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RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves

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Summary

Technologies for reducing the levels of tobacco product constituents that may contribute to unwanted health effects are desired. Target compounds include tobacco-specific nitrosamines (TSNAs), a class of compounds generated through the nitrosation of pyridine alkaloids during the curing and processing of tobacco. Studies have reported the TSNA *N*′-nitrosonornicotine (NNN) to be carcinogenic in laboratory animals. NNN is formed via the nitrosation of nornicotine, a secondary alkaloid produced through enzymatic *N*-demethylation of nicotine. Strategies to lower nornicotine levels in tobacco (*Nicotiana tabacum* L.) could lead to a corresponding decrease in NNN accumulation in cured leaves. The major nicotine demethylase gene of tobacco has recently been isolated. In this study, a large-scale field trial was conducted to evaluate transgenic lines of burley tobacco carrying an RNA interference (RNAi) construct designed to inhibit the expression of this gene. Selected transgenic lines exhibited a six-fold decrease in nornicotine content relative to untransformed controls. Analysis of cured leaves revealed a commensurate decrease in NNN and total TSNAs. The inhibition of nicotine demethylase activity is an effective means of decreasing significantly the level of a key defined animal carcinogen present in tobacco products.

Introduction

Tobacco is consumed primarily in the form of cigarettes by more than one-billion persons worldwide, although cigars, bidis, pipe tobaccos and smokeless products (snuff, snus and chewing tobacco) are also used. A number of compounds present in tobacco products have been reported to contribute to adverse health effects, including cancer. To date, over 60 compounds found in cigarette smoke, and at least 16 contained in unburned tobacco, have been associated with carcinogenesis in laboratory animals (Hecht, 2003, 2006). Although avoidance/cessation is the best way to avert the health risks associated with tobacco use, potential reduced-exposure products might be used within complementary strategies to reduce tobacco-related harm at the population level (Stratton *et al*., 2001; Gartner *et al*., 2007). Technologies to eliminate or reduce carcinogen levels may therefore be justified in order to decrease the risk to the portion of the population that uses tobacco products.

Tobacco-specific nitrosamines (TSNAs) are amongst the most intensively investigated classes of compounds that have been linked to tumour formation in laboratory animals (Hecht and Hoffmann, 1989; Hoffmann *et al*., 1994; Hecht, 1998). These compounds are generated through the nitrosation of naturally occurring alkaloids during the curing and processing of harvested tobacco leaves (Bush *et al*., 2001). *N*′-Nitroso-nornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have been reported to be carcinogenic in laboratory animals (Hecht, 2003; International Agency for Research on Cancer (IARC), 2007), and are formed via the nitrosation of nornicotine and nicotine, respectively (Bush *et al*., 2001).

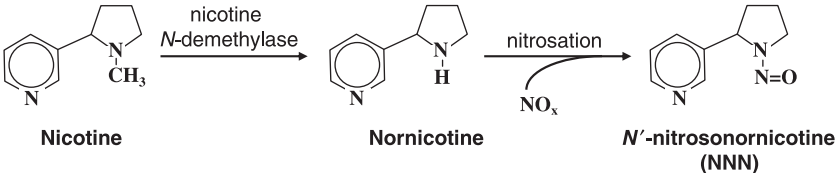

Figure 1 Primary biochemical mechanism for the formation of *N*′-nitrosonornicotine (NNN) in tobacco.

TSNA formation is most prevalent in air-cured tobacco types (i.e. burley and dark tobacco) that are stalk-cut and cured in rain-protected structures for a period of 6–8 weeks. The abundance of TSNAs in air-cured leaves of burley and dark tobaccos is highly variable, but NNN predominates in most cases. Because NNN is formed through the nitrosation of nornicotine (Figure 1), significant positive correlations between nornicotine content and NNN accumulation have been reported (Bush *et al*., 2001). One strategy to reduce the accumulation of NNN in tobacco products might therefore be to lower nornicotine levels in tobacco leaves during growth and curing. Nornicotine is produced via the oxidative *N*-demethylation of nicotine by a nicotine *N*-demethylase enzyme during senescence and curing (Figure 1) (Hao and Yeoman, 1996a,b, 1998). This process of nicotine 'conversion' is controlled, to a large extent, by a single gene at an unstable locus within the tobacco genome (Mann *et al*., 1964; Wernsman and Matzinger, 1970; Siminszky *et al*., 2005). The gene is usually in the inactive state, or in a reduced state of activity, in most tobacco cultivars. Less than 5% of available nicotine is typically metabolized to nornicotine in these plants. The gene can become spontaneously reactivated, however, through a yet-to-be-determined mechanism (Wernsman and Matzinger, 1970) to cause a very high rate of conversion, often greater than 95%. For currently unknown reasons, the frequency of reactivation is much higher in burley tobacco cultivars than in other tobacco types.

Genetic strategies to inhibit nicotine demethylation may lead to reduced NNN formation and total TSNA concentration in tobacco products. The nicotine demethylase gene at the unstable locus has recently been isolated, and demonstrated to encode a cytochrome P450 monooxygenase, designated as CYP82E4, that is expressed during senescence and in response to ethylene treatment (Siminszky *et al*., 2005). Transcript accumulation of *CYP82E4* was found to be enhanced by as much as 80-fold in high-converting vs. low-converting tobacco (Gavilano *et al*., 2006). RNA interference, or RNAi, has been widely used to induce post-transcriptional gene silencing via sequence-specific degradation of target mRNA (Waterhouse and Helliwell, 2004). Preliminary studies have suggested the value of RNAi for suppressing the expression of the nicotine demethylase enzyme through induced silencing of *CYP82E4* and any closely related homologous sequences (Gavilano *et al*., 2006). To test the efficacy of RNAi-mediated suppression of nicotine demethylase activity for reducing NNN and total TSNAs in commercial air-cured tobacco curing situations, an array of transgenic lines of tobacco was generated expressing an RNAi construct targeted towards *CYP82E4*, and this material was evaluated in a multi-environment test for nicotine conversion and TSNA formation.

