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Variable Nornicotine Enantiomeric Composition Caused by Nicotine Demethylase CYP82E4 in Tobacco Leaf

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Supporting Information

ABSTRACT: Nornicotine is the demethylation product of nicotine and the precursor of tobacco-specific nitrosamine *N*′-nitrosonornicotine (NNN) in tobacco (*Nicotiana tabacum* L.). There is an inconsistent enantiomer fraction (EF) of nornicotine reported in the literature. The objective of this study was to explore possible mechanisms to account for the variable EF_{nnic} in tobacco. A survey of tobacco with different demethylating capabilities confirmed that there was variable EF_{nnic}. Experiments of induction and inhibition of the major nicotine demethylase CYP82E4 activity in tobacco demonstrated that CYP82E4 selectively demethylated (*S*)-nicotine and resulted in different EF_{nnic} in tobacco leaves. Results from plants with silenced demethylases by RNAi suggested that other demethylases selectively used (*R*)-nicotine and resulted in high EF_{nnic}. In summary, enantioselective demethylation likely plays an important role in contributing to a large and variable EF_{nnic} observed in tobacco.

KEYWORDS: *nornicotine, N′-nitrosonornicotine, nicotine demethylase, enantiomeric composition, Nicotiana tabacum*

INTRODUCTION

Nornicotine is one of the four major alkaloids in *Nicotiana tabacum* L. Nornicotine is, at least mainly, synthesized by demethylation of nicotine in leaf and root. The nornicotine in root is transported to leaves and accumulates in the vacuole.[1] However, the details of nornicotine biosynthesis and translocation are not clear, and recently published results of nicotine and nornicotine enantiomeric composition cannot be explained on the basis of current knowledge. The enantiomeric fraction of nornicotine (EF_{nnic}) (0.05−0.70), proportion of *R* enantiomer compared to total nornicotine, is much higher than what is expected from enantiomeric fraction of nicotine (EF_{nic}) (0.001−0.004) (Table 1).

Table 1. Enantiomer Fraction, EF, of Nicotine and Nornicotine in Tobacco (*Nicotiana tabacum* L.)

EF	material
Nicotine	
0.001−0.004[34]	leaves
<0.025[38]	leaves
Nornicotine	
predominantly *R*[39]	roots
0.14−0.25[40]	leaves
0.10−0.40[41]	leaves
0.30−0.70[30]	leaves
0.05−0.43[38]	leaves

Investigating nornicotine biosynthesis has both fundamental metabolic and practical applications. Nornicotine has received much attention due to its relationship to tobacco-specific nitrosamine *N*′-nitrosonornicotine (NNN). NNN is carcinogenic in many bioassays[2,3] and is the nitrosation product of nornicotine mainly during the tobacco curing[4,5] and also in human saliva.[6] Understanding nornicotine biosynthesis and accumulation will greatly facilitate the interpretation of the enantiomeric components of nicotine and NNN (Figure 1). Chiral compounds with very similar physical and chemical

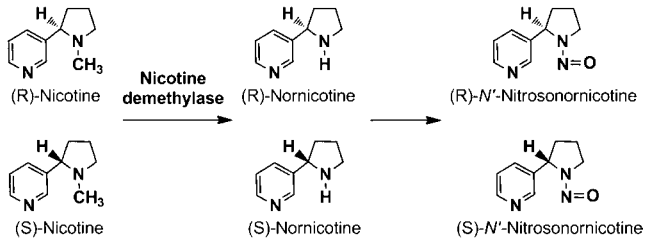

Figure 1. Structures of *R* and *S* enantiomers of nicotine, nornicotine, and *N*′-nitrosonornicotine.

properties in achiral environments generally exhibit different biological and toxicological activities, because each enantiomer can enantioselectively interact with enzymes and biological receptors in organisms.[7] For example, the carcinogenicity of (*S*)-NNN is suggested to be greater than that of (*R*)-NNN in rat esophagus,[8,9] confirmed by the recent rat feeding assay.[10] Nicotine also exhibits different biological activities.[11] (*R*)-Nicotine has many of the same physicochemical properties as (*S*)-nicotine, but (*S*)-nicotine has a greater level of toxicity. LD_{50} values for intravenous administration of (*R*)-nicotine in several species of animals have been approximately 18 times higher than that of (*S*)-nicotine, which means that (*R*)-nicotine is less potent. This suggests a potential application for (*R*)-nicotine as a therapeutic agent.[11] Since nornicotine is the major metabolite of nicotine and precursor of NNN, we may

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minimize the harmful effects of cigarettes through adjusting the enantiomeric ratio of nicotine and NNN.

