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EVOLUTION OF NICOTINE *N*-DEMETHYLASE GENES AND THEIR USE IN REDUCING NORNICOTINE LEVELS IN TOBACCO

Balazs Siminszky, Lily B.Gavilano and Manohar Chakrabarti
University of Kentucky

Ralph E. Dewey, and Steven W. Bowen
North Carolina State University

Abstract

The alkaloid profile of most cultivated tobacco is different from that of its two progenitors, *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Tobacco accumulates nicotine as the most abundant alkaloid, while its ancestors convert nicotine to nornicotine in the green and/or senescing leaf. In a portion of tobacco plants (termed "converters"), however, the alkaloid composition is similar to that found in *N. sylvestris* in that nicotine predominates only in the green leaf, and a large percentage of the nicotine content is converted into nornicotine once the leaves senesce. Nornicotine, the main precursor of *N'*-nitrosonornicotine, is produced by enzymatically-catalyzed nicotine demethylation. The evolution of the nicotine-retaining phenotype of tobacco has been thought to emerge through the inactivation of the conversion loci inherited from its two progenitors. The goals of this project were to elucidate the genetic changes associated with the inactivation of the conversion loci derived from *N. sylvestris* and *N. tomentosiformis* and determine the molecular basis of nicotine *N*-demethylation in converter tobacco. Our results showed that in all three species of *Nicotiana* examined conversion of nicotine to nornicotine is catalyzed by members of a closely-related cytochrome P450 subfamily. Nonfunctionalization of three nicotine *N*-demethylase (NND) genes, designated *NtabCYP82E2*, *NtabCYP82E3* and *NtabCYP82E4*, led to a transition of the alkaloid profile of tobacco from a high nornicotine to a high nicotine phenotype. We also demonstrated that reactivation of the unstable *NtabCYP82E4* locus is responsible for the reversion to the ancestral nornicotine phenotype in the senescing leaves of converter tobacco plants. Transcriptional suppression of the *NtabCYP82E4* gene by RNA interference technology effectively reduced the nornicotine content of tobacco, even in a strong converter background, to levels lower than those typically observed in nonconverter individuals.

Introduction

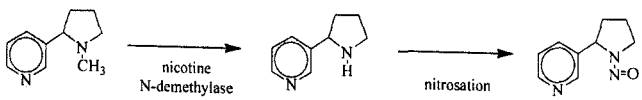
The production of pyridine alkaloids is a characteristic feature of the *Nicotiana* genus, but the alkaloid components and their relative abundance in different species are highly variable (Sisson & Severson, 1990). Although several alkaloids

are present in all species of *Nicotiana*, in the majority of species a single alkaloid predominates. For example, nicotine is the major alkaloid in cultivated tobacco (*N. tabacum*), while nornicotine constitutes the greatest proportion of the alkaloid fraction in *N. tomentosiformis*, and anabasine is the primary alkaloid in *N. glauca*.

In some *Nicotiana* species, the relative abundance of nicotine *versus* nornicotine is dependent on leaf age. For instance, nicotine is the major alkaloid in the green leaves of *N. sylvestris* and *N. gossei*, but nornicotine predominates once the leaves senesce (Sisson & Severson, 1990). Differences in alkaloid profile can even be found among individuals of the same species. In the majority of tobacco plants nicotine is the primary alkaloid in both the green and the senescing leaves, but almost the entire nicotine pool is converted into nornicotine in the senescing leaves of some individuals (Wernsman & Matzinger, 1968). Tobacco plants that accumulate nicotine as their predominant alkaloid in the green and senescing leaves are termed "nonconverters" while the term "converter" is used for those individuals that convert a large percentage of their nicotine to nornicotine in the senescing leaves. Interestingly, the alkaloid phenotype of tobacco plants can change in a single generation, as nonconverter tobacco can give rise to a converter progeny. Once a plant has converted, however, the high nornicotine phenotype is typically stable.

