Generation of tobacco lines with widely different reduction in nicotine levels via RNA silencing approaches

Peng Wang[1], Zhifeng Liang[1], Jia Zeng[1], Wenchao Li[1], Xiaofen Sun[1], Zhiqi Miao[2] and Kexuan Tang[1,2,*]

[1]*State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai 200433, China*

[2]*Plant Biotechnology Research Center, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China*

**Corresponding author (Fax, +8621-65643552; Email, kxtang1@yahoo.com)*

Issues related to the nicotine content of tobacco have been public concerns. Several reports have described decreasing nicotine levels by silencing the *putrescine N-methyltransferase* (*PMT*) genes, but the reported variations of nicotine levels among transgenic lines are relatively low in general. Here we describe the generation in tobacco (*Nicotiana tabacum*) lines with widely different, reduced nicotine levels using three kinds of RNA-silencing approaches. The relative efficacies of suppression were compared among the three approaches regarding the aspect of nicotine level in tobacco leaves. By suppressing expression of the *PMT* genes, over 200 transgenic lines were obtained with nicotine levels reduced by 9.1–96.7%. RNA interference (RNAi) was the most efficient method of reducing the levels of nicotine, whereas cosuppression and antisense methods were less effective. This report gives clues to the efficient generation of plants with a variety of metabolite levels, and the results demonstrate the relative efficiencies of various RNA-silencing methods.

[Wang P, Liang Z, Zeng J, Li W, Sun X, Miao Z and Tang K 2008 Generation of tobacco lines with widely different reduction in nicotine levels via RNA silencing approaches; *J. Biosci.* **33** 177–184]

1. Introduction

Over 2500 compounds have been found in tobacco (*Nicotiana tabacum*), among which alkaloids and terpenoids are the major groups (Nugroho and Verpoorte 2002). Studies on the alkaloids in tobacco cover a wide spectrum due to their important roles in addiction through recreational euphorics (Laviolette and van der Kooy 2004), defensive function as insecticides (Steppuhn *et al* 2004), and potential therapeutic uses (Powledge 2004). Nicotine represents the majority of alkaloids in tobacco leaf tissues, and anatabine, anabasine and nornicotine are the minor components of the alkaloid fraction (Chintapakorn and Hamill 2003; Saitoh *et al* 1985). Nicotine biosynthesis occurs primarily in the tobacco roots, from where it is transported through the xylem to aerial parts of the plant, and deposited primarily in the leaves (Baldwin 1989; Dawson 1941). Nicotine is synthesized through the condensation of two metabolites, 1-methyl-Δ^1-pyrrolinium and nicotinic acid (Hashimoto and Yamada 1994). 1-methyl-Δ^1-pyrrolinium is synthesized from arginine and/or ornithine. Putrescine *N*-methyl transferase (PMT) is the key enzyme in diverting metabolism towards the biosynthesis of nicotine and tropane alkaloids. Since PMT is involved in the first committed step in nicotine synthesis, it is the ideal target for genetic engineering for the purpose of altering the levels of nicotine and other alkaloids in tobacco (Facchini

Keywords. *Nicotiana tabacum*; nicotine; *N*-methyltransferase; plant breeding; putrescine RNA interference; RNA silencing

Abbreviations used: BLASTn, Basic Local Alignment Search Tool of Nucleotides; CaMV, *Cauliflower mosaic virus*; cDNA, complementary DNA; Ct, cycle threshold value; GUS, β-glucuronidase; HPLC, high-performance liquid chromatography; NOS, nitric oxide synthase**;** ntPMT, putrescine *N*-methyl transferase from *Nicotiana tabacum*; OCS, octopine synthase; PDK, pyruvate dehydrogenase kinase; PMT, putrescine *N*-methyl transferase; RNAi, RNA interference; RT-QPCR, real-time quantitative polymerase chain reaction

2001; Hashimoto and Yamada 1994; Nugroho and Verpoorte 2002).

