Phytochemistry 95 (2013) 188–196

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

(*R*)-nicotine biosynthesis, metabolism and translocation in tobacco as determined by nicotine demethylase mutants

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ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form 13 May 2013
Available online 9 July 2013

Keywords:
Nicotiana tabacum L.
Tobacco
Solanaceae
(*R*)-nicotine
Nornicotine
Enantioselectivity
Nicotine demethylase

ABSTRACT

Nicotine is a chiral compound and consequently exists as two enantiomers. Since (*R*)-nicotine consists of less than 0.5% of total nicotine pool in tobacco, few investigations relating to (*R*)-nicotine have been reported. However, previous studies of nicotine demethylases suggested there was substantial amount of (*R*)-nicotine at synthesis in the tobacco plant. In this study, the accumulation and translocation of (*R*)-nicotine in tobacco was analyzed. The accumulation of nicotine and its demethylation product the nornicotine enantiomers, were investigated in different tobacco plant parts and at different growth and post-harvest stages. Scion/rootstock grafts were used to separate the contributions of roots (source) from leaves (sink) to the final accumulation of nicotine and nornicotine in leaf tissue. The results indicate that 4% of nicotine is in the (*R*) form at synthesis in the root. After the majority of (*R*)-nicotine is selectively demethylated by CYP82E4, CYP82E5v2 and CYP82E10 in the root, nicotine and nornicotine are translocated to leaf, where more nicotine becomes demethylated. Depending on the CYP82E4 activity in senescing leaf, constant low (*R*)-nicotine remains in the tobacco leaf and variable nornicotine composition is produced. These results confirmed the enantioselectivity of three nicotine demethylases in *planta*, could be used to predict the changes of nicotine and nornicotine composition, and may facilitate demethylase discovery in the future.

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1. Introduction

Nicotine (**1**) is the most abundant pyridine alkaloid in tobacco (*Nicotiana tabacum* L.), and has important biological functions including antiherbivore defense and smoking addiction (Benowitz, 2008). Nicotine (**1**) has two enantiomers which differ from each other at the 2′-C position on the pyrrolidine ring. (*S*)-nicotine (**1b**) is the predominant form, and (*R*)-nicotine (**1a**) only accounts for 0.2% of total nicotine (**1**) in cured leaf (Armstrong et al., 1998). Therefore, nicotine (**1**) is often considered to be synonymous with (*S*)-nicotine (**1b**) in the literature. However, much higher (*R*)-nicotine accumulated when nicotine demethylation was inhibited by RNAi or mutation (Cai and Bush, 2012; Cai et al., 2012). Furthermore based on this *in vitro* study, it was hypothesized that the reason of low (*R*)-nicotine (**1a**) in tobacco could be due to its selective metabolism by the three nicotine demethylases (Cai et al., 2012). In this study, the (*R*)-nicotine accumulation in tobacco was examined by manipulating nicotine (**1**) demethylation.

The biosynthesis and metabolism of the four main alkaloids in tobacco have been studied extensively (Fig. 1). Several different amino acids are the building blocks for the pyridine, pyrrolidine and piperidine rings, which are used in different combinations to form nicotine (**1**), anabasine (**2**) and anatabine (**3**) (Shoji and Hashimoto, 2011). The fourth main alkaloid nornicotine (**4**) is formed from nicotine (**1**) demethylation. Nicotine synthase has not been genetically or biochemically characterized. Until now, only a putative enzyme mixture (Friesen and Leete, 1990) and genes suspected to be involved in the condensation reaction of pyridine and pyrrolidine ring for nicotine (**1**) synthesis (DeBoer et al., 2009; Kajikawa et al., 2009, 2011) have been identified. Three functional nicotine demethylase genes have, however, been reported for *N. tabacum* L.: *CYP82E4* (accounts for most demethylation in senescing leaf), *CYP82E5v2*, and *CYP82E10* (constitutive expression) (Siminszky et al., 2005; Gavilano and Siminszky, 2007; Xu et al., 2007; Lewis et al., 2010). Additionally, *CYP82E2* and *CYP82E3* are present in the tobacco genome, but they do not encode for active or functional enzymes (Chakrabarti et al., 2007). Besides these three *Nicotiana* species including tobacco and its two parents, four functional CYP82E demethylases were recently identified in *Nicotiana alata*, while in a closely related species *Nicotiana langsdorffii* two *CYP82E* genes were found: one possessing an abolished gene expression, the other one containing a one-nucleotide deletion in the first exon (Pakdeechanuan et al., 2012b).

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http://dx.doi.org/10.1016/j.phytochem.2013.06.012