Nornicotine is an undesirable alkaloid in tobacco because it serves as a precursor to *N'*-nitrosonornicotine (NNN; Figure 1.), a tobacco-specific nitrosamine that has been linked to various types of cancer in laboratory animals (Hecht & Hoffmann, 1989; Hoffmann *et al.*, 1994; Hecht, 1998). In addition, nornicotine has been directly implicated in other adverse health consequences in humans due to its ability to mediate aberrant glycation of proteins and interact with commonly prescribed steroids, such as prednisone (Dickerson & Janda, 2002). Other unwanted health effects of nornicotine may include periodontal disease (Katz *et al.*, 2005), and the onset of various birth defects and age-related macular degeneration (Brogan *et al.*, 2005).

Figure 1: Molecular structures of nicotine, nornicotine and NNN.



Nicotine Nornicotine NNN

Investigations of the inheritance of nicotine to nornicotine conversion began more than 50 years ago. Early work on the genetic regulation of nornicotine production demonstrated that the low nicotine, high nornicotine trait was inherited as a single dominant locus (Griffith *et al.*, 1955; Burk & Jeffrey, 1958), and subsequent studies showed that the converter locus was derived from the *N.*

tomentosiformis progenitor (Mann *et al.*, 1964). Because *N. sylvestris*, the other progenitor species of tobacco, also metabolizes a large percentage of its nicotine content to nornicotine in the senescing leaves, Mann *et al.* (1964) proposed that the evolution of the alkaloid profile of nonconverter tobacco involved the inactivation of both the *N. sylvestris*- and the *N. tomentosiformis*-derived converter loci. These workers also suggested that the mutation that inactivated the converter locus donated by *N. tomentosiformis* (C_T) was unstable, and the high nornicotine phenotype of converter tobacco was a result of the reactivation of C_T. These early observations led to an interesting dichotomy, in that converter tobacco plants display the senescence-specific nornicotine accumulation phenotype typical of the *N. sylverstris* progenitor, yet obtained that phenotype through the activation of a conversion locus derived from the *N. tomentosiformis* progenitor, a species that accumulates nornicotine in the green leaf.

Several decades later, investigations of the molecular mechanism of nicotine conversion suggested that nicotine N-demethylation occurred through an oxidative process in which the catalytic actions of cytochrome P450 (P450) enzymes were implicated (Chelvarajan *et al.*, 1993; Hao & Yeoman, 1996b; Hao & Yeoman, 1996a; Hao & Yeoman, 1998). P450 enzymes constitute a large superfamily of proteins that are ubiquitously present in bacteria, fungi, plants and animals (Schuler, 1996). P450-dependent reactions that have been documented to occur in plants are related to several aspects of plant secondary metabolism, such as phenylpropanoid, terpenoid, and fatty acid biosynthesis. Cytochrome P450-mediated reactions include aliphatic hydroxylation, ring hydroxylation, *N*-dealkylation, *O*-dealkylation, epoxidation, peroxidation, etc...

The objectives of our research were to investigate the molecular basis of nicotine to nornicotine conversion in *Nicotiana* and to identify the genetic mutations involved in the inactivation of the conversion loci donated by progenitors *N. sylvestris* and *N. tomentosiformis* to ancestral tobacco. The results of our experiments provided new insight into the evolution of the alkaloid profile of modern tobacco and valuable information for the effective reduction of nornicotine formation in both converter and nonconverter plants.

Nicotine *N*-demethylation is catalyzed by the *CYP82E* gene subfamily in *Nicotiana*