RNA silencing was discovered in plants many years ago, and has attracted general interest since the discovery of RNA interference (RNAi) (Wingard 1928; Baulcombe 2004). There are several RNA-silencing pathways, but the mechanism of regulation of these pathways is complicated (Baulcombe 2004; Brodersen and Voinnet 2006). Despite the elusive nature of RNA silencing, numerous efforts have been pursued to apply this efficient tool to suppress endogenous gene expression (Hall 2005; Ito *et al* 2005; Shi and Fu 2004). Several studies have realized the potential of RNA silencing in the synthesis of natural plant products to improve plant nutritional value (Ogita *et al* 2003; Allen *et al* 2004; Tang and Galili 2004; Dixon 2005). Due to relatively comprehensive information regarding the nicotine biosynthesis pathway, several reports have been published regarding suppression of the *PMT* genes in *Nicotiana* (Nakatani and Malik 1992; Sato *et al* 2001; Voelckel *et al* 2001; Chintapakorn and Hamill 2003; Steppuhn *et al* 2004). However, variations of suppression efficacies therein lay in a relatively narrow range. Here, we present the generation of transgenic tobacco lines with widely different reduction in nicotine levels, and also systematically compare the results of suppressing the *PMT* genes by using the RNA-silencing approaches of RNAi, antisense and cosuppression.

2. Materials and methods

2.1 *Materials and reagents*

Tobacco seeds, *Escherichia coli* strain DH5*α*, and *Agrobacterium tumefaciens* strain EHA105 were stored in the Fudan-SJTU-Nottingham Plant Biotechonology R&D Center. The gene-silencing vector pHANNIBAL was a gift from Peter Waterhouse at CSIRO Plant Industry, Australia. Xterra MS C_{18} 5 μm 3.9 × 150 mm column was purchased from Waters. Restriction endonucleases were purchased from TaKaRa or New England Biolab. Kits for the molecular biology operation were purchased from Shanghai Watson Biotech Incorporation or TaKaRa. Nicotine was purchased from Sigma-Aldrich. Other chemicals were purchased from Merck.

2.2 *Construction of vectors and plant transformation*

After using BLASTn to ensure the presence of homologous complementary DNA (cDNA) fragments above 30 bp in length, a 540 bp fragment was chosen to be the target of RNA silencing in this study. The DNA fragment was amplified by polymerase chain reaction (PCR), based on the sequence of the target cDNA of putrescine *N*-methyl transferase from *Nicotiana tabacum (NtPMT)*. Appropriate restriction sites were added to the 5′ ends: to amplify the forward segment, *Xho*I and *Kpn*I were added to the primers, respectively, and the resulting primers were 5′-CCGCTC GAGATGACGTGGTAGTTGATGTATCTAG-3′ and 5′-GGGGTACCCGATCATACTTCTGGCGAAAGATG-3′. To amplify the reverse segment, *Bam*HI and *Cla*I were added to primers, respectively, and the resulting primers were 5′-G CGGATCCATGACGTGGTAGTTGATGTATCTAG-3′ and 5′-GGATCGATCGATCATACTTCTGGCGAAAGATG-3′. The DNA was digested using corresponding restriction enzymes and ligated into the pHANNIBAL vector sequentially, with the sequence in the appropriate orientation under the transcriptional control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and octopine synthase (OCS) terminator, which was expected to be expressed in tobacco as an inverted repeat fragment. The entire expression element was then excised by restriction enzymes *Pst*I and *Sac*I and incorporated into pCAMBIA1301. Otherwise, the digested fragments in forward and reverse orientation were ligated into the appropriate position in pHANNIBAL, respectively, and ligated into pCAMBIA1301, which was expected to express in tobacco as forward and reverse fragments compared with the orientation of the cDNA of *PMT* correspondingly. After PCR and confirmation of sequencing, the above three plasmids, together with the construct without the target fragment, were introduced into *Agrobacterium tumefaciens* strain EHA105, which was then used to transform tobacco leaf disc explants.

