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Genetic Engineering of *Nicotiana tabacum* for Reduced Nornicotine Content

LILY B. GAVILANO,[†] NICHOLAS P. COLEMAN,[†] LEIGH-EMMA BURNLEY,[‡] MELISSA L. BOWMAN,[§] NEWTON E. KALENGAMALIRO,[‡] ALEC HAYES,[‡] LOWELL BUSH,[†] AND BALAZS SIMINSZKY*,[†]

Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky 40546, Philip Morris USA Research Center, 4201 Commerce Road, Richmond, Virginia 23234, and Lancaster Laboratories, Inc., 2425 New Holland Pike, P.O. Box 12425, Lancaster, Pennsylvania 17605-2425

Nornicotine is an undesirable secondary alkaloid in cultivated tobacco, because it serves as a precursor to *N*′-nitrosonornicotine (NNN), a tobacco-specific nitrosamine with suspected carcinogenic properties. Nornicotine is produced through the oxidative N-demethylation of nicotine by a nicotine *N*-demethylase enzyme during the senescence and curing of tobacco leaves. While the nornicotine content of most commercial burley tobacco is low, a process termed "conversion" can bestow considerably increased nornicotine levels in a portion of the plants within the population. Previously, we isolated a nicotine *N*-demethylase gene, designated CYP82E4, and demonstrated that RNAi-induced silencing of CYP82E4 and its close homologues is an effective means for suppressing nicotine to nornicotine conversion. In this study, we used real-time polymerase chain reaction to confirm the central role of CYP82E4 in nicotine N-demethylation by demonstrating that the transcript accumulation of CYP82E4 is enhanced as much as 80-fold in converter vs nonconverter tobacco. We also show the design of an optimized RNAi construct (82E4Ri298) that suppressed nicotine to nornicotine conversion from 98% to as low as 0.8% in a strong converter tobacco line, a rate of nornicotine production that is about 3.6-fold lower than typically detected in commercial varieties. Southern blot analysis showed that a single copy of the RNAi transgene was as effective in suppressing nornicotine accumulation as multiple copies. Greenhouse-grown transgenic plants transformed with the RNAi construct were morphologically indistinguishable from the empty vector or wild-type controls. These results demonstrate that the genetic transformation of tobacco with the 82E4Ri298 construct is an effective strategy for reducing nornicotine and ultimately NNN levels in tobacco.

KEYWORDS: Alkaloid; cytochrome P450; gene silencing; nicotine *N*-demethylase; *N*′-nitrosonornicotine; plant genetic engineering; metabolic engineering; *Nicotiana tabacum* L.; real-time PCR; RNA interference; tobacco-specific nitrosamines

INTRODUCTION

Nicotine, the most abundant alkaloid in commercial tobacco (*Nicotiana tabacum* L.), is a naturally occurring insecticide that plays an important role in the plant's antiherbivore defense (*1*). In humans, however, nicotine derivatives have been implicated in causing various detrimental health effects including the increased risk for certain cancers (*2*). In cultivated tobacco, nornicotine is a secondary alkaloid that is produced by the enzymatically catalyzed N-demethylation of nicotine in the senescing leaves during the curing period (*3*−*8*). The adverse health effects associated with nornicotine are primarily attributed to the ability of nornicotine to serve as a precursor of *N*′-nitrosonornicotine (NNN), a tobacco-specific nitrosamine (TSNA), that has been shown to be a potent carcinogen in laboratory animals (*9*−*11*). NNN is produced by the nitrosation of nornicotine during the curing and processing of tobacco products.

Apart from serving as a substrate for NNN production, nornicotine has the potential to confer additional unwanted health-associated effects because of its ability to induce the aberrant glycation of proteins and to alter the pharmacological properties of prednisone and possibly other commonly used steroid drugs in smokers (*12*). Recent findings also indicate that smoking-associated nornicotine exposure may be implicated in the onset of various birth defects and age-related macular degeneration by disrupting retinoid homeostasis (*13*). Furthermore, Katz et al. suggested that nornicotine is one of the

* To whom correspondence should be addressed. Tel: 859-257-5020 ext. 80761. Fax: 859-257-7874. E-mail: balazs@uky.edu.
[†] University of Kentucky.
[‡] Philip Morris USA Research Center.
[§] Lancaster Laboratories, Inc.

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Table 1. Sequences of the Oligonucleotide Primers Used in Various PCR Amplifications

experiment	primer name	primer sequence (5′–3′)[a]	5′ terminus position (bp)[b]
RT-PCR (SYBR)	E4SyFwd	ACGTGATCCTAAACTCTGGTCTG	1248
	E4SyRev	GCCTGCACCTTCCTTCATG	1485
RT-PCR (Taqman)	E4TmFwd	CGGTAATCGGCCATCTTTTC	125
	E4TmRev	CCGAGTTTTCGAGCTAATGGA	191
MGB probe	E4MGB	CAATGACGACGGCGACGA	150
82E4Ri298 design	E4SFwd	**AAGCTT**TGACGCCATTTTTTCCAATCG	297
	E4SRev	**CTCGAG**TTTTCCAGCGATCATCTTCAC	594
Northern blot probe	E4FlFwd	ATGGTTTTTCCCATAGAAGCC	1
	E4FlRev	TTTTTGGGACAATCAGTCAAT	1738
Southern blot probe	KanFwd	TGAATGAACTGCAGGACGAG	NA
	KanRev	AATATCACGGGTAGCCAACG	NA

[a] Restriction enzyme cleavage sites, appended to the 5′-end of the primers to facilitate cloning, are bolded. [b] Position 1 is the first base of the start codon in the CYP82E4 cDNA. NA, not applicable.

constituents of tobacco that causes periodontal disease in smokers (*14*). Finally, high levels of nornicotine have also been shown to adversely affect tobacco quality by producing undesirable flavor and smoking characteristics in tobacco products (*15*).