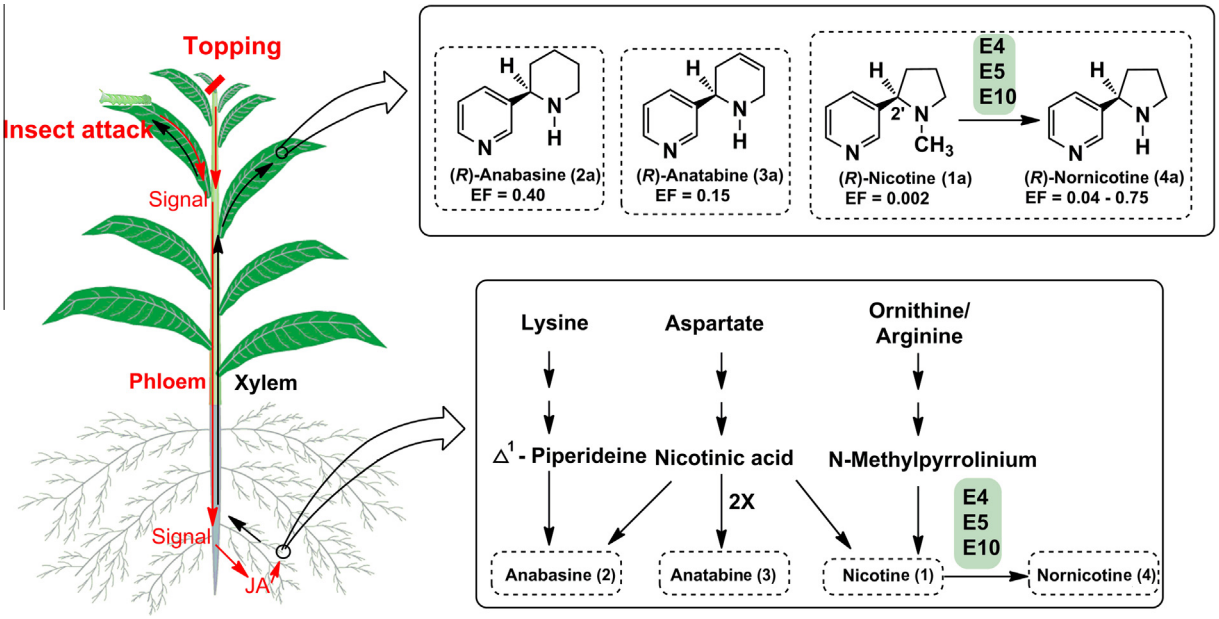

Fig. 1. Biosynthesis and translocation of four main pyridine alkaloids in tobacco plant (*Nicotiana* species) (Leete, 1992; Armstrong et al., 1998, 1999; Lewis et al., 2010; Shoji and Hashimoto, 2011). Insect attack and topping (removal of the terminal inflorescence) will stimulate the biosynthesis of alkaloids in roots. The alkaloids then will be translocated to the leaf through the xylem. (*S*)-nicotine (**1b**) may accumulate to greater than 99% of the total nicotine (**1**) pool in leaves (EF_{nic} = 0.002), few reports on (*R*)-nicotine (**1a**) have been published due to the low level. Our previous study suggested that there was much higher level of (*R*)-nicotine (**1a**) at synthesis. Thus it was essential to understand the accumulation and translocation of (*R*)-nicotine (**1a**) in *planta* by suppressing nicotine (**1**) metabolism. The four major alkaloids (**1–4**) in tobacco are boxed with only the (*R*) form structures depicted. Enantiomer fraction (EF) represents the percentage of (*R*) enantiomer of a substance. E4 = CYP82E4; E5 = CYP82E5v2; E10 = CYP82E10.

After biosynthesis in the tobacco root (Dawson, 1942), nicotine (**1**) is translocated to the leaf via the xylem (Guthrie et al., 1962) and stored in the leaf vacuole with the help of a tonoplast localized transporter (Shitan et al., 2009). Nicotine (**1**) can be demethylated in both leaves (Dawson, 1945) and roots (Mizusaki et al., 1965), but primarily in senescing leaves (Chakrabarti et al., 2008). The accumulation of nicotine (**1**) in tobacco starts 72–96 h after germination and increases until maturity (Weeks and Bush, 1974; Tso, 1990). Alkaloid levels in tobacco are affected by genetic makeup, environment and cultural practices (Bush, 1999). Bush (1999) concluded that any environmental and cultural practices that improve the growth of the plant would increase alkaloid formation and accumulation. For example, topping (remove of the apical meristem) will stimulate the root growth and increase the alkaloid production.

Although there are abundant reports about the biosynthesis, metabolism and translocation of nicotine, there is no report of the accumulation of its (*R*)-enantiomer (**1a**) probably due to its rapid metabolism (Cai et al., 2012). In this paper, the changes of nicotine (**1**) and nornicotine (**4**) enantiomeric compositions were studied by investigating nicotine demethylase mutants at different growth and post-harvest stages. Since nicotine (**1**) metabolism can occur in roots and leaves, scion/stock grafts were used to dissect individual leaf and root contributions to the final nicotine (**1**) and nornicotine (**4**) composition in leaf.

2. Results

2.1. Accumulation of alkaloids in mutant lamina during growth and curing

During tobacco production, levels of alkaloids are significantly changed by two cultural practices, topping and harvest. Topping, a typical practice in tobacco production, stimulates alkaloid production. After harvest, the gene expression of *CYP82E4* is induced and nornicotine (**4**) levels will increase at the expense of nicotine (**1**). Since there is substantial amount of (*R*)-nicotine (**1a**) in nicotine demethylation deficient plants, it was instructed to determine how (*R*)-nicotine (**1a**) accumulates in *planta*.

Nine tobacco lines were chosen for field cultivation during 2011, containing one commercial variety control and eight mutant lines (Fig. S1). Commercial tobacco variety TN 90LC has a functional CYP82E4, but the *CYP82E4* expression is suppressed, probably by an epigenetic mechanism (Chakrabarti et al., 2008). Leaves were sampled at different time periods during the growth and post-harvest process. Individual alkaloid levels in lamina (leaf with midvein removed) were determined (Fig. 2) at five critical times during tobacco production (Fig. S2): after recovery from transplant shock (one month after transplanting), at topping (two months after transplanting), at harvesting (three months after transplanting), two weeks after harvesting, and post-curing.

Alkaloid accumulation in lamina increased dramatically after topping, as expected (Fig. 2). During the first two weeks after harvest, nornicotine (**4**) concentration increased significantly at expense of nicotine (**1**) due to CYP82E4 activity. Although nicotinic acid is shared in the biosynthesis of the four main alkaloids, nicotine (**1**) biosynthesis has been shown to compete with anatabine (**3**) biosynthesis (Chintapakorn and Hamill, 2003; Shoji and Hashimoto, 2008), mutations in the nicotine demethylase genes had no obvious effect on anabasine (**2**) and anatabine (**3**) accumulation (Fig. 2B). Due to nicotine (**1**) demethylation, alkaloid profiles in tobacco changed during tobacco growth and curing, which has been reported before (Sisson and Severson, 1990). For example, the parent tobacco plant (*E4E5E10*) had alkaloid levels at one month after transplant with nicotine (**1**) > anatabine (**3**) > nornicotine (**4**) > anabasine (**2**). At topping, levels were nicotine (**1**) > nornicotine (**4**) > anatabine (**3**) > anabasine (**2**). After harvest, levels were nornicotine (**4**) > nicotine (**1**) > anatabine (**3**) > anabasine (**2**).

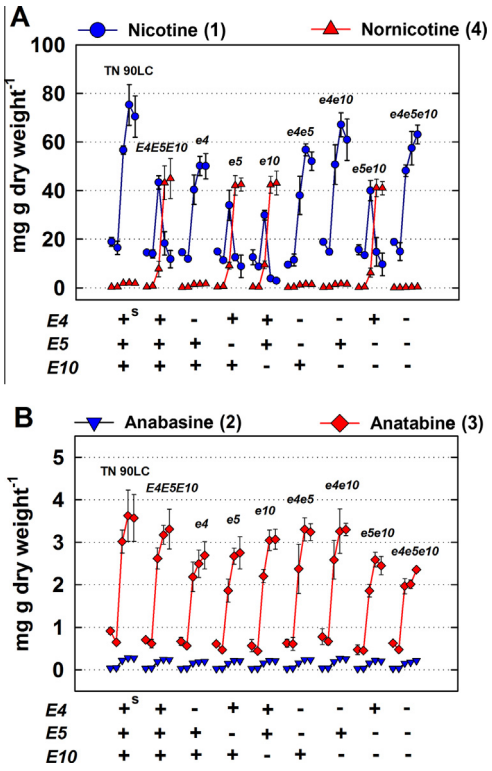

Fig. 2. Alkaloid levels in lamina of different nicotine demethylase mutant lines during growth and post-harvest. TN 90LC (commercial variety, control) and different nicotine demethylase mutants were grown in the field and sampled five times. The sequential sampling times for each line were one month after transplanting, at topping, at harvesting, at two weeks of curing, and post-curing. Each data point is an average of four bulk samples, and each bulk sample consists of five middle leaves, one from each of five plants. The error bars represent standard deviations. +/− below the *x*-axis 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*. The full genotypes of the tobacco lines were summarized in Table 1.