There are several mechanisms potentially responsible for the large and variable EF_{nnic} (Figure 2). Among these putative

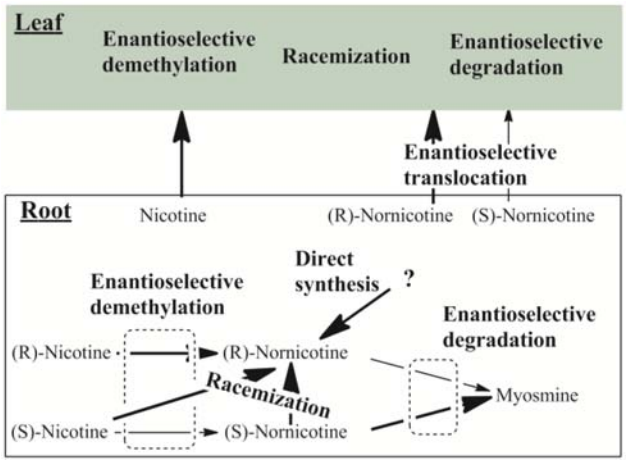

Figure 2. Possible mechanisms to account for the high and variable EF_{nnic} in tobacco leaf.

reasons, enantioselective demethylation likely plays a major role. Stereoselective translocation, enantioselective metabolism, and direct synthesis may contribute along with enantioselective demethylation. Racemization is unlikely a reason for variable nornicotine composition, but we cannot exclude the possibility.

The most plausible explanation for variable composition of nornicotine is enantioselective demethylation of nicotine. Demethylation rates of (*R*)- and (*S*)-nicotine have been reported to be different.[12,13] Exogenous nicotine feeding assays in cell culture[12] and plant tissue[13] demonstrate the rate of (*R*)-nicotine demethylation is higher than that of (*S*)-nicotine. There are three functional nicotine demethylases in tobacco.[14−17] Each demethylase may have its own preference for nicotine enantiomers. Throughout the life cycle of a tobacco plant, different expressions and activities of nicotine demethylases could contribute to the variable values of nornicotine enantiomeric composition.

Also, stereoselective degradation of (*S*)-nornicotine may contribute to the variable EF_{nnic}. Kisaki and Tamaki[18] found that (*R*)-nornicotine was recovered more when feeding (*R*)- and (*S*)-nornicotine to excised leaves of *N. tabacum,* respectively. This result implied the enantioselective degradation of (*S*)-nornicotine. Cell culture feeding assay supported Kisaki and Tamaki's work, but myosmine level, the main product of nornicotine degradation, was the same.[12] This could be due to the possibility that myosmine is degraded as fast as it is formed, or that other intermediates are involved.

Stereoselective translocation may play a minor role. Transporters which are strictly stereoselective are found in plants[19] and animals.[20] The nornicotine accumulated in tobacco leaf comes from the root and from nicotine demethylation in the leaf. Selective translocation of nicotine or nornicotine could change the nornicotine composition. Until now, the transporters reported[21−23]only transport nicotine among different cellular compartments. Recently the long-rang translocation of nicotine and nornicotine is found to be dominantly suppressed in *Nicotiana alata,* [24] but the mechanisms behind the long-distance translocation have not been identified.