Results and discussion

Generation of materials and preliminary evaluation

Burley tobacco breeding line DH98-325-5 was chosen to represent a standard low-nicotine-converting (< 3.0%) genotype of tobacco, and DH98-325-6 was chosen to represent a standard high-converting (> 80%) genotype. To inhibit the expression of nicotine demethylase in these two genotypes, an RNAi gene construct designed to silence *CYP82E4* and its closely related homologues was introduced. Three primary transformants (R_0 generation) per breeding line had been verified previously to exhibit the *CYP82E4*-silenced phenotype based on Northern blots and percentage nornicotine relative to untransformed controls (Gavilano *et al*., 2006). Two selected R_0 individuals per breeding line possessed only single transgene insertions, as determined by Southern blots, and one selected R_0 individual per breeding line possessed multiple transgene insertions (Gavilano *et al*., 2006). Seven R_2 families derived from three independent DH98-325-5 transformants and 14 R_2 families derived from three independent DH98-325-6 transformants were selected for field evaluation to identify those that did not segregate for the silenced condition.

Based on alkaloid data collected from cured fully expanded leaves from immature field-grown plants evaluated near Lexington, KY, USA, three independently transformed R_2 families of DH98-325-5 and two R_2 families of DH98-325-6 were found to stably exhibit an ultra-low-nicotine-converting phenotype (Figure 2). No plant of any of the five selected transgenic RNAi lines (48 plants phenotyped per line) exhibited a nicotine conversion rate exceeding 0.76%, as determined