To identify genes that encode nicotine N-demethylase (NND) activity in converter tobacco, we utilized a cDNA microarray-based gene expression profiling strategy (Siminszky *et al.*, 2005). Randomly selected cDNA clones generated from senescing tobacco leaves were sequenced and the resulting expressed sequence tag (EST) database was analyzed to identify a set of sequences that represented unique genes (unigenes). To isolate genes differentially expressed in the senescing leaves of converter versus nonconverter tobacco, fluorescently labeled cDNA generated from

senescing leaves of near isogenic converter and nonconverter tobacco was hybridized to 6963 unigenes printed onto glass slides. This strategy led to the identification of a small subfamily of closely-related P450 genes that were transcriptionally upregulated in the senescing leaves of converter versus nonconverter plants. Using an anchored PCR strategy, the entire coding regions of three cDNAs, designated *NtabCYP82E2*, *NtabCYP82E3* and *NtabCYP82E4*, were isolated and overexpression constructs were prepared from the full-length cDNAs. Heterologous expression studies using yeast as a host demonstrated that NtabCYP82E4 actively catalyzed the conversion of nicotine to nornicotine, but NtabCYP82E2 and NtabCYP82E3 did not confer NND activity (Table 1). Quantitative real-time PCR analysis confirmed that NtabCYP82E4 is strongly upregulated in the senescing leaves of converter tobacco suggesting that NtabCYP82E4 played a key role in nornicotine production in these tissues (Gavilano *et al.*, 2006).

Table 1: NND activity of CYP82E proteins in tobacco and its two progenitors.

Species	CYP82E2	CYP82E3	CYP82E4
		NND activity	
N. tabacum	No	No	Yes
N. tomentosiformis	N/A	Yes	Yes
N. sylvestris	Yes	N/A	N/A

To determine the ancestral origin of the *NtabCYP82E* genes, we amplified genomic DNA isolated from *N. sylvestris* and *N. tomentosiformis* by PCR using *NtabCYP82E*-specific primers. The results of these experiments revealed that *NtabCYP82E2* was donated by *N. sylvestris* to tobacco, while *NtabCYP82E3* and *NtabCYP82E4* were derived from *N. tomentosiformis* (Chakrabarti *et al.*, 2007; Gavilano *et al.*, 2007). Interestingly, *in vitro* assays using microsomal extracts isolated from yeast overexpressing *NtomCYP82E3* or *NtomCYP82E4* revealed that both of these genes mediated NND activity in *N. tomentosiformis* (Gavilano *et al.*, 2007).

The observation that NtabCYP82E3 and NtomCYP82E3 shared over 99% amino acid identity, yet only one of the two proteins, NtomCYP82E3, catalyzed nicotine N-demethylation, prompted us to investigate the structural determinants of the differential NND activity associated with NtabCYP82E3 and NtomCYP82E3 (Gavilano *et al.*, 2007). DNA sequence analysis revealed that the predicted amino acid residues predicted at position 330 differed between the two orthologous *CYP82E3* genes. Similar to P450 proteins isolated from other organisms, including bacteria, yeast, plants and animals, NtomCYP82E3 contained a highly conserved Trp330 residue, while in NtabCYP82E3 a Cys330 was found. Subsequent experiments using site-directed mutagenesis and *in vitro* NND assays showed that

the Cys330Trp mutation was necessary and sufficient to confer NND activity to NtabCYP82E3 demonstrating that the differential NND activity associated with NtomCYP82E3 and NtabCYP82E3 was a direct result of the variation in amino acid sequence at position 330. Trp330 present in NtomCYP82E3 enables NND activity, while Cys330 found in NtabCYP82E3 prevents catalysis (Table 1).

NtomCYP82E3 and NtomCYP82E4 are genetically linked to the C_T converter locus

Because our investigations showed that at least two NND genes, *NtomCYP82E3* and *NtomCYP82E4* are located in the genome of *N. tomentosiformis*, we sought to determine whether either gene is linked to the C_T converter locus (Gavilano *et al.*, 2007). The C_T converter locus confers nornicotine production in the green leaves of *N. tomentosiformis*, therefore any gene linked to C_T should also be associated with the green-leaf conversion phenotype. To test whether the presence of *NtomCYP82E3* and *NtomCYP82E4* correlated with the green-leaf conversion phenotype, we took advantage of tobacco cultivar SC58(C_TC_T). Cultivar SC58(C_TC_T) was developed by crossing *N. tomentosiformis* with the nonconverter Flue-Cured tobacco cultivar SC58 and the product of the cross was subjected to 8 cycles of backcrosses with the recurrent SC58 parent, cultivar SC58 (Mann *et al.*, 1964). In each backcross generation, senescing-leaf converter individuals were selected and used in the next cross with the recurrent parent. After 8 generations of selective backcrosses, cultivar SC58(C_TC_T) is estimated to contain less than 0.5% of the haploid *N. tomentosiformis* genome including the C_T converter locus.