2.2. Accumulation of nicotine (**1**) and nornicotine (**4**) enantiomers in mutant lamina during growth and curing

Enantiomer levels (Fig. S3) and enantiomer fraction (EF) (Fig. 3) of nicotine (**1**) and nornicotine (**4**) were analyzed and calculated at five sampling times. Triple mutant line *e4e5e10* continuously accumulated (*R*)-nicotine (**1a**) throughout growth and post-harvest stages, and its level in lamina reached about 2.5 mg g^{-1} after harvest, which is higher than the level of the other three alkaloids. The presence of any of the three demethylases resulted in reduced level of (*R*)-nicotine (**1a**), to under 0.1 mg g^{-1} in final lamina sample. Compared with (*R*) pool (the sum of (*R*)-nicotine (**1a**) and (*R*)-nornicotine (**4a**) level) in *e4e5e10* line, tobacco lines with active CYP82E5v2 or CYP82E10 (*e4*, *e4e5* and *e4e10* lines) have a reduced (*R*) pool, while the (*R*) pool was increased in tobacco line with active CYP82E4 (*e5e10* line). (*S*) form of nicotine and nornicotine had the same accumulation pattern as the total nicotine and nornicotine.

The enantiomer fraction of nicotine (**1**) and nornicotine (**4**) changed throughout the sampling periods (Fig. 3). Compared with the triple mutant line (*e4e5e10*), the EF_{nic} of all the other lines decreased significantly. The large decreases in the EF_{nic} occurred at early growth stages in plants with functional *CYP82E5v2* and/or *CYP82E10* gene, and late growth stages in plants without active CYP82E5v2 and/or CYP82E10, which is consistent with the expression time of the three nicotine demethylases. *CYP82E5v2* and *CYP82E10* exhibit constitutive expression, while *CYP82E4* mainly expresses at mature stage (Gavilano and Siminszky, 2007; Chakrabarti et al., 2008; Lewis et al., 2010). The mutants with inactive CYP82E4 had a relatively stable nornicotine enantiomeric composition, while plants with active CYP82E4 had a continuously decreasing EF_{nnic}, due to the larger increase in (*S*)-nornicotine (**4b**).

The field results were rearranged for analyzing the relationship between nicotine (**1**) demethylation and nicotine (**1**) or nornicotine (**4**) enantiomeric composition (Fig. S4). With only CYP82E5v2 or CYP82E10 active (*e4e10* or *e4e5*), plants contained 0.50 to 0.80 EF_{nnic} and nicotine (**1**) demethylation was low. When only *CYP82E4* was active (*e5e10*), demethylation resulted in 0.06 to 0.25 EF_{nnic}. A much wider range of EF_{nnic}, from 0.06 to 0.80, was observed when more than one active demethylase enzyme was present (Fig. S4 insert). Similar EF_{nnic} ranges were found *in vitro* enzyme assays, in which three nicotine demethylases were incubated with nicotine (**1**) (EF_{nic} of 0.03) individually and collectively (Cai et al., 2012).

2.3. Contributions of three nicotine demethylases to the leaf nicotine (**1**) and nornicotine (**4**) enantiomeric composition in the roots and leaves of the tobacco plant

The roots are the site of nicotine (**1**) biosynthesis and also the initial site of nornicotine (**4**) formation. To understand the accumulation of nicotine (**1**) and nornicotine (**4**) enantiomers in the leaves discussed in previous sections, their enantiomeric compositions accumulating in the roots and translocated to the leaves were determined. Grafting is frequently used in tobacco alkaloid research, such as in the experiment which determine nornicotine (**4**) to be a product of nicotine (**1**) (Dawson, 1945) and the

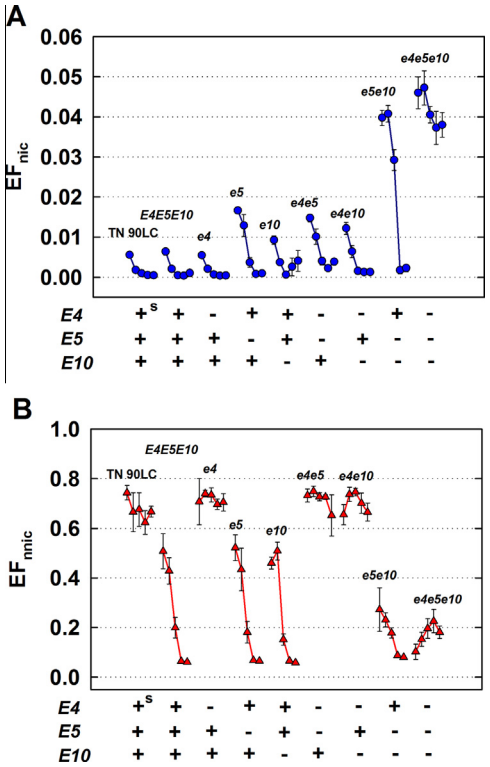

Fig. 3. Nicotine (**1**) (A) and nornicotine (**4**) (B) enantiomeric compositions in lamina of different nicotine demethylase mutant lines during growth and post-harvest. TN 90LC (commercial variety, control) and different nicotine demethylase mutants were grown in the field and sampled five times. The sequential sampling times for each line were one month after transplanting, at topping, at harvesting, 2 weeks of curing, and post-curing. Each data point is an average of four bulk samples, and each bulk sample is a mixture of five middle leaves from five plants. The error bars represent standard deviations. +/− below the *x*-axis indicate the presence/absence of a functional demethylase gene. TN 90LC has functional *CYP82E4* gene, but the gene expression is suppressed ($+^s$).