In most commercial tobacco varieties, nornicotine constitutes a minor portion (<5%) of the total alkaloid pool, but a process termed "conversion" can give rise to individual plants within a population in which as much as 97% of nicotine is metabolized to nornicotine. Plants that convert high percentages of nicotine to nornicotine (converters) may arise in a single generation from parents that accumulate nicotine as their most abundant alkaloid (nonconverters). As compared to flue-cured tobacco varieties, burley cultivars are more prone to conversion affecting as high as 20% of the burley population per generation.

In flue-cured tobaccos, TSNA formation has been largely reduced by retrofitting curing barns with heat exchangers. Ensuring low nornicotine and NNN accumulation in air-cured burley cultivars, however, currently involves the maintenance of clean foundation seed stocks by rouging out converter individuals from the breeding lines, a process that is expensive, labor intensive, and imperfect, as converter plants may arise in a single generation.

We previously demonstrated that (i) a closely related family of P450s (designated CYP82E2 subfamily) plays a major role in nicotine *N*-demethylation; (ii) CYP82E4, a member of the CYP82E2 subfamily, mediates the nicotine to nornicotine conversion in transgenic tobacco and yeast; and (iii) the down-regulation of the CYP82E2 subfamily in transgenic tobacco using RNAi-induced gene silencing can lower nornicotine production, thus providing a new tool in efforts to reduce the accumulation of certain TSNAs in tobacco products (*7*). Here, we provide additional evidence that CYP82E4 is involved in nicotine *N*-demethylation in tobacco and describe the design of an improved RNAi construct that allowed further suppression of nornicotine production in very strong converter lines to levels below those typically found in nonconverter individuals.

MATERIAL AND METHODS

Plant Materials. Double haploid burley tobacco lines DH 98-325-5 (325-5; nonconverter) and DH98-325-6 (325-6; converter) described in ref *7* were used in all experiments, except for the fluorogenic 5′-nuclease (TaqMan) chemistry-based reverse transcription polymerase chain reaction (RT-PCR) assays where the isogenic DH 91-1307-46 (nonconverter) and DH 91-1307-46 (converter) lines were used. All plants were grown in a controlled environment greenhouse equipped with supplemental lighting providing a 14/10 h light/dark cycle. For curing, tobacco leaves were collected from converter and nonconverter plants about 1 month before flowering and treated by dipping each leaf twice for 10 s into 2% ethephon and dried for 2 h. Leaves were cured for up to 2 weeks in plastic bags, under dark conditions, until they turned yellow. Cured leaves were used for the Northern and alkaloid analysis. Samples of cured leaves subjected to gas chromatography (GC) analysis were dried at 50 °C for 2 days. For Southern analysis, green tobacco leaves of adult plants were used. To produce T_1 generation transgenic plants, primary transformants (T_0) were self-pollinated, and the harvested T_1 seed was screened by germinating seedlings on Murashige−Skoog (MS) agar plates containing 100 mg/L kanamycin for 6 weeks. Survivors were transplanted to soil and grown in a greenhouse as described above. Plants were fertilized with Peter's Professional All Purpose Plant Food (20-20-20; Spectrum Brands Inc., Madison, WI) once a week.

RT-PCR Analyses. *SYBR Green I Chemistry.* Total RNA was isolated from cured leaves of converter and nonconverter burley tobacco plants using the TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA). Purified RNA was treated with RNase-free DNase (TURBO DNA-free, Ambion, Austin, TX). First strand cDNA was synthesized using 5 μg of total RNA and the StrataScript First-Strand Synthesis System (Stratagene, Cedar Creek, TX). Relative quantitative RT-PCR was employed for determining the abundance of the CYP82E4 cDNA using SYBR Green I fluorescence chemistry (*16*). A calibration curve was generated with a serial dilution of the CYP82E4 cDNA cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI). The RT-PCR mixture contained 2.5 mM $MgCl_2$, 125 μM each dNTP, 0.5 μM each primer, 0.5× SYBR Green I, 0.5 μg of cDNA (or 1 μL of reference plasmid), and 1.25 U Platinum Taq polymerase (Invitrogen Life Technologies). The sequences of the allele-specific CYP82E4 primers (E4SyFwd and E4SyRev) are shown in **Table 1**. RT-PCR was performed in a BioRad iCycler thermocycler (BioRad Laboratories, Hercules, CA) set to the following protocol: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 50 s followed by final extension at 72 °C for 5 min. A 165 bp fragment of the α-tubulin gene was used as an internal standard. The CYP82E4 cDNA concentration was determined from the transcript-specific calibration curve and normalized to the internal standard. Fold induction was calculated by dividing the normalized fluorescence values of the converter by the nonconverter samples. Melting curve analysis was used to confirm the purity of PCR products as described previously (*17*). Two plants were sampled per treatment, and amplifications were repeated three times.