If *NtomCYP82E3* or *NtomCYP82E4* is located in the vicinity of the C_T converter locus, the gene(s) should be present in the genome of SC58(C_TC_T). Using allele-specific PCR we determined that both *NtomCYP82E3* and *NtomCYP82E4* were introgressed into SC58(C_TC_T) tobacco demonstrating that both genes were linked to the C_T converter locus (Gavilano *et al.*, 2007; Table 2).

Table 2: CYP82E2 and CYP82E3 orthologs present in N. tomentosiformis, N. sylvestris and different tobacco genotypes.

Plant	*CYP82E2* ortholog	*CYP82E3* ortholog
SC58	*NtabCYP82E2*	*NtabCYP82E3*
N. tomentosiformis	N/A	*NtomCYP82E3*
SC58(C_TC_T)	N/A	*NtomCYP82E3*
N. sylvestris	*NsylCYP82E2*	N/A
SC58(C_SC_S)	*NsylCYP82E2*	N/A

Expression of *CYP82E3* and *CYP82E4* is differentially regulated

To establish the transcriptional regulation of *CYP82E3* and *CYP82E4*, we analyzed transcript accumulation of these genes using allele-specific, quantitative real-time PCR in the green and senescing leaves of converter and nonconverter tobacco and *N. tomentosiformis* (Gavilano *et al.*, 2007). The results showed that *CYP82E3* was preferentially expressed in the green leaves of *N. tomentosiformis* and all the tobacco genotypes tested (albeit at much lower levels than in *N. tomentosiformis*). In contrast, *CYP82E4* was strongly induced by ethephon treatment and senescence in both *N. tomenetosiformis* and converter tobacco plants. In nonconverter tobacco, the expression of *NtabCYP82E4* was very low in both green and senescing leaves.

Taken together these results demonstrated that in *N. tomentosiformis* at least two active NNDs, *NtomCYP82E3* and *NtomCYP82E4*, are located on the chromosomal region defined by the C_T locus. Inactivation of the nornicotine accumulating phenotype in tobacco involved the stable Trp330Cys mutation that abolished the NND function of *NtabCYP82E3* and the introduction of an unstable mutation that silenced the transcription of *NtabCYP82E4* in nonconverter tobacco. In converter tobacco the transcriptional reactivation of the *NtabCYP82E4* locus mediates nornicotine production in the senescing leaves.

These results provide a plausible explanation concerning the mechanism by which the conversion locus derived from the green-leaf-converter *N. tomentosiformis* confers a senescing-leaf-specific phenotype to converter tobacco. Because the C_T locus donated by the *N. tomentosiformis* progenitor would be predicted to include both the constitutively expressed *NtomCYP82E3* and senescence-inducible *NtomCYP82E4* genes, the permanent inactivation of *NtabCYP82E3* in tobacco restricted nornicotine production to the senescing leaves.