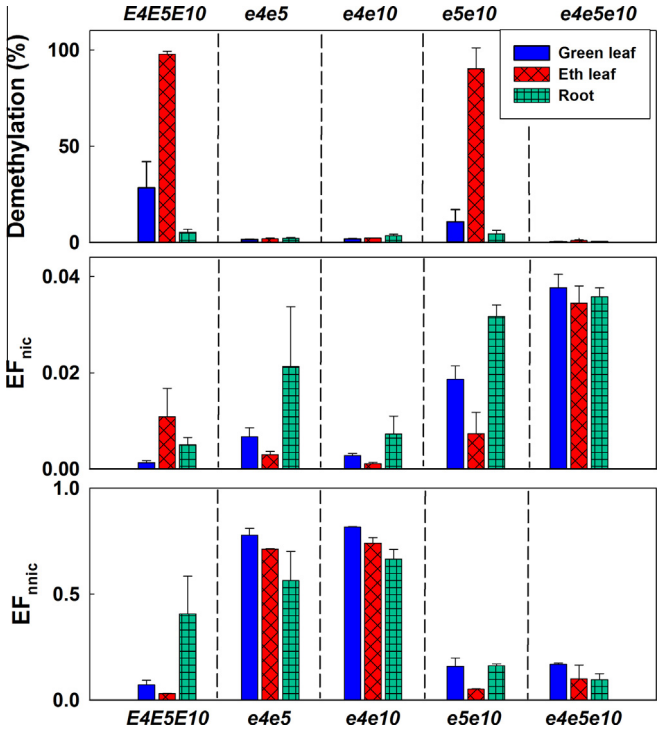

Fig. 4. Demethylation and composition of nicotine (**1**) and nornicotine (**4**) in self-grafted mutant tissues. Eth leaf = ethephon-treated leaves which induce senescence and expression of *CYP82E4*. Each bar is an average of three replicates. The error bars represent standard deviation. Demethylation reflects how much nicotine (**1**) goes through the demethylation process. Demethylation (%) = nornicotine (**4**) concentration (mg g dry weight^{-1}) * 100/sum of nicotine (**1**) and nornicotine (**4**) concentration (mg g dry weight^{-1}).

dominant suppression feature of root-to-shoot translocation of nicotine (**1**) (Pakdeechanuan et al., 2012a). In this study, scion/stock grafts were used to separate the effects of roots and leaves on the final nicotine (**1**) and nornicotine (**4**) enantiomeric composition observed in the leaves (Figs. 4–6).

Tobacco plants were self-grafted to determine the effect of grafting *per se* on nicotine (**1**) and nornicotine (**4**) composition. No significant differences were observed between grafted and intact tobacco plants in terms of their enantiomeric compositions (intact plants results not shown). For grafted plants, green leaves, ethephon treated leaves and roots were analyzed (Fig. 4). Ethephon treatment was used to stimulate *CYP82E4* gene expression (Chakrabarti et al., 2008) and to mimic the aging effects at mature stage. Nicotine (**1**) and nornicotine (**4**) compositions in lamina of grafts were consistent with the enantiomeric compositions observed for field grown plants at topping and at harvest time. Also, after ethephon treatment, grafted plants had nicotine (**1**) and nornicotine (**4**) compositions similar to field-grown mutants during the first two weeks of curing. Since the leaf results were consistent between grafted and field-grown plants, the nicotine (**1**) and nornicotine (**4**) compositions in the roots were further examined.

Mutants with active *CYP82E5v2* or *CYP82E10* exhibited lower EF_{nic} than *e4e5e10* and had predominantly (*R*)-nornicotine (**4a**) in roots, as in Kisaki and Tamaki (1960). The roots of tobacco lines *E4E5E10*, *e4e5* and *e4e10* contained a different nicotine (**1**) and nornicotine (**4**) enantiomeric composition from that of *e5e10* and *e4e5e10* roots, suggesting that CYP82E5v2 and CYP82E10 are major factors affecting their enantiomeric compositions in the roots and that CYP82E4 had little effect on root nicotine (**1**) and nornicotine (**4**) enantiomeric composition. Except for the triple mutant line, lower EF_{nic} levels were found in leaves than in the roots, confirming the notion that the three nicotine demethylase enzymes can influence nicotine (**1**) composition in the leaf through both the roots and leaves.

Since the nornicotine (**4**) ultimately found in leaves is formed both in roots and the leaves, tomato scions were grafted onto mutant roots to measure the nicotine (**1**) and nornicotine (**4**) enantiomeric compositions translocated to the leaves (Fig. 5). Tomato can accumulate small amount of nicotine (**1**) in leaf (Sheen, 1988), but it was found here that the amount of nicotine (**1**) observed in the tomato leaves would increase more than 100 fold when tomato scion grafted onto tobacco roots. No nicotine demethylase activity was measured when nicotine (**1**) was administered to tomato leaves (data not shown). The same nicotine (**1**) enantiomeric

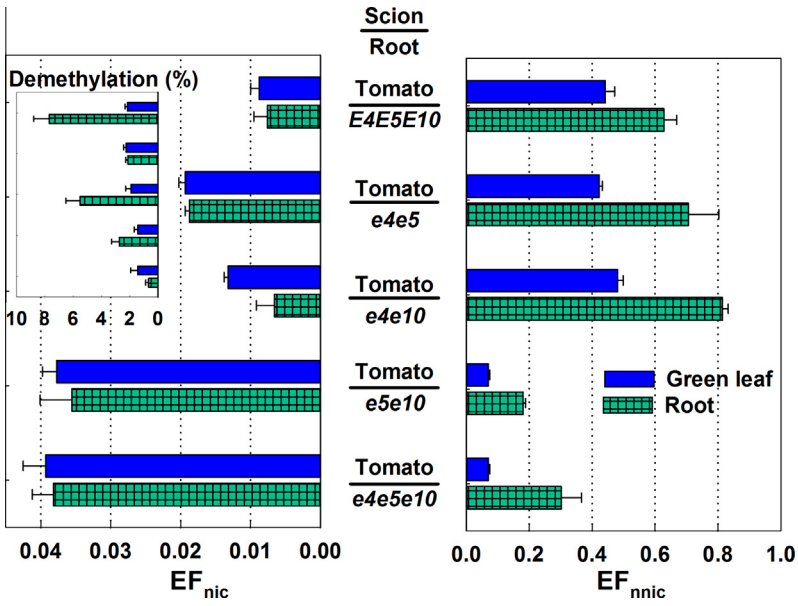

Fig. 5. Nicotine (**1**) and nornicotine (**4**) composition in tomato/tobacco grafts. Each bar is an average of three replicates. The error bars represent standard deviation. Demethylation reflects how much nicotine (**1**) goes through the demethylation process. Demethylation (%) = nornicotine (**4**) concentration (mg g dry weight^{-1}) * 100/sum of nicotine (**1**) and nornicotine (**4**) concentration (mg g dry weight^{-1}).