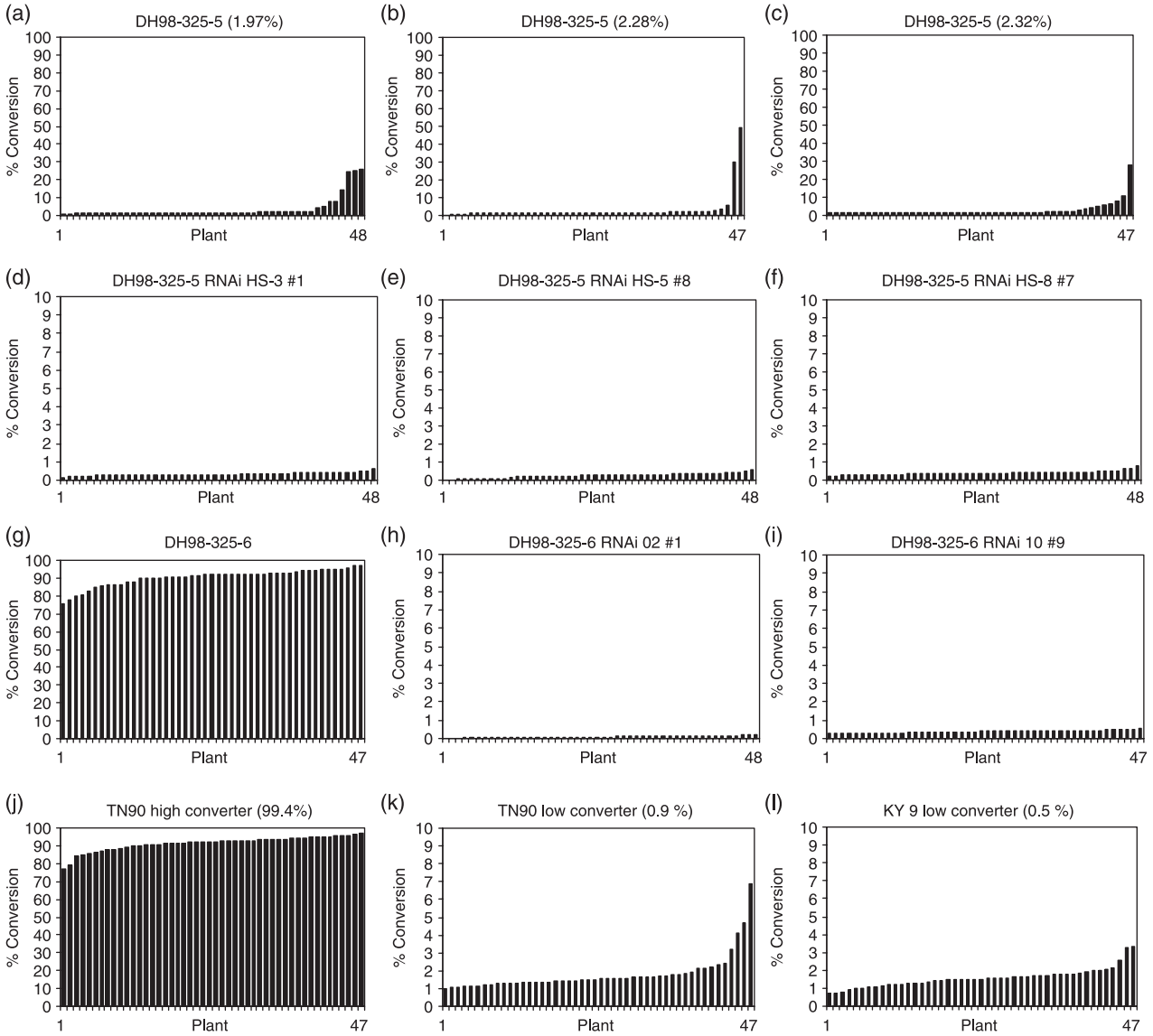

Figure 2 Percentage nicotine conversion for three independent seed lots of low-converting burley tobacco breeding line DH98-325-5 (a–c), three selected independent transgenic lines of DH98-325-5 expressing RNA interference (RNAi) of *CYP82E4* (d–f), high-converting burley tobacco breeding line DH98-325-6 (g), two independent transgenic lines of DH98-325-6 expressing RNAi of *CYP82E4* (h and i), high-converting and low-converting selections of burley tobacco cultivar 'TN90' (j and k, respectively) and a low-converting selection of burley tobacco cultivar 'KY9' (l). The percentage conversion was determined on leaves collected from 47–48 plants when approximately 35 cm tall. Leaves were treated with ethephon and air-cured according to the 'LC-protocol' (http://www.uky.edu/Ag/Tobacco/Pdf/LC-Protocol.pdf) prior to chemical analyses. The numbers in parentheses indicate the percentage nicotine conversion in the parental plant from which the seed lot was derived. The percentage conversion was calculated as [% nornicotine/(% nornicotine + % nicotine)] × 100. Please note the difference in scale for the *y*-axes for (d), (e), (f), (h), (i), (k) and (l).

by gas chromatography. This was in contrast with three non-transformed DH98-325-5 control seed lots (produced by the self-pollination of three individual plants exhibiting nicotine conversion rates of 1.97%, 2.28% and 2.32%), for which the percentage conversion was in the range 0.10%–49.10%. From a total of 142 plants from the three seed lots, 20 exhibited nicotine conversion rates that exceeded 3.0% despite having been derived from controlled self-pollination of individual plants exhibiting less than 3.0% conversion (Figure 2). Eight plants exhibited greater than 10% conversion. These observations are a reflection of the previously reported genetic instability at the major nicotine conversion locus within the *N. tabacum* genome (Wernsman and Matzinger, 1970). All 47 plants of the DH98-325-6 untransformed control exhibited greater than 75% nicotine conversion in this experiment. For comparison, the percentage nicotine conversion ranges for 47–48 plants produced from the self-pollination of low-converting (< 1.0%) single-plant selections of commercial

burley tobacco cultivars 'TN90' and 'KY9' were 0.9664%–6.8944% and 0.6874%–3.3581%, respectively (Figure 2).

Multi-environment evaluation for nornicotine formation and TSNA accumulation

The initial observations discussed above provided support for the hypothesis that the *CYP82E4* RNAi mechanism would be of value for suppressing nornicotine levels in conventional tobacco production/curing situations, and therefore greatly decrease the potential for NNN formation. The five selected transgenic RNAi lines, their non-transgenic controls and three checks (high- and low-converting selections of burley tobacco cultivar TN90, and a low-converting selection of cultivar KY9) were selected for harvesting and air-curing from replicated field experiments in Kentucky, North Carolina and Virginia during 2006. The experimental design was a randomized complete block design with four replications per location. Production and curing regimes were consistent with those used by tobacco growers in these regions. Data were collected for the four principal tobacco alkaloids (percentage nornicotine, percentage nicotine, percentage anatabine, percentage anabasine) and their corresponding nitrosated TSNA derivatives [NNN, NNK, *N*′-nitrosoanatabine (NAT) and *N*′-nitrosoanabasine (NAB), respectively]. Although NAT and NAB are reported to have little to no biological activity (Hecht and Hoffmann, 1989; Hoffman *et al*., 1994; Hecht, 1998), these TSNAs were quantified because of their contribution to total TSNAs (calculated as the sum of NNN, NNK, NAT and NAB). For statistical comparisons, natural logarithmic transformations were performed on all chemical data, except percentage anatabine and percentage anabasine. Back-transformed means for all entries (averaged over all three environments) are presented in Table 1. Means based on non-transformed data are provided in Table 2. Linear statistical contrasts between the mean of the three 'low-converting' non-transgenic DH98-325-5 seed lots and the mean of the three selected DH98-325-5 RNAi lines indicated that the RNAi construct conferred highly significant decreases ($P < 0.0001$) in percentage nicotine conversion and percentage nornicotine (Figure 3). No significant changes were observed for percentage nicotine, percentage anabasine or percentage anatabine.

Table 1 Entry means for untransformed controls, transgenic RNA interference (RNAi) lines and three checks evaluated in three environments during 2006

						TSNA				
Genotype	Nicotine (%)*	Nornicotine (%)	Anabasine (%)	Anatabine (%)	Nicotine conversion (%)†	NNN (ppm)	NNK (ppm)	NAB (ppm)	NAT (ppm)	Total (ppm)
DH98-325-5 (Low Converter) (1.97%)‡	3.535 b	0.116 bc	0.040§	0.126 de	3.142 b	0.725 c	0.164 bc	0.025§	0.495§	1.498 cd
DH98-325-5 (Low Converter) (2.32%)	3.592 b	0.107 bc	0.040	0.129 de	2.884 b	0.574 cd	0.118 cd	0.021	0.379	1.151 def
DH98-325-5 (Low Converter) (2.28%)	3.595 b	0.085 c	0.038	0.126 de	2.293 bc	0.426 d	0.155 bc	0.018	0.392	1.033 def
DH98-325-5 RNAi HS-3 #1	3.717 b	0.023 de	0.037	0.123 de	0.612 d	0.139 ef	0.146 bc	0.019	0.425	0.771 fgh
DH98-325-5 RNAi HS-5 #8	4.090 b	0.020 de	0.038	0.137 d	0.496 d	0.109 f	0.111 cd	0.017	0.361	0.629 gh
DH98-325-5 RNAi HS-8 #7	3.674 b	0.017 e	0.038	0.106 e	0.453 d	0.099 f	0.112 cd	BLD¶	0.293	0.544 h
DH98-325-6 (High Converter)	0.645 d	2.439 a	0.033	0.177 bc	76.951 a	9.610 a	0.078 d	0.020	0.544	10.309 a
DH98-325-6 RNAi 02 #1	3.760 b	0.024 d	0.032	0.119 de	0.645 d	0.144 ef	0.194 ab	0.021	0.494	0.896 efg
DH98-325-6 RNAi 10 #9	3.864 b	0.024 de	0.028	0.121 de	0.605 d	0.168 e	0.217 ab	0.023	0.492	0.937 ef
TN90 (High Converter) (99.4%)	0.833 c	2.901 a	0.039	0.238 a	74.468 a	5.870 b	0.047 e	BLD	0.400	6.366 b
TN90 (Low Converter) (0.90%)	4.762 a	0.129 b	0.039	0.190 b	2.640 b	0.547 cd	0.092 d	0.023	0.586	1.302 cde
KY9 (Low Converter) (0.50%)	5.266 a	0.097 bc	0.038	0.158 c	1.805 c	0.666 c	0.265 a	0.041	0.827	1.865 c
Mean	3.444	0.498	0.037	0.146	13.916	1.590	0.141	0.021	0.474	2.275