Nornicotine may be directly synthesized which would be a possible explanation of the varying EF_{nnic}. In the biosynthetic pathway of nicotine, putrescine is first *N*-methylated by putrescine *N*-methyltransferae. The product *N*-methylputrescine is then deaminated oxidatively to 4-methylaminobutanal, which spontaneously cyclizes to give the *N*-methylpyrrolinium. This oxidative deamination reaction is catalyzed by *N*-methylputrescine oxidase (MPO). The *N*-methylpyrrolinium condenses with nicotinic acid-derived metabolite 1,2-dihydropyridine to give nicotine in tobacco.[25] In addition to its preferred *N*-methylputrescine substrate, recombinant MPO1 enzyme could to a lesser degree utilize putrescine, probably resulting in an unmethylated pyrrolinium salt.[26] If the nicotine synthase can use this unmethylated pyrrolinium salt, nornicotine could be directly produced, bypassing nicotine. Mutant plants with knockouts of all three demethylases still contain some nornicotine, implying the existence of the direct synthesis of nornicotine.[17]

Reports about nicotine and nornicotine racemization are not conclusive. Racemization during[27] or after demethylation[25] was proposed for (*R*)-nornicotine production. (*R*)-Nornicotine may come from the racemization of (*S*)-nornicotine.[25] Chemically nornicotine may be racemized in the presence of pyridoxal,[28] which is present in green plants as natural forms of vitamin B_6. Nornicotine derived from pure (*S*)-nicotine was partially racemized in *N. tabacum* during demethylation, and feeding (*S*)-nornicotine only (*S*)-nornicotine was recovered, implying that the racemization occurs during demethylation.[29] However, feeding one form of nicotine to cell cultures[12] and tobacco leaves[30] only resulted in the corresponding form of nornicotine being recovered which makes racemization the unlikely explanation for (*R*)-nornicotine production.

In this paper, EF_{nnic} values in different tobacco lines and tissues were investigated to validate the variable results in the literature. Induction and suppression of nicotine demethylase CYP82E4 demonstrate that CYP82E4 reduces EF_{nnic} in tobacco and produces a variable EF_{nnic}.

MATERIALS AND METHODS

Plant Materials. All plants used in this study were *Nicotiana tabacum* L. TN 90LC represents low nicotine demethylation (low converter) plants. For several RNAi plants, burley tobacco breeding lines DH98-325-5 and DH98-325-6 were transformed with 298-bp of *CYP82E4* cDNA to silence *CYP82E4* and its closely related homologues. On the basis of PCR and ultralow demethylation phenotype, R2 families of stable expressing the *CYP82E4*-silenced condition were used in this study. Details of generation and growth conditions of low converter and RNAi plant have been described in a previous paper.[31] Details of development of mutants is described in a previous paper.[17] Both low converter and RNAi plants were grown at Spindletop farm in Lexington (KY) in 2006, and were topped and sampled at mature growth stage. RMS2 represents high nicotine demethylation (converter) plants which have high nornicotine accumulation. Converter plants were grown at Spindletop farm in Lexington (KY) in 2007 and were sampled at flowering stage. RNAi lines from different parents (Figure 7 and Table 4S) were grown in Blackstone, VA. All the samples were freeze-dried and ground for further individual alkaloids content and nornicotine enantiomers analysis.

Alkaloids Quantification and Separation of Enantiomers of Nornicotine. Nicotine, nornicotine, anabasine, and anatabine were quantitatively analyzed by gas chromatography (GC) (Perkin-Elmer Autosystem XL with Prevent) according to the LC-Protocol.[32] Alkaloids of ground tobacco samples were extracted by methyl *tert*-butyl alcohol (MTBE) and aqueous sodium hydroxide. The MTBE

extracts was injected into GC, and quantification of alkaloids was against chemical standards.

Nornicotine enantiomer analysis was done by extracting ground tobacco samples with MTBE and aqueous sodium hydroxide. Nornicotine in MTBE extract was purified by thin layer chromatography (TLC). TLC plates were TLC Silica Gel 60 F254 (EMD Chemicals Inc.). Developing solvent for TLC was chloroform/methanol/ammonia hydroxide (85:15:2, v/v/v). Nornicotine band was scraped from TLC plates and the TLC powder was directly derivatized by camphanic acid chloride solution for 30 min. The reaction was stopped by addition of saturated sodium carbonate solution, and the solution was extracted by MTBE. MTBE extracts were dried with anhydrous sodium sulfate and then were injected into GC (6890 Agilent GC, Agilent Technologies) for R/S nornicotine analysis. Samples were injected in splitless mode at 250 °C. The oven temperature program was initially 120 °C, increased 30 °C min^{-1} to 215 °C, then 0.2 °C min^{-1} to 220 °C held for 10 min, then 3 °C min^{-1} to a final temperature 300 °C, and held for 20 min. Temperature of flame ionization detector (FID) was 320 °C . GC column was DB1 (60 m (L) × 320 um (D) × 0.25 um (FT)) (J&W Scientific). The carrier gas was helium, and the flow was 1.7 mL min^{-1}. *R*/*S* ratio of nornicotine was calculated on the basis of peak area of each isomer. Nornicotine isomer amount was calculated on the basis of total nornicotine amount and *R*/*S* ratio. The data were analyzed by Sigmaplot 12.0.