TaqMan Chemistry. Total RNA was isolated from tobacco lines using TRIzol reagent. Purified RNA was treated with RNase-free DNase (TURBO DNA-free). First strand cDNA was synthesized using 10 μg of total RNA and the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The RT-PCR mixture contained 1× Taqman Universal PCR Master Mix (Applied Biosystems), a 400 nM concentration of each primer (E4TmFwd and E4TmRev), 250 nM Taqman minor groove binder (MGB) probe (E4MGB), 2 ng of cDNA, and nuclease-free water (*18*). The primer and probe sequences are shown in **Table 1**. RT-PCR was performed in an ABI 7500 Real-Time System (Applied Biosystems) set to the following protocol: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1

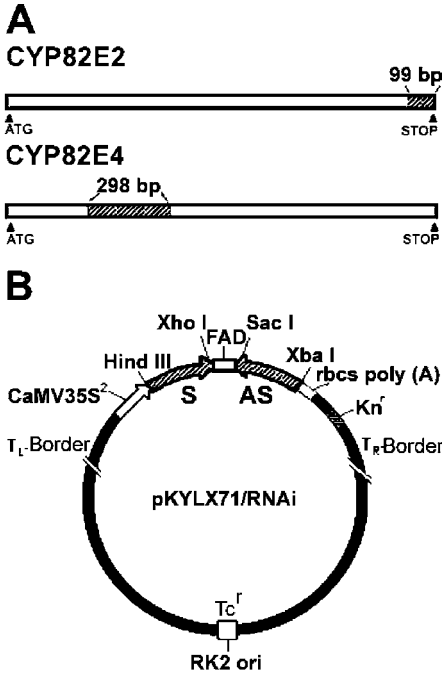

Figure 1. (**A**) Schematic representations of the CYP82E2 and CYP82E4 cDNAs. Regions used to create the inverted repeats in the 82E2Ri99 and 82E4Ri298 RNAi constructs are boxed. (**B**) Map of the 82E2Ri99 and 82E4Ri298 RNAi constructs. S, sense arm of the inverted repeat; AS, antisense arm of the inverted repeat.

min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as the endogenous control to normalize the amount of cDNA template in the reactions. The fold change was determined by dividing the normalized fluorescence values of each sample by those obtained from a nonconverter or uncured control sample. For each treatment, RNA was isolated from three independent plants and amplifications were repeated three times per RNA sample.

Preparation of the 82E2Ri99 and 82E4Ri298 RNAi Constructs. Construct 82E2Ri99 is identical to the CYP82E2/RNAi plasmid described in ref *7*. The assembly of the 82E4Ri298 expression cassette followed the same basic steps as those outlined for CYP82E2/RNAi. Briefly, 298 bp sense and antisense strands of the CYP82E4 cDNA (accession no. DQ131886) corresponding to the region between nucleotide positions 297 and 594 were ligated into the pKYLX80I vector downstream and upstream of the 151 bp, soybean ω-3 fatty acid desaturase intron, respectively. The primers (E4SFwd and E4SRev) used for the isolation of the 298 bp sense and antisense arms are listed in **Table 1**. The RNAi cassette was excised from pKYLX80I and placed between a strong CaMV35S[2] promoter and a rubisco small subunit terminator of the binary plant expression vector, pKYLX71 (*19*; **Figure 1**).

Tobacco Transformation. Transgenic tobacco plants were generated via *Agrobacterium*-mediated transformation following standard procedures (*20*). Briefly, transformed burley tobacco plants were regenerated from calli on MS medium supplemented with 100 mg/L kanamycin and plant hormones in a growth room maintained at 25 °C under a 16/8 h light/dark cycle. Calli were transferred to fresh selection media every 2−3 weeks until shoots appeared. Small shoots were transferred to rooting media to allow root development for 2 weeks. Fully regenerated plants were transferred to a greenhouse and grown under standard conditions.

Northern and Southern Blot Analyses. Total RNA was isolated from cured tobacco leaves using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). Total RNA samples were separated on 1.2% TBE agarose gel and transferred to positively charged nylon membranes by electroblotting with 2× TBE buffer. Membranes were UV cross-linked and washed in 2× SSC for 5 min. Northern blot hybridization, washing, and detection were carried out using the digoxigenin (DIG) System as described by the manufacturer (Roche Diagnostics Corp., Indianapolis, IN). The 1.8 kb full-length ORF of the CYP82E4 cDNA was labeled with DIG and used as a probe.

Genomic DNA was extracted with DNAzol (Invitrogen, Life Technologies) from green tobacco leaves according to manufacturer's protocol. After incubation with EcoRI or NcoI restriction enzymes overnight, 15 μg of the digested DNA was separated on 0.7% TBE agarose gel, depurinated with 0.25 M HCl for 10 min, and denaturated with 0.5 N NaOH for 30 min. DNA was blotted overnight by capillary transfer onto positively charged nylon membranes (Roche Diagnostics Corp.) and hybridized at 65 °C overnight with a 515 bp, DIG-labeled fragment of the neomycin phosphotransferase II (NPT II) gene. Hybridization, washing, and detection were performed according to the protocols supplied with the DIG System. The primers used for the amplifications of the Northern and Southern hybridization probes (E4FlFwd, E4FlRev, KanFwd, and KanRev) are listed in **Table 1**.

