with *in planta* results. The EF_{nnic} would be predicted to decrease even further as CYP82E4 catalyzed demethylation continued. Based on the *in vitro* and *in planta* results, we propose a model to explain the variable nornicotine enantiomeric compositions observed in tobacco leaves (Fig. 5). In tobacco, expression of CYP82E5v2 and CYP82E10 is low level constitutive, whereas in plants with an activated *CYP82E4* gene, CYP82E4 expression levels are high, but very specific to senescence. So newly synthesized nicotine with a high EF_{nic} could have the (*R*)-nicotine preferentially demethylated by CYP82E5v2 and CYP82E10 prior to induction of CYP82E4 and thus initially yield EF_{nnic} values that are very high. During senescence, however, CYP82E4 demethylates both (*R*)- and (*S*)-nicotine and reduces the EF_{nnic}. Adding to this complexity is the fact that tobacco plants can display varying degrees of *CYP82E4* activation, ranging from completely inactive to strongly expressed, as evidenced by the variable range of nornicotine accumulation phenotypes documented in tobacco. Therefore, the relative contributions of CYP82E5v2 and CYP82E10 *versus* CYP82E4 could also vary considerably among plants within a given tobacco population. In sum, the high and variable EF_{nnic} relative to EF_{nic} observed in tobacco can be reasonably explained by the combined actions of CYP82E4, CYP82E5v2, and CYP82E10.

In tobacco, *CYP82E5v2* and *CYP82E10* are dominant, but not additive (6). It is possible that this phenomenon could also be explained by the observation of the enantioselectivities of CYP82E5v2 and CYP82E10. Because CYP82E5v2 and CYP82E10 almost exclusively use (*R*)-nicotine, and the (*R*)-isomer only accounts for ~3% of the initial nicotine pool, the expression of either gene individually may be enough to deplete that amount of (*R*)-nicotine present in the plant. In addition, the promiscuity of CYP82E4 toward both (*R*)- and (*S*)-isomers could explain the results that addition of a functional *CYP82E5v2* and *CYP82E10* to plants with a highly activated *CYP82E4* gene does not cause further increase in observed nicotine demethylation (6). Finally, the selectivity of CYP82E4, CYP82E5v2, and CYP82E10 may also explain the choice of a 3% conversion limit in what has been termed the LC protocol. The LC protocol is a standard practice used by tobacco breeders to remove plants with a high propensity for nicotine demethylation during seed production to minimize the number of progeny that will accumulate high levels of nornicotine (and therefore the NNN carcinogen). Of the three nicotine demethylases, CYP82E4 is responsible for >90% of the nicotine demethylation in plants with an activated *CYP82E4* gene, so such plants should be excluded from seed production. The results from this study suggest that CYP82E5v2 and CYP82E10 can use no more than ~3% of the available nicotine, so any plant with >3% demethylation would be predicted to contain a *CYP82E4* gene that is expressed to some degree. Thus, although the selection of a 3% conversion threshold in the LC protocol was chosen in the absence of knowledge regarding the function and expression profiles of the nicotine demethylase gene family, it appears that this percentage roughly represents the level of nornicotine production that could be attributed to CYP82E5v2 and CYP82E10 alone. In contrast, plants displaying nornicotine levels higher than 3% are likely to have a heritable *CYP82E4* gene that has been activated to some extent and can thus be selected against by eliminating all plants from the seed production populations with nornicotine levels above 3%.

Racemization has been proposed to explain the high (*R*/*S*) ratios of nornicotine, but racemization would unlikely be responsible for variable nornicotine enantiomeric composition in *planta*. The nicotine demethylases CYP82E4, CYP82E5v2, and CYP82E10 are cytochrome P-450 enzymes (1, 2, 5, 6), and nicotine demethylation proceeds by oxidation of the *N*-methyl group (29). Based on *N*-dealkylation reactions catalyzed by P-450s (30), it has been proposed that demethylation occurs without any alteration at the 2′-carbon of the pyrrolidine ring; thus (*S*)-nicotine should yield (*S*)-nornicotine, and the demethylation of (*R*)-nicotine should yield (*R*)-nornicotine (supplemental Fig. S7). Both the *in vitro* and leaf disc feeding assays support the concept that racemization during or after demethylation is not a mechanism involved in influencing the enantiomeric composition of nornicotine (supplemental Fig. S3).