Enantiomer fraction (EF) is calculated as follows:[33]

$$EF = R \text{ enantiomer}/(R \text{ enantiomer} + S \text{ enantiomer})$$

RESULTS

EF_{nnic} in Different Tobacco Lines and Tissues. To verify the literature reports, tobacco varieties with different nicotine demethylation capabilities were chosen. Nornicotine composition of different tissues from burley tobacco lines TN 90LC, L8, RM52, and RNAi were analyzed. TN 90LC is a widely used commercial variety. L8 is a breeding line for root disease resistance. RM52 is a high nicotine tobacco line. The RNAi line (P2 RNAi #2−8, see Figure 7 and Table S4) had nicotine demethylases silenced by RNAi technology. A wide range of EF_{nnic} was measured (Figure 3), which confirms the earlier literature reports (Table 1). Unlike RNAi, L8, and TN 90LC plants, RM52 has a variable EF_{nnic} and demethylation across different tissues. There is an inverse correlation between EF_{nnic} and demethylation in conventional tobacco lines, L8, TN 90LC, and RM52. Lamina from lower leaves (referred to as bottom lamina) from RNAi and RM52 plants have lower EF_{nnic} values than bottom lamina from L8 and TN 90LC. Reasons for these results are different. RNAi plants had a lower (*R*)-nornicotine level than L8 and TN 90LC, while RM52 had a much higher (*S*)-nornicotine level (Figure S1). Considering the 0.002 EF_{nic},[34] one may wonder what is the source of the additional (*R*)-nornicotine, resulting in the elevated EF_{nnic}. There is similar EF_{nnic} and demethylation in stalk and root of all four tobacco lines, suggesting no enantioselective translocation of nornicotine.

Ethephon-Inducted *CYP82E4* Expression Associated with Decreased EF_{nnic}. As mentioned in the Introduction, several reasons can account for the high and variable EF_{nnic}. To investigate how demethylation affects EF_{nnic}, tobaccos with different nicotine demethylating capability were chosen and treated with ethephon. Ethephon has been shown to promote leaf senescence and stimulate nicotine demethylation[32] and *CYP82E4* expression.[35] Compared to freeze-dried leaves, ethephon treated converter leaves had increased nicotine demethylation (dotted line in Figure 4), and decreased EF_{nnic}.

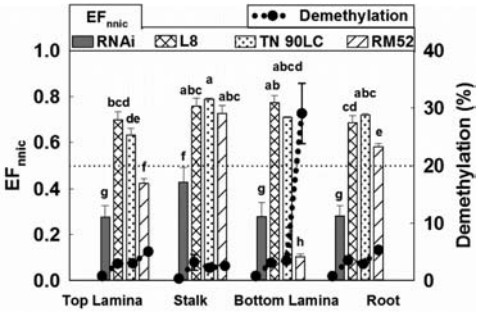

Figure 3. There is wide range of EF_{nnic} in different tobacco lines. Four tobacco lines have different nicotine demethylation abilities. All samples were from mature stage of plant growth and were analyzed for alkaloid levels and nornicotine composition. EF_{nnic} is calculated on the basis of (*R*)-nornicotine and (*S*)-nornicotine levels, and demethylation is calculated on the basis of nicotine and nornicotine levels. L8 is a tobacco breeding line for disease resistance. TN 90LC is a commercial tobacco cultivar. RM52 is a tobacco line with high nicotine demethylation ability. Nicotine demethylases in RNAi plants are silenced by RNAi technique.[36] Lamnia represents the leaf without midrib. Each bar is an average of four plants. Error bar represents the standard deviation. The data were analyzed with one-way ANOVA by Sigmaplot 12.0. Values with different letters are significantly different (Holm−Sidak test, $p < 0.05$).