A natural logarithmic transformation was performed on all measured characters, except percentage anabasine and percentage anatabine, prior to mean separation procedures. Means were back-transformed for presentation. Means were separated according to Duncan's multiple range procedure. Any two means having a common letter are not significantly different at the $P = 0.05$ level of significance.

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.

*All percentages are reported on a dry tobacco weight basis.

†Percentage nicotine conversion was calculated as [% nornicotine/(% nornicotine + % nicotine)] × 100.

‡Numbers in parentheses indicate the percentage nicotine conversion of the parental plant from which the seed lot was obtained.

§Mean separation was not performed because a significant *F*-test ($P = 0.05$) was not observed for this measured character.

¶Indicates below the limit of detection.

Table 2 Entry means for untransformed controls, transgenic RNA interference (RNAi) lines and three checks evaluated in three environments during 2006. Means are based on untransformed data

Genotype	Nicotine (%)*	Nornicotine (%)	Anabasine (%)	Anatabine (%)	Nicotine conversion (%)†	TSNA				
						NNN (ppm)	NNK (ppm)	NAB (ppm)	NAT (ppm)	Total (ppm)
DH98-325-5 (Low Converter) (1.97%)‡	3.619	0.148	0.040	0.126	3.990	1.172	0.179	0.043	0.578	1.972
DH98-325-5 (Low Converter) (2.32%)	3.719	0.132	0.040	0.129	3.363	1.067	0.137	0.042	0.493	1.739
DH98-325-5 (Low Converter) (2.28%)	3.664	0.097	0.038	0.126	2.689	0.585	0.176	0.023	0.462	1.246
DH98-325-5 RNAi HS-3 #1	3.819	0.024	0.037	0.123	0.632	0.179	0.182	0.030	0.548	0.939
DH98-325-5 RNAi HS-5 #8	4.186	0.021	0.038	0.137	0.511	0.145	0.126	0.024	0.448	0.744
DH98-325-5 RNAi HS-8 #7	3.705	0.020	0.039	0.106	0.518	0.127	0.122	BLD§	0.326	0.592
DH98-325-6 (High Converter)	0.717	2.517	0.033	0.177	77.568	12.317	0.088	0.027	0.649	13.081
DH98-325-6 RNAi 02 #1	3.845	0.026	0.032	0.119	0.661	0.190	0.216	0.029	0.592	1.027
DH98-325-6 RNAi 10 #9	3.894	0.024	0.028	0.121	0.614	0.249	0.393	0.039	0.740	1.422
TN90 (High Converter) (99.4%)	0.979	2.977	0.039	0.238	75.233	6.608	0.055	BLD	0.458	7.135
TN90 (Low Converter) (0.9%)	4.834	0.148	0.039	0.190	2.842	0.713	0.095	0.027	0.666	1.501
KY9 (Low Converter) (0.5%)	5.363	0.104	0.038	0.158	1.860	1.164	0.498	0.082	1.260	3.004

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.

*All percentages are reported on a dry tobacco weight basis.

†Percentage nicotine conversion was calculated as [% nornicotine/(% nornicotine + % nicotine)] × 100.

‡Numbers in parentheses indicate the percentage nicotine conversion of the parental plant from which the seed lot was obtained.

§Indicates below the limit of detection.

TSNA formation is known to vary widely between environments. In our study, *F*-tests revealed significant differences ($P < 0.01$) between environments for all quantified TSNAs. Highly significant differences ($P < 0.0001$) were observed amongst experimental entries within each of the three curing environments for NNN and total TSNAs, however. More importantly, in comparing the mean of the three DH98-325-5 RNAi lines (averaged over all three environments) with the mean of the three non-transgenic DH98-325-5 seed lots, there was a decrease in NNN from 0.941 to 0.150 ng/mg (means based on non-transformed data). This 84% decrease was significant at the $P < 0.0001$ level (Figure 3). For this genetic background, no significant increase in NNK was associated with the RNAi mechanism. The amount of total TSNAs was consequently decreased from 1.652 to 0.758 ng/mg (based on non-transformed data). This 54% decrease was found to be significant at the $P = 0.0012$ level (Figure 3).

The impact of the *CYP82E4* RNAi mechanism was much greater in the high-nicotine-converting background of DH98-325-6. In comparisons between the means of the two selected RNAi lines and untransformed DH98-325-6 (averaged over all three environments), the percentage nicotine conversion was decreased from 77.6% to 0.6% (means based on non-transformed data). This difference was significant at the $P < 0.0001$ level (Figure 4), and demonstrated the effectiveness of the RNAi mechanism in dramatically decreasing nicotine demethylase activity at either low or high levels of *CYP82E4* transcription. Because considerably less nicotine was being converted to nornicotine, the transgenic DH98-325-6 lines exhibited significantly greater ($P < 0.0001$) nicotine content relative to the corresponding non-transformed control (Figure 4). A significant decrease in percentage anatabine ($P = 0.0048$) was also observed in the RNAi lines.