***NsylCYP82E2* is linked to the C_S converter locus**

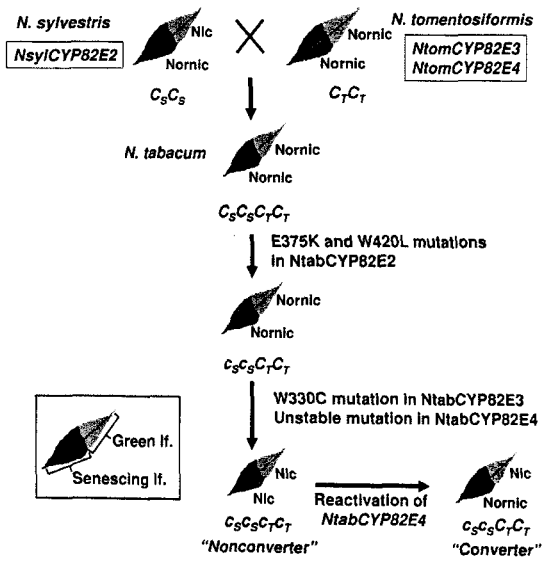
Following the genetic characterization of the C_T locus, we extended our investigation to the molecular structure of the conversion locus derived from *N. sylvestris* (C_S) (Chakrabarti *et al.*, 2007). DNA sequence analysis of the PCR products obtained by the amplification of a cDNA library isolated from the senescing leaves of *N. sylvestris* using *CYP82E*-specific primers revealed that the *CYP82E* gene expressed in senescing leaf tissues was *NsylCYP82E2*. *In vitro* assays conducted with yeast microsomes showed that *NsylCYP82E2* exhibited NND activity (Table 1), and expression profile analysis determined that the transcription of *NsylCYP82E2* was sharply upregulated in ethephon treated, senescing leaves. Because our previous studies indicated that NtabCYP82E2 did not catalyze nicotine *N*-demethylation (Table 1), the discovery of the *NsylCYP82E2* NND gene suggested that *NsylCYP82E2* may represent the C_S converter locus that became inactivated during the evolution of tobacco. To test this hypothesis, we sequenced the *CYP82E2* locus in the SC58(C_SC_S) tobacco line that was developed by a breeding scheme identical to that

described for the SC58(C_TC_T), except *N. sylvestris* was used as the donor parent. DNA sequence analysis showed that the chromosomal fragment derived from *N. sylvestris* carried the *NsylCYP82E2* ortholog in SC58(C_SC_S) tobacco demonstrating that *NsylCYP82E2* was linked to the C_S converter locus (Table 2). Site-directed mutagenesis analyses specifically established that two mutations (Glu375Lys and Trp422Leu) were responsible for the inactivation of *NtabCYP82E2* in tobacco (Chakrabarti *et al.*, 2007).

Proposed model for the evolution of the alkaloid profile of modern tobacco

Collectively, our data support the following model for the evolution of the alkaloid composition found in cultivated tobacco (Chakrabarti *et al.*, 2007; Figure 2). The allotetraploid genome of tobacco originated from the hybridization of the ancestral S and T genomes from species most closely related to modern day *N. sylvestris* and *N. tomentosiformis*, respectively. Both *N. sylvestris* and *N. tomentosiformis* contain a conversion locus, respectively designated C_S and C_T, which are responsible for the conversion of nicotine to nornicotine in the senescing leaves of *N. sylvestris* and in green and senescing leaves of *N. tomentosiformis*. The chromosomal fragment defined by the C_T conversion locus contains at least two NND genes, *NtomCYP82E3* and *NtomCYP82E4*. *NtomCYP82E3* is expressed highly in green leaves, while *NtomCYP82E4* is preferentially transcribed in the senescing leaves of *N. tomentosiformis*. The C_S locus of *N. sylvestris* encodes *NsylCYP82E2*, a likely ortholog of *NtomCYP82E4*, which also mediates the conversion of nicotine to nornicotine during leaf senescence. Because the original ancestral tobacco likely inherited the dominant C_T and C_S conversion alleles, these plants are predicted to have accumulated nornicotine as the predominant alkaloid in green and yellowed leaves alike.

Figure 2: Proposed model for the evolution of the alkaloid profile of tobacco. The lower (black) and upper (grey) regions of the schematic leaf represent the senescent and green developmental stages, respectively. Nic and Nornic denote predominant alkaloids. Abbreviations: C_S and c_S, dominant and recessive alleles of the C_S conversion locus derived from N. sylvestris; C_T and c_T, dominant and recessive alleles of the C_T conversion locus derived from N. tomentosiformis; lf, leaf; Nic, nicotine; Nornic, nornicotine.