2.3 *Regeneration of plants and confirmation of transgenic lines*

All the wild-type and transgenic tobacco plants were grown in a growth chamber at 25°C with a 14 h light period. Twenty days after transformation, leaves of explants showed positive *β*-glucuronidase (GUS) assay as described previously (Jefferson *et al* 1987), and were screened for hygromycin resistance in Murashige and Skoog medium. After ~40 days, this was further confirmed by PCR of the target fragments in which the following primers were used: 5′-CTCCTCTCAGAGCAGAATC-3′, and 5′-TCTGTTAGATCCTCGATTTGA-3′, and a partial fragment of the CaMV 35S promoter was expected to be amplified in each positive sample.

2.4 *Extraction of alkaloids and HPLC analysis*

The high-performance liquid chromatography (HPLC) system consisted of a Waters 2695 separations module, a Waters 2996 photodiode array detector, and an Xterra MS C18 5 μm 3.9 × 150 mm column. The column temperature

was 30°C. After two months of growth, when the plants were about 40 (37–43) cm high, three mature leaves were taken from the middle portion of 2–3 pre-flowering plants of each line. Lines out of the desired height range were not analysed further. The leaves were dried at 60°C, ground and dried again for 24 h to constant dry weight. The dried samples were divided into 0.05 g lots and extracted with 1 ml of 25 mM sodium phosphate buffer (pH 7.8) at 30°C for 24 h with constant agitation. The aqueous extract was filtered and diluted 10-fold with water, and filtered through a millipore filter. The procedure for routine HPLC determination of alkaloid and nicotine concentrations was as described by Saunders and Blume (1981).

2.5 *Expression profiling of the* PMT *genes by real-time quantitative PCR (RT-QPCR)*

After extraction from the roots, all RNA templates were digested with DNase I (RNase-free) prior to use. Aliquots of 0.2 μg total RNA were employed in the RT-quantitative polymerase chain reaction (QPCR) reaction using random hexamer primers. First-strand cDNA was synthesized using the TaKaRa reverse transcriptase kit (TaKaRa, Shiga, Japan). RT-QPCR was performed on an iCycler iQTM real-time PCR machine (BioRad, Watford, UK) with PMT- and QPRT-specific primers, and the SYBR ExScript RT-PCR kit (Takara, Shiga, Japan) protocol was used to confirm changes in gene expression. The target messages, actin and PMT in unknown samples were quantified 3 times by measuring the cycle threshold value (Ct) and concentrations were determined using standard curves constructed with serial dilution templates of known concentration. Melt curve analysis and agarose gel electrophoresis were performed after each RT-QPCR to assess product specificity. A comparative method for quantification and delta Ct was adopted. The Ct values corresponding to the actin endogenous housekeeping control gene expression were subtracted from the Ct values for expression of the target gene *PMT* in order to calculate delta Ct. The values were measured in triplicate samples.

3. Results

3.1 *RNA-silencing target sequences, plasmid construction and transformation into tobacco*

Five genes make up the *PMT* gene family, and all of their coding regions contain 8 exons (Riechers and Timko 1999). The coding sequence alignment of *N. tabacum PMTs* revealed that the sequences are highly conserved, except for differences in exons 1 and 2, and a couple of single-base changes in other exons (data not shown). The chosen RNA-silencing target sequences span exons 3 and 7. For *NtPMT2* (Genbank Accession No.: AF126809), the non-conserved region of the nucleotide sequence was between the start codon and the 134th base pair, and the target sequence covered 540 bp, between the 515th and 1055th nucleotide from the beginning of the sequence (figure 1a).

Plasmids were constructed to suppress the five members of the *PMT* gene family. The target fragment was incorporated into the vector (figure 1b) in sense, antisense and inverted-repeat orientations (RNAi), respectively, under the transcriptional control of the CaMV 35S promoter. The vectors also contained the *gusA* gene driven by the CaMV 35S promoter with a hygromycin selection marker, resulting in three plasmids. A control plasmid, which contained the expression elements without the *PMT* target fragment, was used as reference. These plasmids were used separately to transform tobacco using the *Agrobacterium*-mediated leaf disc transformation method.