These aforementioned modifications in alkaloid profiles for the transgenic DH98-325-6 lines led to a very significant ($P < 0.0001$) decrease in NNN (Figure 4) from 12.317 to 0.220 ng/mg (means based on non-transformed data). The significant increase ($P = 0.0014$) in NNK that was observed was primarily a result of the very low nicotine level of the untransformed high-nicotine-converting DH98-325-6 control. Despite the corresponding increase in nicotine and NNK that accompanied the silencing of nicotine demethylase in the RNAi lines, there was a very significant decrease ($P < 0.0001$, Figure 4) in total TSNAs (from 13.081 to 1.225 ng/mg, based on non-transformed data). The tertiary amine status of nicotine may contribute to a lower reactivity and an apparent lower predisposition for nitrosation to form NNK in burley tobacco, when compared with the propensities of nornicotine, anatabine or anabasine (all secondary amines) to form their corresponding TSNAs. For example, in the non-transgenic

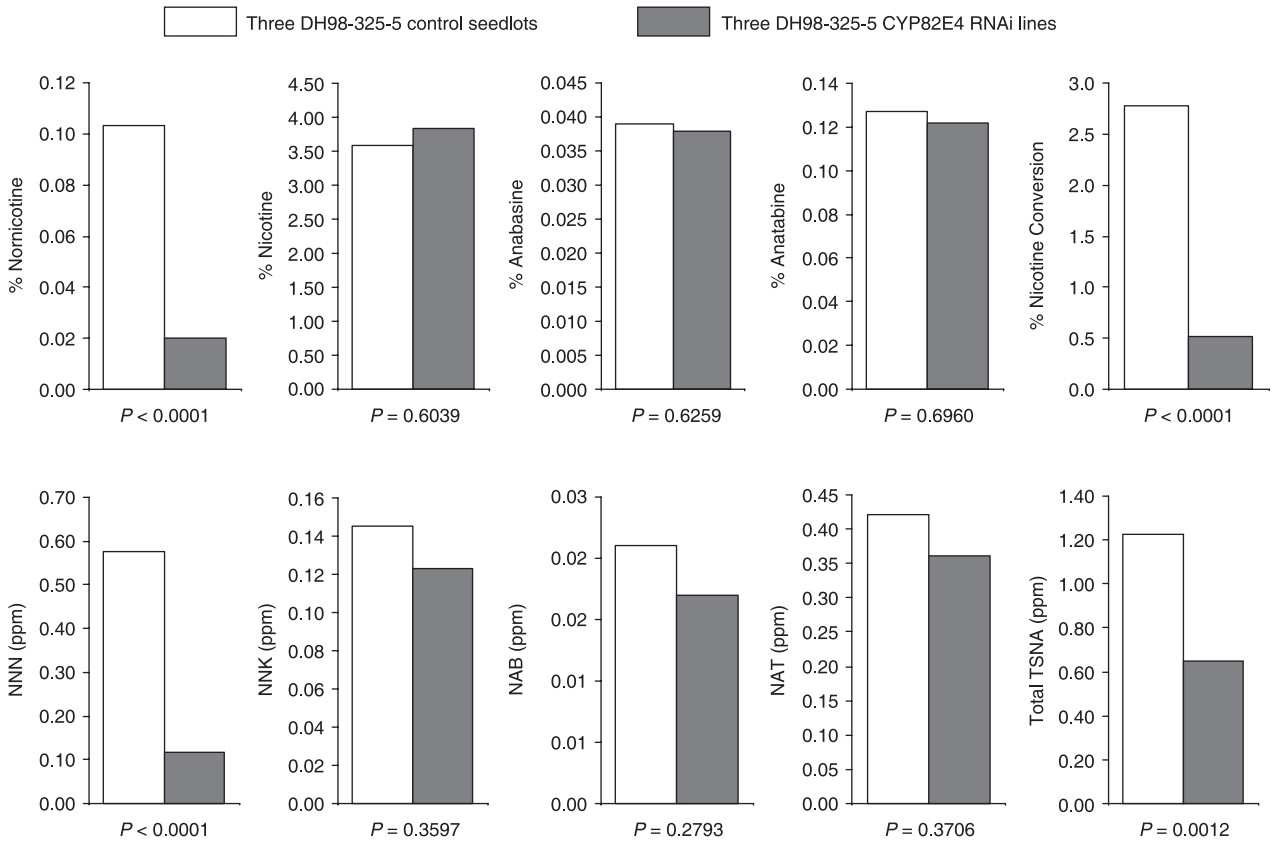

Figure 3 DH98-325-5 non-transformed controls (mean of three independently derived seed lots) vs. DH98-325-5 *CYP82E4* RNA interference (RNAi) lines (mean of three transgenic lines). *P* values were generated from single degree of freedom contrasts of the group means using PROC GLM of SAS. A natural logarithmic transformation was performed on all measured characters, except percentage anabasine and percentage anatabine, prior to the analyses. Back-transformations were performed prior to mean presentation in histograms. Percentages are based on a dry tobacco weight basis. The percentage nicotine conversion was calculated as [% nornicotine/(% nornicotine + % nicotine)] × 100. NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.

DH98-325-5 materials evaluated in the current investigation, NNN was at much higher levels than NNK, despite the fact that nicotine was by far the most prevalent alkaloid (Table 1). Strong relationships have been reported previously between nornicotine and NNN in burley tobacco, whereas relationships between nicotine and NNK are weaker (Bush *et al*., 2001; Li *et al*., 2006). The formation of NNK from nicotine may also be more complicated at the biochemical level when compared with the pathway of formation for NNN, NAB and NAT. NNK formation may be a multistep process requiring a chemical intermediate, possibly pseudo-oxynicotine (Bush *et al*., 2001).