Several molecular events involving the duplicated NND genes shaped the evolution of the alkaloid profile of tobacco from its ancestral form to its present phenotype. The stable Glu375Lys and Trp422Leu loss-of-function mutations inactivated the *N. sylvestris*-derived *NtabCYP82E2* and the Trp330Cys amino acid replacement disabled the *N. tomentosiformis*-derived *NtabCYP82E3* gene. Though the specific mechanism is unclear, silencing the transcription of *NtabCYP82E4* eliminated the third major conversion factor of tobacco. The inactivation of *NtabCYP82E2* and *NtabCYP82E3* by debilitating point mutations, combined with the transcriptional silencing of *NtabCYP82E4* led to the emergence of modern cultivated tobacco (nonconverter). The reactivation of *NtabCYP82E4* is responsible for the restoration of ancestral type in converter individuals.

RNAi-mediated suppression of nornicotine production

Identification of *NtabCYP82E4* as the major nicotine N-demethylase gene allowed us to design strategies for suppressing nicotine conversion in tobacco. To silence the expression of *NtabCYP82E4*, an RNA interference (RNAi) construct (82E4Ri) was assembled using a 298 bp fragment of the *NtabCYP82E4* coding

region. Transgenic tobacco lines transformed with the 82E4Ri construct showed sharply reduced nornicotine levels compared to individuals carrying an empty vector (Table 3). In greenhouse-based growth studies, expression of the 82E4Ri construct in nonconverter tobacco conferred as low as 1.2% nicotine conversion compared to the 3-5% conversion rate typically found in untransformed burley varieties used by seed producers. The reduction in nicotine conversion was even more dramatic in converter tobacco. Suppression of *NtabCYP82E* expression in the 82E4Ri#2 line of the strong converter burley variety DH98-325-6 resulted in 1.0% nicotine conversion in contrast to the more than 97 % conversion rate observed in the untransformed controls. These results indicated that silencing the expression of the *NtabCYP82E* gene subfamily with the 82E4Ri construct conferred lower nornicotine content than normally found in commercial burley varieties demonstrating the effectiveness of using molecular tools for reducing the levels of harmful constituents in tobacco.

Table 3: Alkaloid content of T_1 generation DH98-325-5 (nonconverter) and DH98-325-6 (converter) plants transformed with the 82E4Ri construct[a,b]

Line	% Nicotine[c]	% Nornicotine[c]	% Conversion[d]
DH98-325-5 (nonconverter)			
82E4Ri#3			
Mean	1.764	0.024	1.4a
STE	0.456	0.004	0.3
82E4Ri#5			
Mean	1.500	0.020	1.3a
STE	0.306	0.006	0.3
82E4Ri#8			
Mean	1.772	0.020	1.2a
STE	0.409	0.003	0.3
Vector Control#1[e]			
Mean	1.466	0.203	11.6b
STE	0.713	0.161	9.7
DH98-325-6 (converter)			
82E4Ri#2			
Mean	1.970	0.019	1.0a
STE	0.536	0.004	0.3
82E4Ri#8			
Mean	1.623	0.022	1.3a
STE	0.300	0.002	0.2
82E4Ri#10			
Mean	1.419	0.017	1.3a
STE	0.515	0.004	0.3
Vector Control#2[e]			
Mean	0.028	1.170	97.6c
STE	0.006	0.234	0.5

[a]Tobacco leaves were treated with ethephon and cured for 2 weeks at 25°C.
[b]Means and standard errors (STE) represent 9 and 4 T_1 progenies of the 82E4Ri construct- and empty vector-transformed (vector control) lines, respectively.
[c]Percentage of leaf dry weight.
[d][% nornicotine/(% nicotine + % nornicotine)] x 100; Values followed by different letters are significantly different according to Fisher's Protected LSD (0.05).
[e]Tobacco plants transformed with only pKYLX71 vector were used as controls.

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