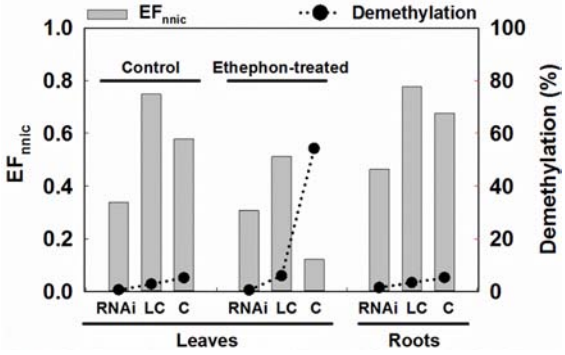

Figure 4. EF_{nnic} decreases after the induction of nicotine demethylation. All tobacco lines were grown in greenhouse. Two top leaves and roots from each line were sampled at two weeks after topping. One leaf was freeze-dried used as control, and the other one was sprayed 0.1% ethephon and air-dried. All dried samples were analyzed for alkaloid levels and nornicotine composition. Demethylation is calculated on the basis of nicotine and nornicotine levels, and EF_{nnic} is calculated on the basis of (*R*)-nornicotine and (*S*)-nornicotine levels. RNAi: P2 RNAi #2−8 (see Figure 7 and Table S4); LC, low converter DH98-325-5 (P1); C, converter DH98-325-6 (P2).

For individual nornicotine isomer levels (Figure 5), both (*R*)- and (*S*)-nornicotine amounts increased after ethephon induction. However, (*S*)-nornicotine increased much more than (*R*)-nornicotine, which makes the relative (*R*)-nornicotine level decrease. Since CYP82E4 is the major demethylase in converter plants and *CYP82E4* gene expression is dramatically induced by senescence,[36] we can infer that, at mature growth stage, (*S*)-nornicotine in tobacco is produced more than (*R*)-nornicotine which results in increased demethylation and decreased EF_{nnic}.

Nicotine Demethylase CYP82E4 Mutant Results in Increased EF_{nnic}. To further confirm CYP82E4 effects, *e4* mutants[17] were analyzed for nornicotine enantiomeric composition. Tobacco line DH98-325-6 has high nicotine demethylation ability and was chosen as parent for EMS

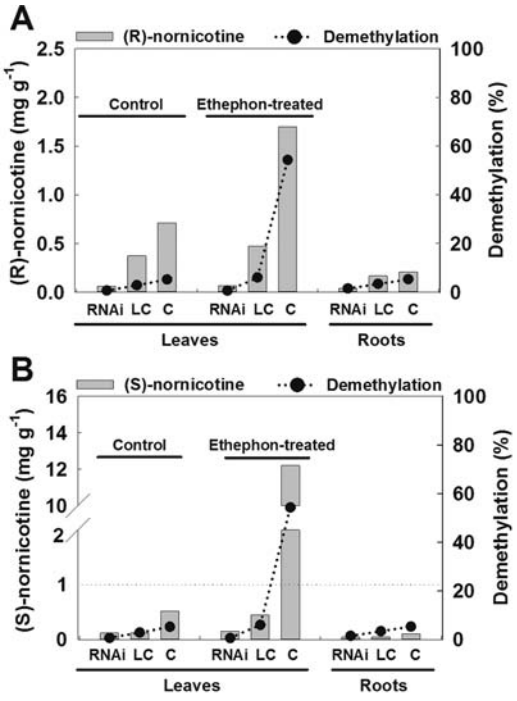

Figure 5. (*R*)-Nornicotine (A) and (*S*)-nornicotine (B) increase differently after ethephon induction of nicotine demethylase. All tobacco lines were grown in greenhouse. Two top leaves and roots from each line were sampled at two weeks after topping. One leaf was freeze-dried and used as control, and the other one was sprayed 0.1% ethephon and air-dried. All dried samples were analyzed for alkaloid levels and nornicotine composition. RNAi: P2 RNAi #2−8 (see Figure 7 and Table S4); LC, low converter DH98-325-5 (P1); C, converter DH98-325-6 (P2).

mutation ("P" in Figure 6). Mutants with homologous mutation in *CYP82E4* gene and their backcross with parental line were grown in the field, and air-cured leaves were analyzed for alkaloid levels and nornicotine composition. Demethylation is used to confirm that the *CYP82E4* gene is effectively silenced.