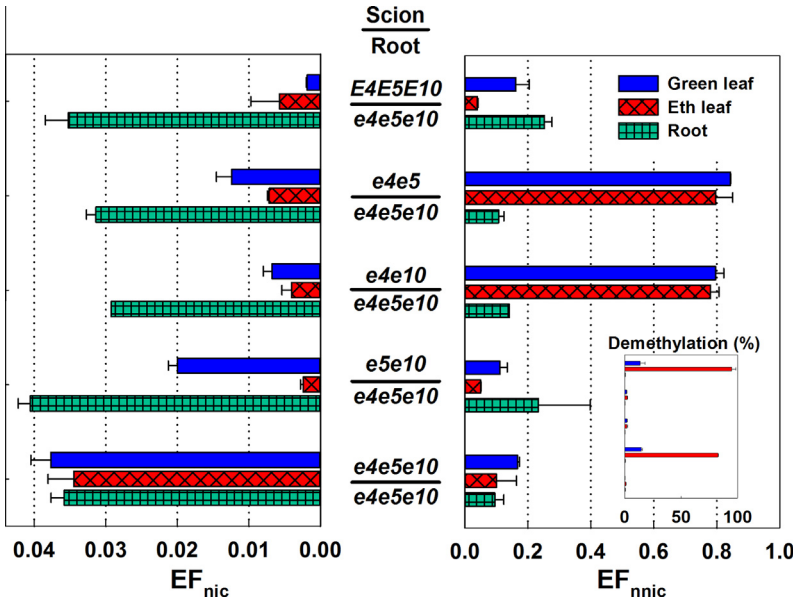

Fig. 6. Nicotine (**1**) and nornicotine (**4**) composition in tobacco/*e4e5e10* grafts. Each bar is an average of two replicates, except that root of *e4E5e10*/*e4e5e10* only has one replicate. The error bars represent standard deviation. Demethylation reflects how much nicotine (**1**) goes through the demethylation process. Demethylation (%) = nornicotine (**4**) concentration (mg g dry weight^{-1}) $*$ 100/sum of nicotine (**1**) and nornicotine (**4**) concentration (mg g dry weight^{-1}).

composition was found in both tomato leaves and tobacco roots, demonstrating that its enantiomeric composition in the roots reflected that translocated to the leaves and that no selective translocation of nicotine (**1**) occurs. Consistently lower EF_{nnic} levels were found in tomato leaves than in tobacco rootstock, implying an enantioselective translocation of nornicotine (**4**). This result is inconsistent with our previous report where no difference was found in the nornicotine (**4**) enantiomeric composition in tobacco stem and root tissues (Cai and Bush, 2012). Comparison of EF_{nic} of the leaves from tomato/tobacco grafts to EF_{nic} of leaves from self-grafted tobacco indicates that over 75% of the (*R*)-nicotine (**1a**) was demethylated in the roots, and that the remainder was demethylated in the leaves.

To investigate the function of the three nicotine demethylase enzymes in tobacco leaves, five tobacco scions (the parent line, three double mutant lines and the triple mutant line) were grafted onto triple mutant (*e4e5e10*) rootstocks (Fig. 6). In this setup, the same enantiomeric composition of nicotine **1** (EF_{nic} = 0.03 to 0.04) and nornicotine **4** (0.10–0.25 EF_{nnic}) was supplied to mutant leaves via translocation. In the leaves, CYP82E5v2 and CYP82E10 were found to convert nicotine (**1**) (EF_{nic} = 0.03) into nornicotine (**4**) (EF_{nnic} = 0.80), while CYP82E4 produced nornicotine (**4**) (EF_{nnic} = 0.15). These results are consistent with *in vitro* assays demonstrating selectivity of each demethylase enzyme.

3. Discussion

A quantitative study of nicotine (**1**) and nornicotine (**4**) enantiomer accumulation in different plant parts and over time has not been reported. In this study, their alkaloid accumulation and enantiomeric composition were investigated using genetic stocks carrying different combinations of nicotine demethylase mutants during growth and post-harvest. It had previously been shown that the three nicotine demethylase enzymes had different selectivity for (*R*)-nicotine (**1a**) *in vitro* experiments (Cai et al., 2012). In this study, the nicotine demethylase mutant genetic stocks were used to confirm previous *in vitro* observations (Fig. S4).

Based on grafting results, a previous model was expanded to explain the nicotine (**1**) and nornicotine (**4**) enantiomeric composition in *N. tabacum* L. (Fig. 7). In the root tissues, 4% of newly synthesized nicotine (**1**) is the (*R*) form (EF_{nic} = 0.04). *CYP82E5v2* and *CYP82E10* predominantly determine the nicotine (**1**) and nornicotine (**4**) enantiomeric composition in roots, whereas *CYP82E4* has little impact. Soon after being synthesized, 0.04 EF_{nic} is reduced to 0.01 EF_{nic}, resulting in 0.60 EF_{nnic}. Based on these changes in EF_{nic}, more than three fourths of (*R*)-nicotine (**1a**) is demethylated in the root. After that, nicotine (**1**) and nornicotine (**4**) are translocated to the leaves, where the remainder of the (*R*)-nicotine (**1a**) is subjected to demethylation by all three demethylase enzymes. *CYP82E4* expression is induced dramatically during senescence, and (*S*)-nicotine (**1b**) is largely demethylated by CYP82E4 into (*S*)-nornicotine (**4b**) due to the limiting amount of (*R*)-nicotine (**1a**) present. Depending on *CYP82E4* expression (intensity and time), a range of EF_{nnic} was measured due to the large amount of (*S*)-nornicotine (**4b**) present. This explains the wide range of EF_{nnic} that can be observed in tobacco leaves. The general conclusion is that nicotine (**1**) in tobacco initially consists of approximately 4% of the (*R*)-enantiomer (**1a**) and that this may become essentially pure (*S*)-nicotine (**1b**) due to the selective demethylation of the former. High selectivity by CYP82E5v2 and CYP82E10 for (*R*)-nicotine (**1a**) explains the presence of high EF_{nnic} present in some tobacco leaves. Variable *CYP82E4* expression in the leaves of different genotypes and subsequent demethylation of (*S*)-nicotine (**1b**) to (*S*)-nornicotine (**4b**) results in a broad range of EF_{nnic}.