Alkaloid Analysis. Tobacco leaves were harvested and air-dried in an oven at 50 °C for 2 days. A 100 mg sample of crushed, dried leaf was added to 0.5 mL of 2 N NaOH in a 20 mL scintillation vial. The sample was mixed and allowed to incubate for 15 min at room temperature. Alkaloids were extracted by the addition of 5 mL of extraction solution [0.04% quinoline (wt/vol) dissolved in methyl-*tert*-butyl ether] and gently rotated on a linear shaker for 3 h. Following phase separation, an aliquot of the organic phase was transferred to a sample vial. Samples were analyzed using a PerkinElmer Autosystem XL (PerkinElmer, Boston, MA) gas chromatograph equipped with a flame ionization detector, a 4 mm split/splitless glass liner, and a 30 m × 0.53 mm i.d. DB-5 column. Chromatographic conditions were as follows: detector temperature, 250 °C; injector temperature, 250 °C; helium flow at 120 °C, 20 mL/min; injection volume, 2 μL; and column conditions, 120 °C, hold 1 min, 120−280 °C at 30 °C/min ramping rate, and hold at 280 °C for 2 min. The alkaloid composition was determined by the TotalChrome Navigator software using a calibration curve. Means of the alkaloid measurements were separated according to Fisher's protected least significant difference (LSD) (PROC MIXED; *21*).

RESULTS

RT-PCR Analysis of CYP82E4 Expression in Converter and Nonconverter Tobacco. In previous experiments, we have shown that the expression of CYP82E4 in transgenic yeast and tobacco results in enhanced nicotine N-demethylation (*7*). To further characterize the role of CYP82E4 in nicotine N-demethylation, we designed experiments to demonstrate that the regulation of CYP82E4 expression is consistent with the levels of nicotine N-demethylation activity observed in converter vs nonconverter tobacco. If CYP82E4 is the primary gene involved in the conversion of nicotine to nornicotine, its expression level would be expected to be much higher in converter than nonconverter individuals, assuming that the conversion phenomenon is a result of a change in gene regulation rather than a mutation of the nicotine *N*-demethylase protein. This assumption was partially supported by expression analysis of the CYP82E2 subfamily by microarray analysis that revealed an increase in transcription of about 2-fold in converter vs nonconverter tobacco (*7*). However, the considerable difference between the modest change in transcription of the CYP82E2 subfamily (≈2-fold) and the dramatic increase in nornicotine production (≈20-fold) warranted further investigations. One possible explanation for this discrepancy is that CYP82E4 is the only member of the CYP82E2 subfamily whose transcription is upregulated in converter vs nonconverter tobacco and that the specific changes in the expression of CYP82E4 were obscured by cross-hybridization to the other closely related CYP82E2 members in the microarray assays.

To determine the rate of CYP82E4 mRNA accumulation in converter and nonconverter tobacco, we employed an allele-

specific real-time PCR (RT-PCR) strategy. Because RT-PCR involves the detection and measurement of the amplification products of a PCR template, the use of allele-specific primers allows the quantification of a single isoform among a group of highly homologous sequences. For accurate quantification of the CYP82E4 transcript, we amplified two different segments of the CYP82E4 coding region and used both SYBR Green I and Taqman chemistries to generate fluorescence signals. RT-PCR analysis using the SYBR Green I chemistry revealed an 80-fold increase in the levels of the CYP82E4 transcript in the cured leaves of converter vs nonconverter tobacco (**Figure 2A**). The single peak melting curve and gel electrophoretic analyses of the amplicons confirmed the homogeneity of the PCR products (**Figure 2B**,**C**).

In the Taqman chemistry-based RT-PCR experiment, CYP82E4 transcript levels were quantified in untreated and ethephon-treated converter and nonconverter tobacco leaves that were cured for 0, 1, or 5 days (**Figure 2D**). Low levels of CYP82E4 transcripts were detected in the uncured leaves or following a 1 day curing period regardless of conversion type or ethephon treatment. Similarly, baseline levels of CYP82E4 transcription were observed in converter or nonconverter leaves that were cured for 5 days without ethephon treatment. In contrast, a 7.5-fold increase in CYP82E4 transcript accumulation was detected in the cured leaves of converter vs nonconverter tobacco, and a 70-fold increase was observed in the uncured vs cured leaves of a converter tobacco variety when ethephon treatment preceded the 5 day curing period (**Figure 2D**). Together, these results provided further evidence to the notion that CYP82E4 is a major contributor to nicotine *N*-demethylation and is strongly inducible by ethylene in senescing tobacco leaves.

Design of the 82E4Ri298 RNAi Construct. RNAi is a widely used technology for posttranscriptional gene silencing (PTGS) in plants (*22*). Of the numerous constructs described for the induction of RNAi-mediated PTGS, those encoding intron-spliced hairpin RNAs have been shown to be particularly efficient (*23*). Intron-spliced hairpin RNAs are generated by transforming plants with an RNAi construct containing an intron flanked by an inverted repeat of a fragment of the targeted gene (*23*). At the time of our original (82E2Ri99) RNAi construct design, we were unaware which member(s) of the CYP82E2 subfamily encoded nicotine *N*-demethylase activity, and we used a 99 bp sense and antisense fragment of the CYP82E2 gene to create the necessary inverted repeat (**Figure 1**). The 99 bp fragment of CYP82E2 used in the design of the RNAi construct contains seven single nucleotide polymorphisms relative to the nucleotide sequence of CYP82E4, the only member of the CYP82E2 subfamily that exhibited nicotine *N*-demethylase activity when expressed in transgenic plants or yeast (*7*).

To engineer an improved RNAi construct capable of suppressing the nicotine *N*-demethylase activity in converter tobacco to levels below those attained with 82E2Ri99, we implemented two changes in the construct design. First, the inverted repeat was assembled from a sense and antisense fragment of the CYP82E4 cDNA to increase the degree of homology with the target nicotine *N*-demethylase gene (**Figure 1**). Second, the two arms of the inverted repeat were extended in size to 298 bp, because gene fragments in the range of 100−300 bp have been shown to induce PTGS at maximal efficiency (*24*, *25*). The new construct was named 82E4Ri298.