To date, efforts to reduce TSNAs in air-cured tobaccos have focused on the management of nornicotine levels at the stage of foundation seed production by harvesting seed only from plants that exhibit less than 3.0% nicotine conversion (Jack *et al*., 2007). The extent to which this 'intracultivar selection' can have an impact on nornicotine and NNN levels in burley tobacco is indicated by the means (averaged over all three environments) of low-converting selections of cultivars KY9 and TN90 (Table 1). These seed lots were generated from the self-pollination of single plants exhibiting ultra-low levels of nicotine conversion (0.50% and 0.90%, respectively), and represent the best that has been achieved via intracultivar selection. Commercial seed lots of these cultivars typically exhibit greater levels of nicotine conversion because an extra reproductive generation is required before seed is made available to growers. Transgenic RNAi lines of both DH98-325-5 and DH98-325-6 exhibited significantly lower levels ($P < 0.05$) of nornicotine, nicotine conversion, NNN formation and total TSNA accumulation relative to these conventionally derived seed lots (Table 1).

Conclusions

In summary, the utility of a biotechnological tool to dramatically decrease the levels of a key potential carcinogen in

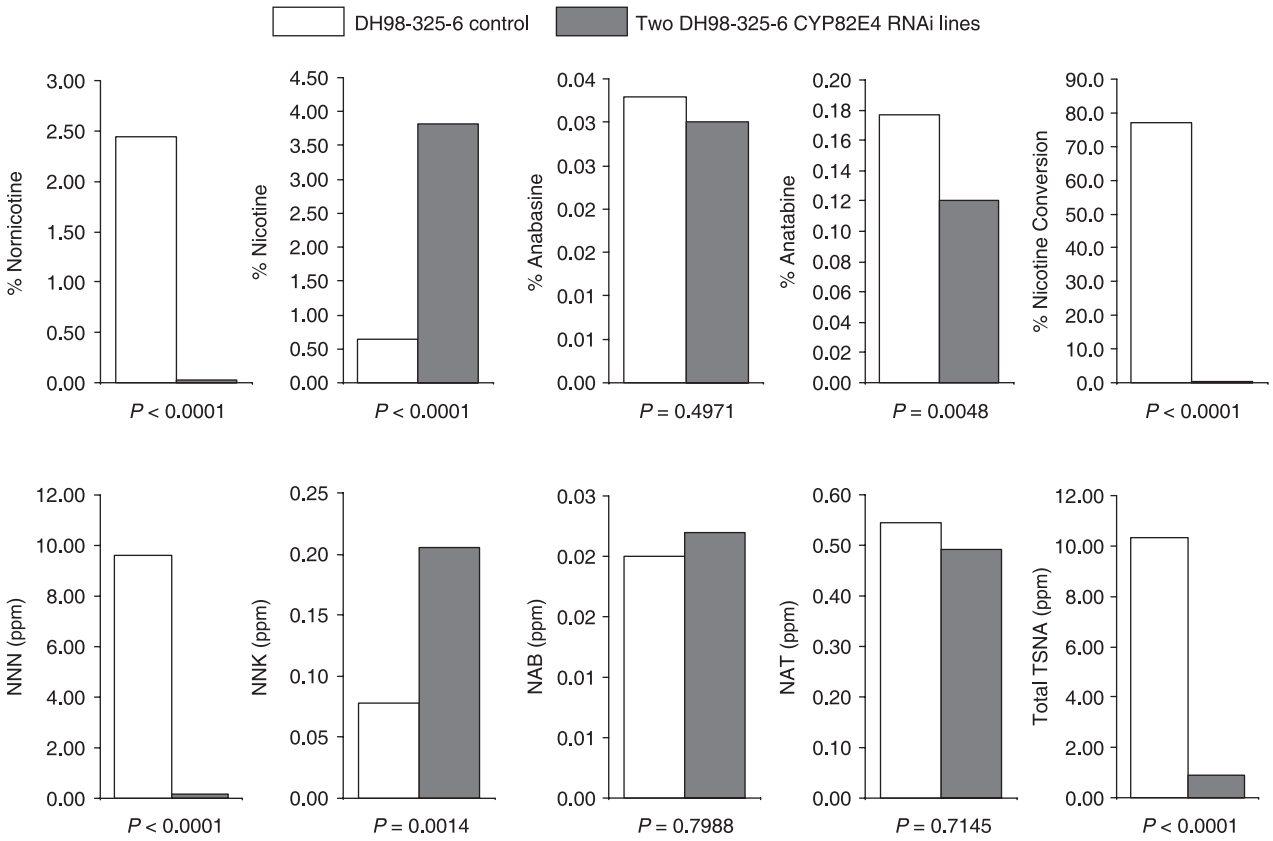

Figure 4 DH98-325-6 non-transformed control vs. DH98-325-6 *CYP82E4* RNA interference (RNAi) lines (mean of two transgenic lines). *P* values were generated from single degree of freedom contrasts of group means using PROC GLM of SAS. A natural logarithmic transformation was performed on all measured characters, except percentage anabasine and percentage anatabine, prior to the analyses. Back-transformations were performed prior to mean presentation in histograms. Percentages are based on a dry tobacco weight basis. The percentage nicotine conversion was calculated as [% nornicotine/(% nornicotine + % nicotine)] × 100. NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.

air-cured tobacco leaves has been demonstrated. As cured tobacco leaves are the primary raw material used in the manufacture of tobacco products, it seems reasonable to expect this technology to translate into the development of potential reduced-exposure products. Although the method may contribute to a decreased risk for smokers, the greatest overall effect could be on smokeless tobacco products in which TSNAs are the primary reputed carcinogens (Hecht, 2003, 2006). Application of the technology, in its current form, would be subject to commercial acceptance of transgenic tobacco varieties, however. Although tobacco is perhaps the easiest plant to manipulate using recombinant DNA techniques, there has been a reluctance to deploy transgenic commercial tobacco cultivars. The data reported here demonstrate the utility of RNAi technology for reducing the level of an undesirable metabolite in an important crop species to a value that has not yet been achieved using conventional methods. Perhaps transgenic crop cultivars with worldwide human health implications, such as that described here, may increase opportunities for the deployment of transgenic cultivars of internationally important commodities.