The nicotine (**1**) and nornicotine (**4**) profiles in *N. tabacum* have likely changed over time (Fig. 8). Cultivated tobacco (*N. tabacum* L.) is an allotetraploid species derived from the hybridization of *Nicotiana sylvestris* and something closely related to modern-day *Nicotiana tomentosiformis* (Clarkson et al., 2005). Both parents have high nicotine (**1**) demethylating ability, so ancestral tobacco must also have possessed this characteristic. Few of tobacco genotypes available today exhibit the strong nicotine (**1**) demethylation ability of the diploid progenitor species. This change has been proposed to be due to the selection for high nicotine (**1**) content by humans (Chakrabarti et al., 2007). Because nornicotine (**4**) contributes to undesirable smoke quality and because a harmful smoke constituent (NNN) may be nornicotine (**4**) derived, researchers are trying to reduce nornicotine (**4**) levels through diminishing nicotine demethylation (Jack and Bush, 2007; Lewis et al., 2008,

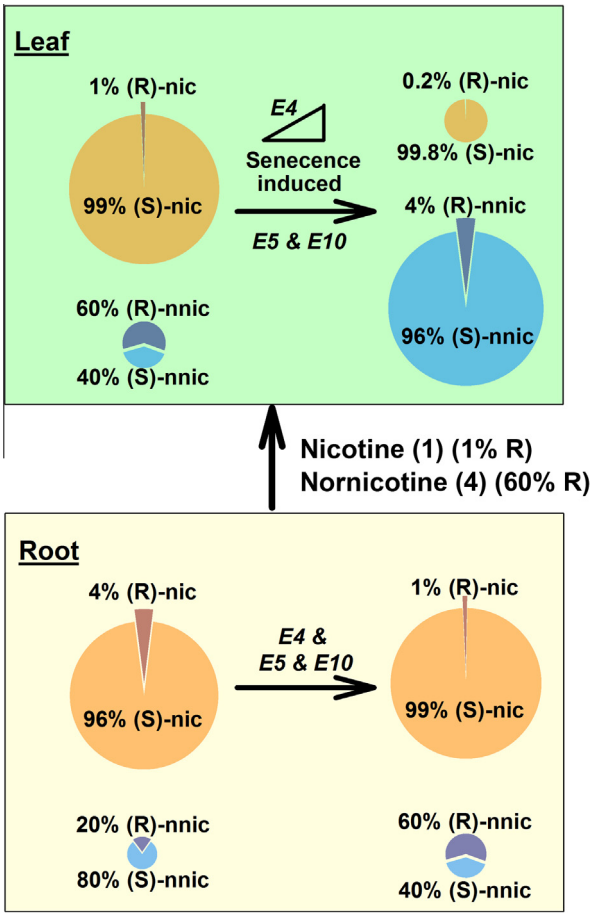

Fig. 7. Schematic diagram showing nicotine (**1**) enantiomer accumulation and translocation in tobacco with three nicotine demethylases. When being synthesized (*-E4-E5-E10*) in the root, EF_{nic} is 0.04. The small amount of nornicotine (**4**) could be the leakage of E4 or other unidentified demethylase. Soon after synthesis, (*R*)-nicotine (**1a**) is selectively demethylated into (*R*)-nornicotine (**4a**), resulting in 0.60 EF_{nnic} from 0.04 EF_{nic}. Over three fourths of (*R*)-nicotine (**1a**) is selectively demethylated in the root, and the rest is translocated to the leaf, where it is further demethylated. *E4* expression is mainly in the leaf tissues, especially during senescence, and has little effect on the nicotine enantiomeric composition in roots. Depending on *E4* expression, 0.04 to 0.60 EF_{nnic} is accumulated in leaves. The pies are representative of the enantiomeric composition. The sizes of the pies represent the relative abundance of (*R*)- and (*S*)- nicotine (**1a** and **1b**) and nornicotine (**4**). E4, E5 and E10 represent CYP82E4, CYP82E5v2 and CYP82E10, respectively.

2010). As mutant alleles in the nicotine demethylase genes are incorporated into commercial tobacco cultivars (Li et al., 2011), It is possible to study another significant change in nicotine (**1**) and nornicotine (**4**) composition, this time driven by selection for low nornicotine (**4**) content.

The accumulation pattern of nicotine (**1**) and nornicotine (**4**) enantiomers in this study could be used to explain the nornicotine (**4**) composition in other *Nicotiana* species and tobacco products, or even used to help the discovery of new demethylases. In *N. alata*, all four functional nicotine demethylases have more similar amino acid sequences to CYP82E4 than CYP82E5v2 and CYP82E10. Therefore, it is predicted that the nornicotine (**4**) enantiomer accumulation pattern in *N. alata* would be similar to tobacco mutant line *e5e10* (Fig. 2, Fig. 3 and Fig. S3). If *N. alata* has nornicotine (**4**) enantiomer accumulation pattern similar to the tobacco mutant line *e5* or *e10*, there is a possibility that one or more functional *CYP82E* genes similar to *CYP82E5v2* and *CYP82E10* have escaped previous identification. The accumulation pattern of nicotine (**1**) and nornicotine (**4**) enantiomers might be used beyond the biology field, such as in archaeology. Zagorevski and Loughmiller-Newman (2012) analyzed the inside contents of more than 50 Maya vessels (~700 AD) and found nicotine (**1**) (EF_{nic} = ~0.005) in one vessel with special hieroglyphic texts. The composition of nicotine (**1**) confirmed that this vessel was used as a tobacco container, not as an ash tray or a container for heating the tobacco. This finding provided unequivocal evidence for the interpretation of a specific iconographic or hieroglyphic representation on the vessel.

The effects of *CYP82E5v2* and *CYP82E10* gene activity appeared to be not additive (Lewis et al., 2010). In this study, it was shown that CYP82E5v2 or CYP82E10, alone, could demethylate almost all (*R*)-nicotine (**1a**) by harvest time (Fig. S3). Therefore, selectivity of these two demethylases along with (*R*)-nicotine (**1a**) being a limited substrate explains why CYP82E5v2 and CYP82E10 in tobacco show no additive effect on nicotine (**1**) demethylation. Substrate limitation may also be the reason why CYP82E5v2 and CYP82E10 are the minor demethylases in tobacco plants.

Gene *CYP82E10* was identified from root-specific cDNA libraries (Lewis et al., 2010), and was not found during study of *CYP82E* genes expressed in leaf tissue (Siminszky et al., 2005; Gavilano et al., 2007; Xu et al., 2007). This suggested that *CYP82E10* is expressed only in root tissues. In the current study, however, the graft *e4e5/ e4e5e10* (Fig. 6) clearly showed the functionality of CYP82E10 in the leaf.