Four *e4* mutants, *e4* #1−4, have much lower demethylation than the parent line (P), which means they are effective mutant lines (Figure 6). All these effective mutant lines have high EF_{nnic}, like control TN 90LC. Four effective mutants were backcrossed with converter parent to produce four heterozygous lines (F1 plants). These four backcross lines have increased nicotine demethylation and decreased EF_{nnic}. The profile of four effective *e4* mutants and their backcross lines demonstrate that CYP82E4 can increase demethylation and decrease EF_{nnic}.

Selectivity of Other Nicotine Demethylases for (*R*)-Nicotine. Nicotine demethylation was also measured in individual tobacco plants with *CYP82E4* silenced by RNAi (Figure 7). Being different from EMS mutantion, RNAi inhibits the enzyme activity not only of CYP82E4, but also its related family members as well. Therefore, we would expect to see complex effects in transgenic RNAi-suppression plants. Two parent lines were chosen for RNAi knockdown: DH98-325-5 (P1 L) has low demethylating ability, and DH98-325-6 (P2 H) has high demethylating ability. The two parents are full-sib doubled haploid burley lines.[37] RNAi plants were grown in the field, and the leaves were sampled after being air-cured. Most RNAi lines from the low converter parent had lower demethylation and EF_{nnic} than their parent. This demonstrates

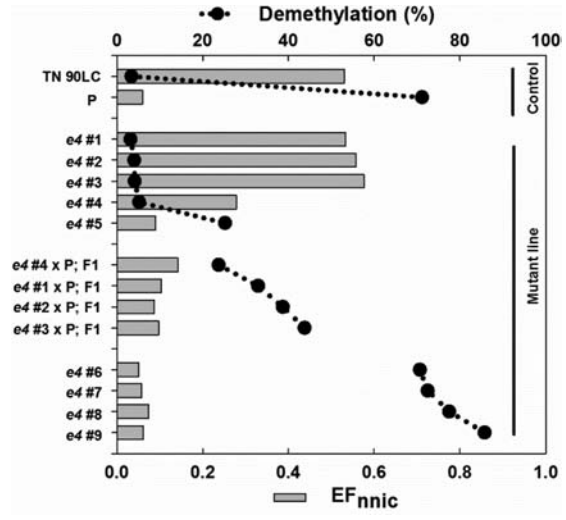

Figure 6. Changes of EF_{nnic} and demethylation due to the mutation of nicotine demethylase CYP82E4. Tobacco lines were grown in the field, and top leaves were sampled from air-cured plants. All dried samples were analyzed for alkaloid levels and nornicotine composition. EF_{nnic} is calculated on the basis of (*R*)-nornicotine and (*S*)-nornicotine levels, and demethylation is calculated on the basis of nicotine and nornicotine levels. Controls were the parent DH98-325-6 (P) and the commercial line TN 90LC. Details of how the mutant lines were created are described by Lewis et al.[17]