Experimental procedures

Generation of transgenic plant material

Agrobacterium tumefaciens strain LBA4404, carrying construct 82E4Ri298 (Gavilano *et al*., 2006), was used to transform burley tobacco breeding lines DH98-325-5 and DH98-325-6 with an RNAi mechanism designed to silence *CYP82E4* and its closely related homologues. The introduced T-DNA carried a cauliflower mosaic virus (CaMV) 35S promoter-driven expression cassette that encoded an intron-spliced hairpin RNA engineered from a 298-bp sequence corresponding to the region between nucleotide positions 297 and 594 of the *CYP82E4* cDNA (accession no. DQ131885) and the 151-bp soybean ω-3 fatty acid desaturase intron. The construct contained neomycin phosphotransferase II (*npt*II) to

allow for the selection of transformed plants in the presence of the antibiotic kanamycin.

Three primary transformants (R_0 generation) per breeding line, previously verified to exhibit the *CYP82E4*-silenced phenotype (Gavilano *et al*., 2006), were selected for the development of lines homozygous for at least one transgene insertion. R_0 individuals were self-pollinated to produce R_1 families. Multiple individuals per R_1 family were evaluated for the silenced phenotype. R_1 individuals expressing very low levels of nicotine conversion were self-pollinated to produce R_2 families. Analysis of R_2 progeny was used to identify R_1 individuals homozygous for at least one transgene insertion and R_2 families stably expressing the *CYP82E4*-silenced condition. Preliminary selection was based on polymerase chain reaction (PCR)-based testing for the presence of *npt*II and seedling segregation for kanamycin resistance. Subsequent selection was based on field evaluation of R_2 progeny for stability of the ultra-low-nicotine-converting phenotype (see below).

Field evaluation and curing

Seven R_2 familes derived from three independent DH98-325-5 transformants were selected for field evaluation during 2006. Fourteen R_2 families derived from three independent DH98-325-6 transformations were also selected. Seven check entries were also included (Table 1). These included three independent seed lots of untransformed DH98-325-5 (produced by self-pollination of single plants that exhibited less than 3.0% nicotine conversion as determined by gas chromatography), untransformed DH98-325-6 (stable, high-converting, untransformed control), low- and high-converting forms of cultivar 'TN90' (produced by self-pollination of single plants exhibiting 0.90% and 99.4% nicotine conversion, respectively), and a low-converting version of cultivar 'KY9' [produced by self-pollination of an individual plant exhibiting very low levels of nicotine conversion (0.50%)].

The evaluation of genetic materials was conducted at three locations during 2006: Blackstone, VA; Lexington, KY; and Waynesville, NC. The experimental design at each location was a randomized complete block design with four replications. Experimental units consisted of single 12-plant rows. End plants served as guard plants and were removed prior to harvest. Production practices were consistent with those used for commercial burley tobacco production for each area.

The identification/verification of R_2 families stably expressing the RNAi mechanism was conducted at the Lexington, KY location. Two fully expanded leaves from each plant per plot were collected from plants approximately 35 cm high, cured, and analysed for percentage nicotine conversion via gas chromatography according to the 'LC-Protocol' (Jack *et al*., 2007). Based on these data, three independent DH98-325-5 and two independent DH98-325-6 R_2 families were selected for harvesting and curing (in addition to the seven checks mentioned above) at all three locations. At maturity, 10 interior plants per row were stalk cut and air-cured in outdoor shelters consistent with those used for commercial production in the given regions. After curing, the amount of cured leaf for each plot was weighed, and the fourth leaf from the top of each plant was collected. The leaf midribs were immediately separated from the lamina. For each plot, the collected lamina was placed into a single paper bag and allowed to air-dry under ambient glasshouse or laboratory conditions. Samples were subsequently ground to pass through a 40-mesh screen and analysed for percentage nicotine, nornicotine, anatabine, anabasine, NNK, NNN, NAT and NAB using the procedures described below.

Chemical analyses

Quantitative determinations of nicotine, nornicotine, anabasine and anatabine in cured leaf samples were made using a Perkin-Elmer Autosystem XL Gas Chromatograph (Perkin-Elmer, Shelton, CT, USA) with Prevent™ according to the 'LC-Protocol' (Jack *et al*., 2007). Ground tobacco samples were treated with aqueous sodium hydroxide, followed by extraction with methyl *tert*-butyl alcohol. Quantification was based on the chromatographic peak response at the retention times of the alkaloid standards. The results were reported as percentages on a dry tobacco weight basis. The percentage nicotine conversion was calculated as: [% nornicotine/(% nornicotine + % nicotine)] × 100.

The concentrations of the four primary TSNAs (NNN, NNK, NAT and NAB) were determined by Lancaster Laboratories (Lancaster, PA, USA) using liquid chromatography with tandem mass spectrometry detection (Micromass Quattro Micro triple quadrupole LC/MS/MS; Waters, Milford, MA, USA) operated under a positive ion electrospray ionization (ESI) source. Freeze-dried cured tobacco samples were extracted with 30 mL of 100 mM ammonium acetate. The mixture was shaken on a wrist action shaker at room temperature for 30 min. Ten microlitres of filtered supernatant were then analysed by LC/MS/MS using multiple reaction monitoring with internal standard calibration. Samples were eluted with a linear gradient of water and 0.15 acetic acid in methanol. The results were reported as ng/mg on a dry tobacco weight basis. The minimum limit of detection for each TSNA was 0.020 ng/mg.