In this study, it was also found that (*R*)-nornicotine (**4**) accumulation increased dramatically in mutants with active CYP82E4 during the first two weeks of curing (Fig. S3A). Since *CYP82E4* expression reached a peak during the same time period and because CYP82E4 can use nicotine (**1**) analogues as substrates *in vitro* assays (Cai et al., 2012), it is proposed that the (*R*)-nornicotine (**4a**) spike observed 2 weeks after harvest may come from nornicotine (**4**) derivatives catalyzed by CYP82E4 through *N*-dealkylation reactions (Fig. S5A). The nornicotine (**4**) derivatives could come from (*R*)-nornicotine (**1a**) derivatization, or alternatively via *de novo* biosynthesis. One piece of evidence to support these possibilities is the observation that plants with active CYP82E5v2 or CYP82E10 but without active CYP82E4 have lower amounts of (*R*)-nicotine (**1**) and (*R*)-nornicotine (**4**) than that in triple mutant plants, implying that (*R*)-nornicotine (**4a**) may be further metabolized (Fig. S5B). Those metabolites of (*R*)-nornicotine (**1a**) could be source materials for conversion back to (*R*)-nornicotine (**4a**). Besides nornicotine derivatives, (*S*)-nicotine (**1b**) theoretically could be the source of (*R*)-nornicotine (**1a**), but the possibility of racemization was ruled out by incubating (*R*) (**1a**) or (*S*)-(**1b**) nicotine with three demethylases and tobacco tissues and only finding the corresponding (*R*) (**1a**) or (*S*) (**1b**)-nornicotine (Cai et al., 2012).

The accumulation pattern of nicotine (**1**) and nornicotine (**4**) enantiomers suggests the possibility of future manipulations of their enantiomeric compositions in tobacco. Nornicotine (**4**) is the precursor of *N*′-nitrosonornicotine (NNN), one of the two most abundant carcinogenic tobacco-specific nitrosamines (TSNAs) identified in tobacco products (Hecht, 1998). Enantiomers of nicotine (**1**) and nitrosated nornicotine (NNN) have different activities in animal and human systems (McIntee and Hecht, 2000; Pogocki et al., 2007; Balbo et al., 2012).

4. Conclusions

Nicotine (**1**) and nornicotine (**4**) enantiomeric composition was investigated in mutant genetic stocks during growth and post-harvest stages. Based on studies of these mutant lines and through grafting studies, approximately 4% of nicotine (**1**) is the (*R*) form **1a** when being synthesized in root tissues. The nicotine demethylase enzymes CYP82E5v2 and CYP82E10 selectively demethylate (*R*)-nicotine (**1a**) to (*R*)-nornicotine (**4a**), resulting in 0.01 EF_{nic} and 0.75 EF_{nnic}, while CYP82E4 decreases EF_{nnic} due to lower preference for (*R*)-nicotine (**1a**) than the other two demethylases. Most

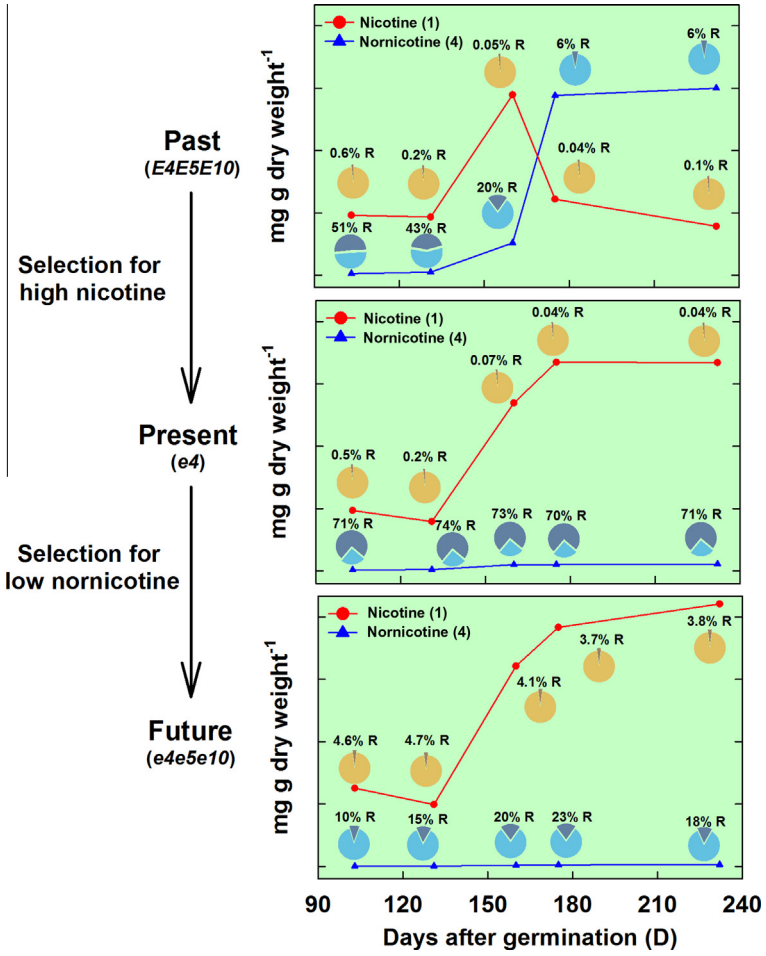

Fig. 8. Changes of nicotine (**1**) and nornicotine (**4**) enantiomeric composition in tobacco leaf over time, probably driven by human selection. All the results are from field tobacco leaf in 2011. "Past" in the figure relates to the time briefly after the formation of allotetraploid tobacco.

(*R*)-nicotine (**1a**) is demethylated in root tissues, while the remainder is metabolized in the leaves. In the leaves, CYP82E5v2 and CYP82E10 can still selectively act on (*R*)-nicotine (**1a**), which keeps EF_{nnic} high, but depending on CYP82E4 activity during senescence, 0.04 to 0.60 EF_{nnic} will be produced.

5. Experimental

5.1. Plant materials

TN 90LC is a standard commercial burley tobacco cultivar with a low tendency to demethylate nicotine (**1**) to form nornicotine (**4**). Burley tobacco breeding line DH98-325-6 (with a very high propensity to demethylate nicotine (**1**) to form nornicotine (**4**)) was used as parent in a mutation breeding experiment to develop genetic stocks possessing different combinations of knockout mutations in *CYP82E4*, *CYP82E5v2* and *CYP82E10*. Development of these mutant lines has been described previously (Lewis et al., 2010) and a scheme for their selection is presented in Fig. S1. The experimental designs in this study are summarized in Table 1.