Suppression of Nicotine to Nornicotine Conversion by the 82E2Ri99 and 82E4Ri298 Constructs. To compare the extent to which 82E2Ri99 and 82E4Ri298 mediate the suppression of nornicotine production, converter and nonconverter burley tobacco plants were transformed with the two gene silencing vectors. Because the level of transgene expression can be highly

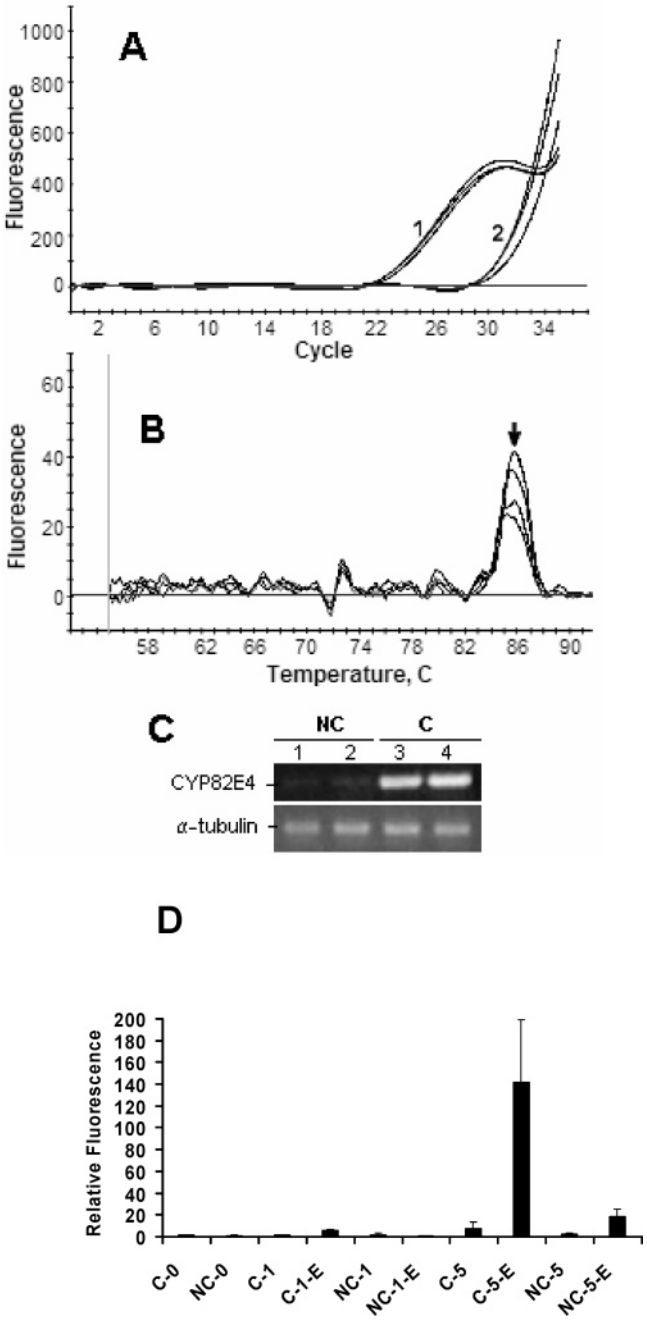

Figure 2. RT-PCR detection of CYP82E4 expression in nonconverter and converter *N. tabacum*. (**A**) Amplification plots of the 239 bp CYP82E4 cDNA fragment of converter (1) and nonconverter (2) tobacco plants used in the SYBR Green chemistry-based RT-PCR experiment. Three replications are shown. (**B**) Melting curve analysis of RT-PCR products of nonconverter and converter samples. The single peak (arrow) indicates the homogeneity of the PCR products. Two replications are shown. (**C**) Agarose gel electrophoretic analysis of the CYP82E4 cDNA fragment produced by 35 cycles of RT-PCR amplification of cDNA isolated from nonconverter (NC) and converter (C) senescing leaves. Two replications are shown. A 165 bp α-tubulin fragment is shown as an internal gene reference. (**D**) Taqman chemistry-based RT-PCR analysis of CYP82E4 expression in converter and nonconverter tobacco following 0, 1, and 5 days of curing with or without ethephon treatment. Error bars represent standard error ($n = 9$). *X*-axis labels: C, converter; NC, nonconverter; E, ethephon; and numbers, curing period in days.

Table 2. Alkaloid Content of T_0 Generation DH98-325-5 (Nonconverter) Tobacco Transformed with the 82E2Ri99 and 82E4Ri298 Constructs[a,b]

line[c]	% nicotine[d]	% nornicotine[d]	% conversion[e]
	82E2Ri99		
1	1.693	0.034	2.0
2	1.435	0.031	2.1
3	2.095	0.043	2.0
4	2.868	0.053	1.8
5	0.947	0.025	2.6
6	2.357	0.043	1.8
7	2.599	0.043	1.6
8	0.796	0.020	2.4
9	2.178	0.039	1.8
10	3.162	0.061	1.9
mean	2.013	0.039	2.0 a
STE[f]	0.748	0.012	0.3
	82E4Ri298		
3	1.806	0.020	1.1
4	1.948	0.027	1.4
5	2.061	0.020	1.0
6	2.704	0.040	1.5
8	2.652	0.023	0.9
9	1.074	0.015	1.3
mean	2.041	0.024	1.2 b
STE	0.550	0.008	0.2
	vector control[g]		
1	1.206	0.052	4.2
2	1.265	0.038	2.9
3	1.752	0.058	3.2
4	1.230	0.072	5.6
5	1.777	0.060	3.3
6	1.536	0.044	2.8
mean	1.461	0.054	3.6 c
STE	0.240	0.011	1.0