3.2 *Suppression of nicotine production and generation of tobacco lines with a continuous spectrum of nicotine levels*

In total, 239 transgenic tobacco lines were regenerated, which were confirmed by GUS activity and PCR analysis (figure 2). Among these, 112 were RNAi lines, 82 were antisense lines, 39 were cosuppression lines, and 6 were the control lines. The transformation efficiencies were between 35.9% and 45.5%, as shown in table 1. The phenotype of the transgenic tobacco lines appeared to be normal as compared with the control plants (data not shown), which was similar to the report by Chintapakorn and Hamill (2003).

After two months of growth, when the aerial lengths of the plants were between 37 and 43 cm, three mature leaves were taken from the middle portion of 2–3 pre-flowering plants of each line. The leaves were dried and powdered, and the alkaloid levels were determined by HPLC. Control lines transformed with empty vectors had a similar level of nicotine to wild-type lines, which was 2.6% of the dried weight. Seventy-nine per cent of RNAi lines, 59% of antisense lines and 56% of cosuppression lines showed evidence of at least 50% reduction in the nicotine level. Seven per cent of RNAi lines and 3% of antisense lines showed over 90% reduction in nicotine concentration compared with the control lines (table 1, figure 3). Continuous spectra of nicotine levels were found in lines from all three kinds of suppression approaches. However, the tobacco line with the lowest nicotine concentration was obtained via the RNAi approach, which was only 3.3% that of the wild-type. The range of variations in nicotine levels of RNA-silencing lines relative to that of control lines were as follows: RNAi lines, 3.3–71.0%; antisense lines, 7.9–87.6%; cosuppression lines,