5.2. Mutants grown in the field

To study the nicotine (**1**) and nornicotine (**4**) enantiomer profiles, TN 90LC, the DH98-325-6 parental line, and the seven mutant lines were grown at Spindletop Farm in Lexington, KY in 2011. The design was a complete randomized block with four replications and 18 plants per plot. Seedlings were grown in a greenhouse at Spindletop Farm, and transplanted to the field on June 1, 2011, and subsequently grown according to standard tobacco cultivation protocols for the state of Kentucky. Plants were sampled five times: one month after transplanting (Jul. 5), at topping (removal of the terminal inflorescence) (Aug. 2), at harvest (Aug. 31), after 2 weeks of curing (Sep. 15) and post-curing (Nov. 11) (Fig. S2). According to the CORESTA guide for tobacco growth stages (CORESTA, 2009), the growth stages of the tobacco at the five sampling times were stage 1105–1108 (one month after transplanting), stage 50–65 (at topping), stage 1112–1123 (at harvest), stage 9101–9103 (at two weeks of air-curing) and stage 9310 (post-curing), respectively. Knockout mutation in gene *CYP82E10* was observed to negatively affect tobacco growth in this study. Plants were harvested and hung on a wagon in an air-curing barn to facilitate leaf sampling. Temperature and relative humidity were monitored during curing (Fig. S6). Lamina (leaf with midvein removed) from middle stem (stalk) position of five plants were sampled for each replicate per tobacco line and bulked. Some plants were sampled at more than one sampling time due to the shortage of plants. Since the nicotine (**1**) level will return to control level 14d after the damage (Tso, 1990) and the time between any two sampling in this study is more than 14d, the repetitive sampling would not influence nicotine (**1**) levels. All samples were oven-dried (55 °C), and ground to pass a 1 mm sieve for alkaloid and R/S enantiomer analysis.

5.3. Graft study

Tobacco lines used for grafting were: the DH98-325-6 parental line, three double mutants (*e4e5*, *e4e10* and *e5e10*) and triple

Table 1
Summary of the experimental designs.

Experiment	Plant selections used*	
Nicotine (**1**) and nornicotine (**4**) accumulation in the lamina of field-grown plants	TN 90LC = (*E4E4E5E5E10E10*)[a] *E4E5E10* = (*E4E4E5E5E10E10*)[b] *e4* = (*e4e4E5E5E10E10*) *e5* = (*E4E4e5e5E10E10*) *e10* = (*E4E4E5E5e10e10*) *e4e5* = (*e4e4e5e5E10E10*) *e4e10* = (*e4e4E5E5e10e10*) *e5e10* = (*E4E4e5e5e10e10*) *e4e5e10* = (*e4e4e5e5e10e10*)	
Dissection of the contribution of the leaf and root to the final nicotine and nornicotine enantiomeric compositions (scion/rootstock)	Self-graft	*E4E5E10*/*E4E5E10* *e4e5*/*e4e5* *e4e10*/*e4e10* *e5e10*/*e5e10* *e4e5e10*/*e4e5e10*
	Tomato/tobacco	Tomato/*E4E5E10* Tomato/*e4e5* Tomato/*e4e10* Tomato/*e5e10* Tomato/*e4e5e10*
	Tobacco/*e4e5e10*	*E4E5E10*/*E4E5E10* *e4e5*/*e4e5* *e4e10*/*e4e10* *e5e10*/*e5e10* *e4e5e10*/*e4e5e10*

* Full genotypes of the tobacco lines were shown in the parenthesis in the top portion of the table, and the corresponding abbreviations were used instead for the simplicity of discussions.
[a] TN 90LC has a functional CYP82E4, but *CYP82E4* expression is suppressed.
[b] Parental line.

mutant (*e4e5e10*). The tomato cultivar 'Rutgers' was used for tomato/tobacco grafts according to Jeffrey and Tso (1964). Cleft grafting was used instead of approach grafting to minimize the alkaloid contamination in the shoot from the stock. The grafts were shaded in high humidity environment for a two-week recovery period. After four new leaves emerged, the plants were topped to induce nicotine (**1**) production. Two weeks after topping, two tobacco leaves were sampled from each grafted plant as one replicate. One leaf was directly oven-dried, and the other was treated with ethephon for 2 days to induce nicotine (**1**) demethylation before oven-drying. Ethephon can promote senescence and induce *CYP82E4* gene expression. The roots were washed (H_2O) to remove potting medium, and then oven-dried.

5.4. Alkaloids quantification and separation of enantiomers of nicotine (*1*) and nornicotine (*4*)

Nicotine (**1**), nornicotine (**4**), anabasine (**2**) and anatabine (**3**) were quantitatively analyzed by GC (Perkin-Elmer Autosystem XL with Prevent^{TM}) according to the 'LC-Protocol' (Jack and Bush, 2007). Alkaloids were extracted from 0.1 ground tobacco samples with 0.5 ml 2M NaOH and extracted by 5 ml MTBE three times at room temperature. Combined MTBE extracts from a sample were injected into the GC, and quantification of alkaloids was conducted against alkaloid standards and a quinoline internal standard. Demethylation reflects how much nicotine (**1**) is demethylated to nornicotine (**4**). Demethylation (%) = nornicotine concentration (mg g dry weight^{-1}) $*$ 100/sum of nicotine (**1**) and nornicotine (**4**) concentration (mg g dry weight^{-1}).

Nicotine (**1**) and nornicotine (**4**) isomers were analyzed using HPLC (Mesnard et al., 2001). Ground tobacco leaves were extracted by MTBE and aqueous sodium hydroxide as described above. Alkaloids from the MTBE extracts were dissolved into a hydrochloric acid solution, and cleaned by MTBE wash to remove chlorophyll. The cleaned acid solution was neutralized by 2M sodium hydroxide solution and extracted with MTBE. Nicotine (**1**) and nornicotine (**4**) from the MTBE extracts were purified by TLC. TLC plates were TLC Silica Gel 60 F254 (EMD Chemicals Inc.). The developing solvent mixture for TLC was $CHCl_3$: MeO_4: NH_4O_4 (85:15:2, v/v/v) (Hu et al., 1974). Purified nicotine was further separated into (*R*) and (*S*) forms **1a** and **1b** by HPLC (Perkin-Elmer series 200) using a Chiralcel OD-H column (0.46 cm (D) $\times$ 25 cm) (Chiral Technologies Inc.), eluted with hexanes/MeOH (98:2, v/v) at 1.0 ml min^{-1}, with detection at 252 nm. Nornicotine (**4**) was methylated to nicotine (**1**) by incubating for 30 min with 50 µl HCO_2H and HCHO (100 µl) 110 °C. The enantiomeric fraction of nicotine (**1**) and nornicotine (**4**) was calculated from the peak areas of the two isomers with absolute amounts of the isomers calculated based on total amount of each alkaloid present and their R/S ratio. Data were analyzed with one-way ANOVA by Sigmaplot 12.0 (Systat Software, San Jose, CA).

Enantiomer fraction (EF) was calculated as: EF = (*R*)-enantiomer/((*R*)-enantiomer + (*S*)-enantiomer) (Harner et al., 2000).

Acknowledgements

This work was supported in part by Altria Client Services and Philip Morris International.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.06.012.

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