In planta there may be other factors contributing to the nornicotine enantiomeric composition that are not reflected in the

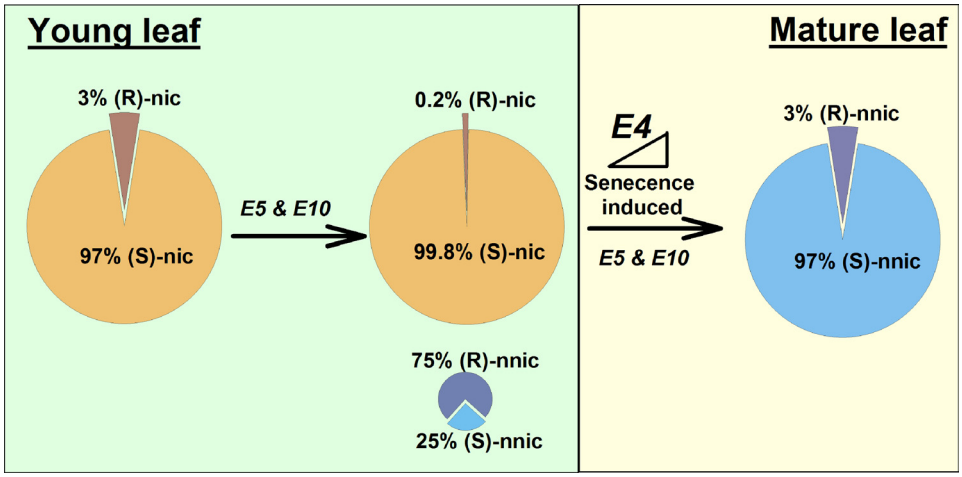

FIGURE 5. **Proposed influences of the CYP82E4 (*E4*), CYP82E5v2 (*E5*), and CYP82E10 (*E10*) nicotine demethylases on nicotine and nornicotine enantiomeric compositions in tobacco leaves.** The sizes of *pies* represent the relative abundance of (*R*)- and (*S*)-nicotine and nornicotine.

in vitro system employed in this study. Although nornicotine is mainly synthesized in the leaf (31), some nornicotine can also be produced in the root (32) and be translocated to the leaf-like nicotine. It is possible that differential selectivities of the various transporters involved in the translocation of nornicotine from the root to leaf could influence the nornicotine composition in leaf. Furthermore, mutant plants with all three demethylases knocked out still contained some nornicotine, which suggests the possibility of direct synthesis of a low level of nornicotine via a route other than nicotine demethylation (6). Reports that (*S*)-nornicotine is degraded faster than (*R*)-nornicotine could also account for some of the increased (*R*/*S*)-nornicotine ratios and introduces yet another level of complexity *in planta* (17, 33).

It has been shown in tobacco cell cultures and leaf feeding assays that a wide range of nicotine analogues can be used as substrates, yielding *N*-dealkylation products. Methylanabasine, one of the analogues metabolized in tobacco, was shown to be substrate of the nicotine demethylases. Therefore, the demethylation of nicotine could be one specific example of a general *N*-dealkylation reaction catalyzed by the tobacco nicotine demethylases. Similar situations for nicotine demethylases are reported in human. In addition to demethylating nicotine, recombinant human CYP2A6, CYP2B6, and CYP2A13 enzymes expressed in baculovirus-infected insect cells can catalyze the demethylation of a broad range of substrates (34).

(*S*)-Nicotine is more physiologically potent than the (*R*)-enantiomer (9), and it has been speculated that from an evolutionary point of view nicotine accumulates in tobacco to deter herbivores (35). Therefore, an evolutionary selection could be operative for plants that produced the more potent form, (*S*)-nicotine with lower demethylation of the (*S*) than (*R*)-enantiomers being favored. The enantiomer-specific synthesis of nicotine is believed to be catalyzed by nicotine synthase, an enzyme that has not been genetically or biochemically characterized. To date, only a putative enzyme mixture (36) and two candidate, yet unconfirmed genes, *A622* (37, 38) and a gene encoding berberine bride enzyme-like protein (39), have been reported. The results presented here demonstrate a selective demethylation of (*R*)-nicotine *in vitro* and the presence of an initial 0.03 EF_{nic} as determined using nicotine demethylase-deficient plants (Fig. 4). These findings and observations suggest that the originally synthesized (*R*)-nicotine is sufficient to account for the reported 0.2% of total nicotine (0.002 EF_{nic}) typically reported in tobacco.

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