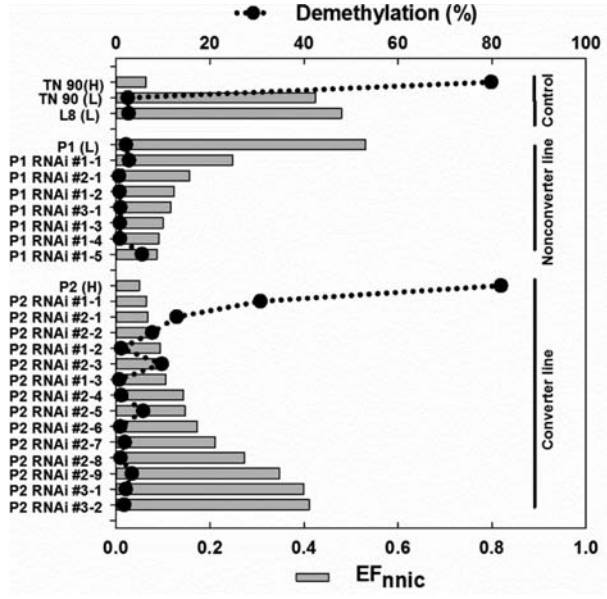

Figure 7. EF_{nnic} and demethylation in different RNAi lines. All tobacco lines were grown and air-cured in Blackstone, VA. All dried leaf samples were analyzed for alkaloid levels and nornicotine composition. EF_{nnic} is calculated on the basis of (*R*)-nornicotine and (*S*)-nornicotine levels, and demethylation is calculated on the basis of nicotine and nornicotine levels. TN 90 is a commercial cultivar. DH98-325-5 (P1) and DH98-325-6 (P2) are full-sib doubled haploid burley lines, which are the parents of all other RNAi lines. Details of how RNAi plants were created are described by Lewis et al.[31]

that in these lines demethylation is further inhibited (lower demethylation), and (*R*)-nicotine demethylation is inhibited more than (*S*)-nicotine demethylation (lower EF_{nnic}). RNAi lines have similar demethylation but lower EF_{nnic} than effective *e4* mutants, suggesting that other demethylases can use (*R*)-

nicotine more readily than (*S*)-nicotine. This could be CYP82E5, CYP82E10, or other unidentified demethylases. RNAi lines from the converter parent had striking differences in demethylation and EF_{nnic}. Line P2 RNAi #1−1 behaved like a converter, which has high demethylation and low EF_{nnic}. Line P2 RNAi #1−2 behaves like an RNAi plant from the low converter parent, which had low demethylation and low EF_{nnic}. Line P2 RNAi #3−2 behaved like a low converter with low demethylation and high EF_{nnic}.

DISCUSSION

The discrepancy between nicotine and nornicotine composition has puzzled researchers for a long time. It has been reported that there is a wide range of EF_{nnic}. In this study, we found that 60−80% of nornicotine in root of conventional tobacco was the *R* form, and 5−80% of nornicotine in leaf was the *R* form (Figure 3). These results are consistent with previous reports (Table1). Demethylation induction by ethephon treatment is inversely correlated with EF_{nnic}. Both (*R*)- and (*S*)-nornicotine accumulation is increased, but (*S*)-nornicotine accumulated much more than (*R*)-nornicotine, which results in reduced EF_{nnic}. The effects of CYP82E4 on nornicotine composition were confirmed by *CYP82E4* gene mutants and their backcross to parent. There are two other functional nicotine demethylases in tobacco, CYP82E5v2 and CYP82E10, besides CYP82E4. RNAi plants which have all nicotine demethylases silenced have lower EF_{nnic} than only the *e4* mutants, suggesting that combinations of CYP82E5v2 and CYP82E10, or other unidentified demethylases, have a high selectivity for (*R*)-nicotine. On the basis of the results reported above, a model to explain the nornicotine composition is proposed. CYP82E5v2, CYP82E10, or combination with other undefined demethylases have high selectivity for (*R*)-nicotine, and can produce 0.80 EF_{nnic} from low EF_{nic}. CYP82E4 produced more (*S*)-nornicotine than (*R*)-nornicotine at the mature growth stage, resulting in a reduced and variable EF_{nnic}.

Potentially there are several reasons responsible for the high and variable EF_{nnic} (Figure 2). We show in this study that the enantioselectivity of nicotine demethylases plays a pivotal role in nornicotine enantiomer accumulations and no selective translocation of nornicotine is found. Exogenous nicotine feeding assays in cell culture[12] and plant tissue[13] demonstrate the rate of (*R*)-nicotine demethylation is higher than that of (*S*)-nicotine. In this study, we suggest that the more (*R*)-nornicotine was produced by CYP82E5v2 and CYP82E10 and that CYP82E4 resulted in more (*S*)-nornicotine. These three demethylases have been biochemically studied.[15,17] In the future, the selectivity of these three demethylases for nicotine enantiomers should be characterized.

In summary, high and variable EF_{nnic} is found in tissues of tobacco with different demethylating capabilities. Experiments of induction and inhibition of CYP82E4 activity in tobacco demonstrate that CYP82E4 results in more (*S*)-nornicotine production and causes a variable EF_{nnic} in tobacco leaf. Results from RNAi silenced demethylation plants suggest that enantioselective demethylation has an important role in the high and variable EF_{nnic} .

ASSOCIATED CONTENT

Supporting Information

Additional tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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