[a] Tobacco leaves were treated with ethephon and cured for 2 weeks at 25 °C. [b] Of the plants transformed with an RNAi construct, only silenced individuals are shown. Alkaloid data represent the means of two measurements. [c] Numbers represent independently transformed individuals. [d] Percentage of leaf dry weight. [e] [% nornicotine/(% nicotine + % nornicotine)] × 100. Values followed by different letters are significantly different according to Fisher's protected LSD (0.05). [f] STE, standard error. [g] Tobacco plants transformed with only pKYLX71 vector were used as controls.

Table 3. Alkaloid Content of T_0 Generation DH98-325-6 (Converter) Tobacco Transformed with the 82E2Ri99 and 82E4Ri298 Constructs[a,b]

line[c]	% nicotine[d]	% nornicotine[d]	% conversion[e]
	82E2Ri99[f]		
1	3.419	0.100	2.8
2	2.569	0.193	7.0
3	2.175	0.064	2.9
4	3.517	0.125	3.4
8	2.268	0.128	5.3
9	2.197	0.133	5.7
10	2.434	0.112	4.4
mean	2.654	0.122	4.5 a
STE[g]	0.573	0.039	1.6
	82E4Ri298[h]		
1	2.043	0.020	1.0
2	3.427	0.026	0.8
3	2.603	0.020	0.8
5	2.427	0.030	1.2
6	2.106	0.021	1.0
7	1.412	0.015	1.1
8	3.328	0.028	0.8
9	1.493	0.015	1.0
10	2.065	0.018	0.8
mean	2.323	0.021	0.9 b
STE	0.669	0.005	0.1
	vector control[h,i]		
1	0.126	1.550	92.5
2	0.330	2.604	88.8
3	0.060	1.419	95.9
4	0.114	1.267	91.7
5	0.119	1.303	91.6
mean	0.150	1.628	92.1 c
STE	0.093	0.498	2.3

[a] Tobacco leaves were treated with ethephon and cured for 2 weeks at 25 °C. [b] Of the plants transformed with an RNAi construct, only silenced individuals are shown. [c] Numbers represent independently transformed individuals. [d] Percentage of leaf dry weight. [e] [% nornicotine/(% nicotine + % nornicotine)] × 100. Values followed by different letters are significantly different according to Fisher's protected LSD (0.05). [f] As shown in ref *7*. [g] STE, standard error. [h] Alkaloid data represent the means of two measurements. [i] Tobacco plants transformed with only pKYLX71 vector were used as controls.

variable among independently transformed individuals, we regenerated 10 transgenic plants per RNAi construct. About 80% of tobacco plants overexpressing either the 99 or the 298 bp inverted repeat showed reduced nornicotine levels as compared to the empty vector controls (**Tables 2** and **3**). In the remaining plants, changes in nornicotine content were not evident, an observation that is consistent with the 65−100% silencing frequency commonly associated with intron-spliced hairpin-mediated RNAi (*26*, *27*). In the nonconverter genotype, 82E2Ri99 and 82E4Ri298 expression reduced nicotine to nornicotine conversion by about 1.8-fold (2.0%) and 3.0-fold (1.2%), respectively, in comparison to the rate of conversion detected in the vector controls (3.6%) (**Table 2**). Among the silenced nonconverter plants, the lowest conversion level of 0.9% was achieved using the 82E4Ri298 construct (**Table 2**).

Relative to nonconverter tobacco, nornicotine accumulation was suppressed even more dramatically in the silenced individuals of the strong converter plants (**Table 3**). Previously, we showed that nicotine conversion was reduced to levels as low as 4.5% in 82E2Ri99-transformed 325-6 tobacco plants in sharp contrast to the 325-6 control plants exhibiting about 98% conversion rates (*7*; **Table 3**). Even greater reductions were achieved using the 82E4Ri298 construct (**Table 3**). Four 82E4Ri298-transformed individuals converted as low as 0.8% of their nicotine to nornicotine, and the arithmetic mean across the nine silenced transformants was 0.9% conversion. Regardless of the RNAi construct used, all silenced plants were morphologically indistinguishable from both the empty vector and the wild-type controls (data not shown).

To test the heritability of nornicotine suppression in the 82E4Ri298-transformed plants, a set of 82E4Ri298 converter and nonconverter lines that displayed the lowest levels of nicotine conversion were advanced to the T_1 generation (**Table 4**). Because segregation of the transgene(s) occurs in the T_1 progeny, transgenic individuals were identified by selecting seedlings capable of growing on kanamycin-containing media. Nine kanamycin-resistant progenies of each selected T_0 generation 82E4Ri298 and four kanamycin-resistant individuals from each selected vector control line were analyzed for alkaloid content. The results revealed that the rate of nicotine conversion did not differ significantly between the primary 82E4Ri298 transformants and their T_1 progeny indicating high heritability of the nornicotine suppression trait (**Tables 2**−**4**). In contrast, advancing the "nonconverter" vector control line by a single generation increased the nicotine to nornicotine conversion rate from 4.2 to an average value of 11.6% illustrating the high degree of instability of the conversion locus in transgenic plants lacking the CYP82E4-specific RNAi construct (**Tables 2** and **4**). Overall, these results show that RNAi-mediated silencing of the CYP82E2 gene subfamily is a highly effective means of lowering nornicotine production in both nonconverter and strong