Statistical analyses

An analysis of variance appropriate for analysing a randomized complete block design evaluated over locations (McIntosh, 1983) was conducted using PROC GLM of SAS (SAS Institute, Cary, NC, USA), where the entries and environments were considered fixed and random effects, respectively. Censored observations (those below the minimum limit of detection) were replaced by one-half the detection limit according to Atwood *et al*. (1991). Natural logarithmic data transformations were performed for nicotine, nornicotine, percentage conversion, NNN, NNK, NAT, NAB and total TSNAs because of heterogeneous error variances and to approximate normal distributions. Means were back-transformed for presentation. Separation of entry means was performed using Duncan's multiple range test ($\alpha = 0.05$) with a preliminary *F*-test to reduce Type I errors (Gomez and Gomez, 1984). Single degree of freedom linear contrasts were made between group means using CONTRAST statements in PROC GLM according to Steel *et al*. (1997).

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References

Atwood, C.L., Blackwood, L.G., Harris, G.A. and Loehr, C.A. (1991) *Recommended Methods for Statistical Analysis of Data Containing Less-than-Detectable Measurements. EGG-SARE-9247, Rev. 1*. Idaho Falls, ID: Idaho National Engineering Laboratory, EG&G Idaho, Inc. URL http://www.osti.gov/energycitations/product.biblio.jsp?osti_id=6026384 [accessed August 2007].

Bush, L.P., Cui, M., Shi, H. and Burton, H.R. (2001) Formation of tobacco specific nitrosamines in air-cured tobacco. *Rec. Adv. Tob. Sci*. **27**, 23–46.

Gartner, C.E., Hall, W.D., Vos, T., Bertram, M.Y., Wallace, A.L. and Lim, S.S. (2007) Assessment of Swedish snus for tobacco harm reduction: an epidemiological study. *Lancet*, **369**, 2010–2014.

Gavilano, L.B., Coleman, N.P., Burnley, L.-E., Bowman, M.L., Kalengamaliro, N.E., Hayes, A., Bush, L. and Siminszky, B. (2006) Genetic engineering of *Nicotiana tabacum* for reduced nornicotine content. *J. Agric. Food Chem*. **54**, 9071–9078.

Gomez, K.A. and Gomez, A.A. (1984) *Statistical Procedures for Agricultural Research*. New York, NY: John Wiley & Sons.

Hao, D.Y. and Yeoman, M.M. (1996a) Mechanism of nicotine N-demethylation in tobacco cell suspension cultures. *Phytochemistry*, **41**, 477–482.

Hao, D.Y. and Yeoman, M.M. (1996b) Nicotine N-demethylase in cell-free preparations from tobacco cell cultures. *Phytochemistry*, **42**, 325–329.

Hao, D.Y. and Yeoman, M.M. (1998) Evidence in favour of an oxidative N-demethylation of nicotine to nornicotine in tobacco cell cultures. *J. Plant Physiol*. **152**, 420–426.

Hecht, S.S. (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem. Res. Toxicol*. **11**, 559–603.

Hecht, S.S. (2003) Tobacco carcinogens, their biomarkers and tobacco-induced cancers. *Nat. Rev. Cancer*, **3**, 733–744.

Hecht, S.S. (2006) Cigarette smoking: cancer risks, carcinogens, and mechanisms. *Langenbecks Arch. Surg*. **391**, 603–613.

Hecht, S.S. and Hoffmann, D. (1989) The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surv*. **8**, 273–294.

Hoffmann, D., Brunnemann, K.D., Prokopczyk, B. and Djordjevic, M.V. (1994) Tobacco-specific *N*-nitrosamines and areca-derived *N*-nitrosamines – chemistry, biochemistry, carcinogenicity, and relevance to humans. *J. Toxicol. Environ. Health*, **41**, 1–52.

International Agency for Research on Cancer (IARC) (2007) Smokeless tobacco and some tobacco-specific nitrosamines. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **89**. Lyon: IARC.

Jack, A., Fannin, N. and Bush, L.P. (2007) Implications of reducing nornicotine accumulation in burley tobacco: Appendix A – The LC Protocol. *Rec. Adv. Tob. Sci*. **33**, 58–79.

Li, Q., Krauss, M.R. and Hempfling, W.P. (2006) Wounding of root or basal stalk prior to harvest affects pre-harvest antioxidant accumulation and tobacco-specific nitrosamine formation during air curing of burley tobacco (*Nicotiana tabacum* L.). *J. Agron. Crop Sci*. **192**, 267–277.

Mann, T.J., Weybrew, J.A., Matzinger, D.F. and Hall, J.L. (1964) Inheritance of the conversion of nicotine to nornicotine in varieties of *Nicotiana tabacum* L. and related amphiploids. *Crop Sci*. **4**, 349–353.

McIntosh, M.S. (1983) Analysis of combined experiments. *Agron. J*. **75**, 153–155.

Siminszky, B., Gavilano, L., Bowen, S.W. and Dewey, R.E. (2005) Conversion of nicotine to nornicotine in *Nicotiana tabacum* is mediated by CYP82E4, a cytochrome P450 monooxygenase. *Proc. Natl. Acad. Sci. USA*, **102**, 14 919–14 924.

Steel, R.G., Torrie, J.H. and Dickey, D.A. (1997) *Principles and Procedures of Statistics, a Biometrical Approach*. New York, NY: McGraw-Hill.

Stratton, K.R., Shetty, P., Wallace, R. and Bondurant, S. (2001) *Clearing the Smoke: Assessing the Science Base for Tobacco Harm Reduction*. Washington DC: National Academy Press.

Waterhouse, P.M. and Helliwell, C.A. (2004) Exploring plant genomes by RNA-induced gene silencing. *Nat. Rev. Genet*. **4**, 29–38.

Wernsman, E.A. and Matzinger, D.F. (1970) Relative stability of alleles at the nicotine conversion locus of tobacco. *Tob. Sci*. **14**, 34–36.