Table 4. Alkaloid Content of T_1 Generation DH98-325-5 (Nonconverter) and DH98-325-6 (Converter) Plants Transformed with the 82E4Ri298 Construct[a,b]

line	% nicotine[c]	% nornicotine[c]	% conversion[d]
DH98-325-5 (nonconverter)			
82E4Ri298 #3			
mean	1.764	0.024	1.4 a
STE	0.456	0.004	0.3
82E4Ri298 #5			
mean	1.500	0.020	1.3 a
STE	0.306	0.006	0.3
82E4Ri298 #8			
mean	1.772	0.020	1.2 a
STE	0.409	0.003	0.3
vector control #1[e]			
mean	1.466	0.203	11.6 b
STE	0.713	0.161	9.7
DH98-325-6 (converter)			
82E4Ri298 #2			
mean	1.970	0.019	1.0 a
STE	0.536	0.004	0.3
82E4Ri298 #8			
mean	1.623	0.022	1.3 a
STE	0.300	0.002	0.2
82E4Ri298 #10			
mean	1.419	0.017	1.3 a
STE	0.515	0.004	0.3
vector control #2[e]			
mean	0.028	1.170	97.6 c
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 nine and four T_1 progenies of the 82E4Ri298 construct- and empty vector-transformed (vector control) lines, respectively. [c] Percentage of leaf dry weight. [d] [% nornicotine/(% nicotine + % nornicotine)] × 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.

converter tobacco. Furthermore, transforming tobacco with the 82E4Ri298 construct conferred a 3.6-fold reduction in nicotine conversion relative to typical nonconverter control plants without affecting plant growth and development.

RNAi triggers the sequence-specific degradation of target mRNA. To demonstrate that the down-regulation of nornicotine production in 82E4Ri298-transformed tobacco was concomitant with a reduction of the CYP82E2 gene subfamily transcripts, we hybridized a CYP82E4 cDNA probe to the total RNA isolated from cured leaves of nonconverter and converter plants. Only a weak hybridization signal was generated by the RNA isolated from 82E4Ri298 transformants displaying low nornicotine content in contrast to the strong signal produced by the RNA extracted from plasmid control or wild-type plants (**Figure 3**). These results suggest that the down-regulation of nicotine conversion was a result of RNAi-mediated gene silencing of the nicotine *N*-demethylase gene(s).

Determination of Transgene Copy Number. Although *Agrobacterium*-mediated transformation typically generates a relatively low number (1−5) of insertion events in the plant genome, the substantial variation in gene expression observed within the population of transplants transformed by the same transgene is frequently associated with differences in transgene copy number (*28*, *29*). To determine whether the integration of multiple 82E4Ri298 copies was required for producing transplants displaying very low nicotine *N*-demethylase activity, we performed Southern analysis on selected individuals exhibiting <1.5% nornicotine accumulation. Southern blot analysis of genomic DNA digested with the EcoRI restriction enzyme showed that the number of transgenes varied widely among these

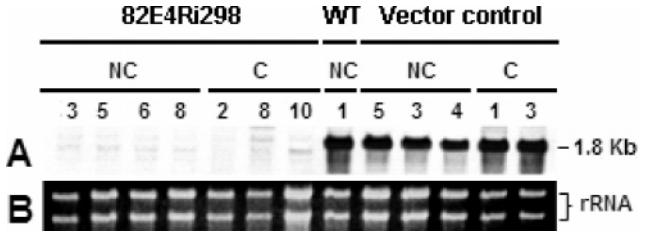

Figure 3. (**A**) Northern blot analysis of tobacco plants using the CYP82E4 cDNA as a probe. Numbers represent plant lines. Transgenic plants transformed with an empty vector pKYLX71 were used as controls. WT, wild-type; C, Converter; and NC, nonconverter. (**B**) Ethidium bromide-stained agarose gel used to fractionate RNA for the Northern blot analysis. 18S and 28S rRNA are shown as loading controls.

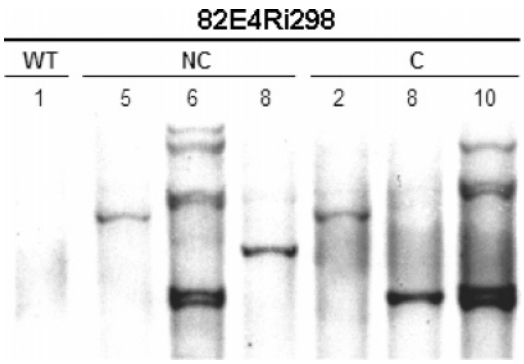

Figure 4. Southern hybridization of genomic DNA isolated from the wild-type (WT), 82E4Ri298-transformed nonconverter (NC), and converter (C) tobacco leaves. A 515 bp fragment of the *NPT II* gene was used as a probe. Numbers represent tobacco lines.

plants including individuals containing one copy (325-5, lines 5 and 8; 325-6, lines 2 and 8), five copies (325-6, line 10), and six copies (325-5, line 6) of the 82E4Ri298 construct (**Figure 4**). Transgene copy number was confirmed using NcoI digested DNA (data not shown). These results indicate that the integration of a single 82E4Ri298 construct into the genome of a strong converter tobacco is sufficient for suppressing nornicotine production to very low levels.

DISCUSSION

Transcript analysis of individual members of the closely related CYP82E2 gene subfamily can be problematic due to the indiscriminate hybridization of the DNA probe to mRNA of multiple homologous isoforms. The difficulties that may be encountered are well-illustrated by our microarray experiments in which hybridization of cDNA isolated from a nonconverter tobacco generated only about twice as much signal as that detected when cDNA from converter plants was used as a probe (*7*). These results could have been falsely interpreted as evidence for only a modest up-regulation of CYP82E4 transcription in converter vs nonconverter tobacco that is out of scale with the sharp increase observed in converter plants. The reason for this apparent discrepancy is the ability of the large number of closely related isoforms of the CYP82E2 gene subfamily to simultaneously hybridize to the CYP82E4 probe, thereby "masking" the hybridization signal of a single individual.

To circumvent the problems associated with the Northern analysis of the CYP82E2 gene subfamily, we employed allele-specific RT-PCR to compare CYP82E4 expression between converter and nonconverter plants. The SYBR Green chemistry-based RT-PCR assay revealed a ≈80-fold enhancement of CYP82E4 expression in converter vs nonconverter plants in

contrast to the 7.5 up-regulation showed by the Taqman chemistry-based RT-PCR experiment (**Figure 2A**,**D**). A likely explanation for the discrepancy in induction values is provided by the differential nornicotine levels (and thus nicotine N-demethylase activity) observed between the "nonconverter" references used in the two assays. While the DH 91-1307-46 tobacco variety used in the Taqman chemistry-based RT-PCR experiment exhibits low to moderate levels of nicotine conversion, the DH98-325-5 nonconverter plants used in the SYBR Green-based RT-PCR assay consistently convert a very low percentage of their nicotine to nornicotine (unpublished data). Regardless of the experimental conditions, these results strongly suggest that the control of the "conversion" phenomenon occurs at the level of nicotine *N*-demethylase transcription and confirms the catalytic involvement of CYP82E4 in nornicotine production. Consistent with other reports (*30*, *31*), our results also demonstrate that the expression of the CYP82E4 gene is strongly induced by ethylene in senescing leaves of converter tobacco (**Figure 2D**).

Because of the positive correlation between nornicotine production and NNN formation, considerable efforts have been made to minimize nornicotine content in commercial tobacco. Currently, a commonly used method for ensuring low nornicotine levels in burley tobacco cultivars involves alkaloid analysis by GC and the systematic removal of individuals that convert more than 3% of their nicotine to nornicotine from the breeding lines. Although an effective means for lowering nornicotine formation, cleaning up the foundation seed stocks does not completely eliminate conversion, because converter individuals can arise in each generation. As a result, nicotine conversion in most burley varieties exceeds the 3% threshold (after ethephon treatment) even after performing alkaloid screens for several generations (Anne Jack, personal communication).

In this study, we described the development of an RNAi construct that allowed the suppression of nornicotine production in a strong converter tobacco line below the levels normally found in nonconverter plants. The expression cassette of the construct encoded an intron-spliced hairpin RNA in which the stem region was engineered from the 298 bp fragment of the CYP82E4 cDNA inserted as an inverted repeat. The loop of the hairpin was created by placing a 151 bp intron of the *FAD* gene between the two sides of the palindromic sequences. An arm length of 298 bp was chosen for the inverted repeats, because the 99 bp arms of the 82E2Ri99 construct used in our previous experiments represented the bottom threshold of the effective size range and fragments longer than 300 bp have been shown not to enhance gene silencing (*24*, *32*).

Our results demonstrated that the 82E4Ri298-transformed plants accumulated less nornicotine than those harboring the 82E2Ri99 construct (**Tables 2** and **3**). Increasing the arm length of the inverted repeats from 99 to 298 bp and using a sequence perfectly matching the CYP82E4 cDNA are likely to be the most important changes in construct design that led to the enhanced silencing of the nicotine *N*-demethylase gene(s). In contrast, no correlation was found between the number of copies of the 82E4Ri298 construct and the nornicotine production (**Tables 2** and **3** and **Figure 4**). This observation is consistent with other reports indicating that the expression of only a few double-stranded RNA molecules is sufficient to induce gene silencing (*33*, *34*). The improved silencing efficiency of the 82E4Ri298 expression cassette enabled us to produce tobacco with a conversion rate as low as 0.8%, which is well below the 3−5% rate typically detected in burley lines used by seed producers. Suppression of nornicotine production showed a high degree of heritability in the T_1 progeny of the primary transformants (**Table 4**). Because of the positive correlation observed between nornicotine content and NNN formation, especially at low nornicotine levels, it is likely that the observed reduction in nornicotine levels will lower NNN production as well assuming that the levels of nornicotine observed in the greenhouse grown plants are faithfully recapitulated in field environments. While the question whether CYP82E4 represents the only member of the CYP82E2 gene subfamily that encodes nicotine *N*-demethylase activity remains open, the observation that only minute levels of residual nornicotine are detected in the 82E4Ri298-transformed plants clearly establishes the CYP82E2 gene subfamily's central role in controlling this process.

In summary, we employed an RNAi-induced gene silencing strategy to minimize nornicotine accumulation in nonconverter and converter burley tobacco. Suppression of the CYP82E2 gene subfamily with an optimized RNAi construct conferred a lower nornicotine content than typically found in commercial burley varieties even in a strong converter background. Therefore, this technology could be a valuable tool in efforts to lower nornicotine and possibly NNN levels in commercial tobacco varieties and products derived from them.

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