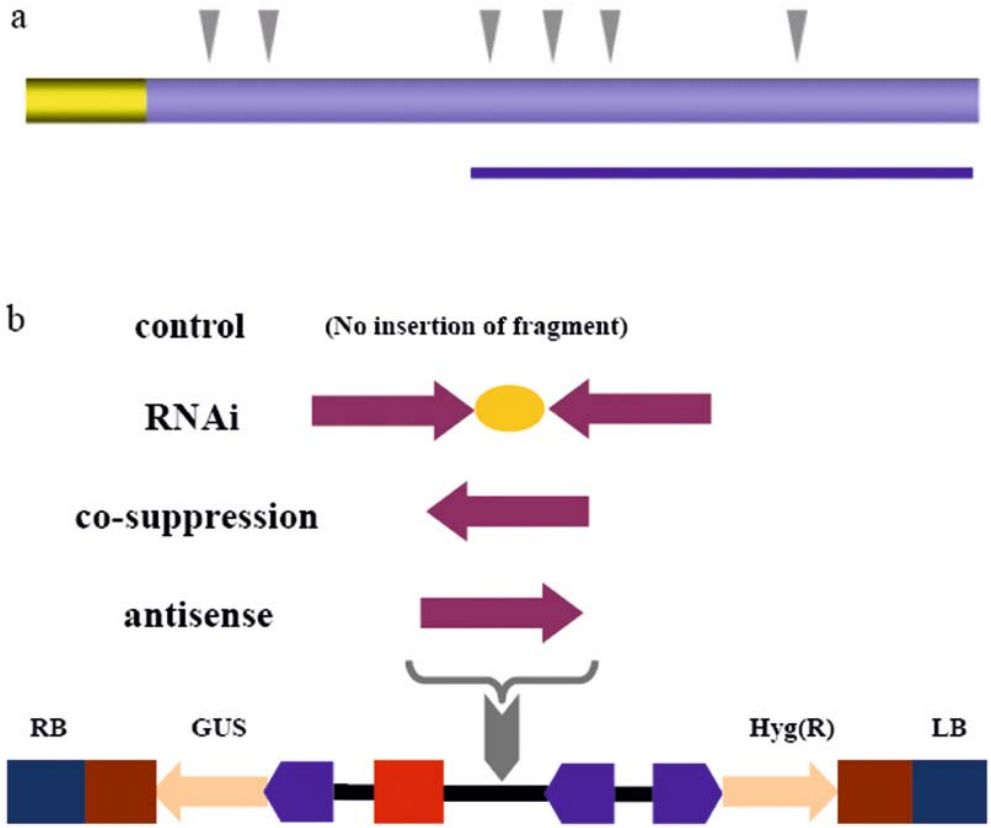

Figure 1. Construction of PMT gene and RNA silencing vectors. (**a**) Framework of coding sequence of NtPMT2 (Genbank Accession No. AF126809). Non-conservative region of 5′ ends is marked by a yellow bar, and the violet bar represents the conservative region. The grey triangles denote location of introns, and the slim blue bar represents the target sequence region of RNA silencing in this study. (**b**) Schematic structure of T-fragments that were used to transform tobacco. The left- and the right-bound arrows represent the silencing fragment and its antisense structure, respectively. The yellow oval represents the PDK intron. In the bottom, the blue arrows represent the CaMV 35S promoter, the brown squares represent the NOS terminator, and the blue squares represent the OCS terminator.

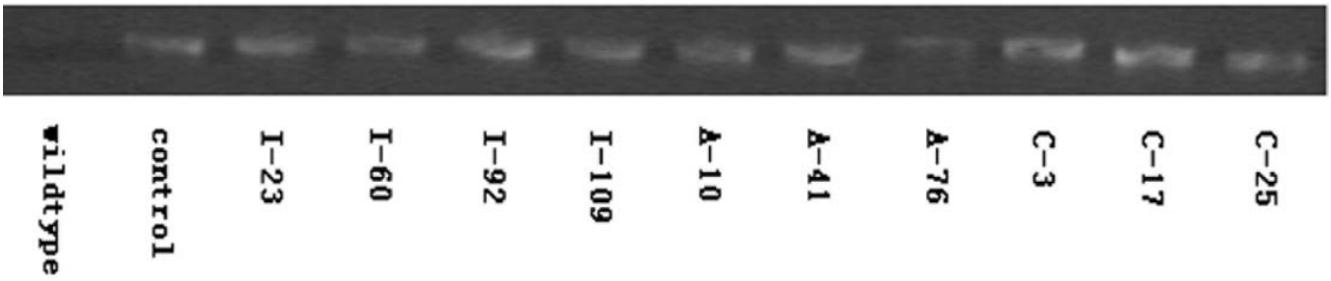

Figure 2. Detection of insertion of silencing fragments into the tobacco genome. DNA was extracted from the leaves of all tobacco lines, followed by PCR manipulation and electrophoresis of PCR products. Shown in the figure are uniform ~800 bp bands of representatives of transgenic lines, along with that of the control line and the wild-type line. The code number of lines is indicated under each band.

14.3–90.9%. In conclusion, the RNAi approach showed a dramatic suppression effect, in which all lines had at least 25% reduction of the nicotine level as compared with the wild-type. Thus, RNAi is the most efficient approach in suppressing the *PMT* gene, whereas antisense and cosuppression approaches were less efficient.

Table 1. Statistical data of independent T_0 transgenic lines via three kinds of RNA-silencing techniques

	RNAi	Antisense	Cosuppression	Total
No. of transformed lines	324	237	84	645
GUS- and PCR-positive lines	121	85	38	244
Lines with nicotine levels measured	109	76	32	217
Percentage of lines with nicotine levels less than 20% of the control line	28%	29%	13%	26%
Percentage of lines with nicotine levels less than 10% of the control line	7%	3%	0%	5%
The line with the lowest nicotine level	I-92 3.3% of the control	A-55 7.9% of the control	C-3 14.4% of the control	I-92 3.3% of the control

RNAi, RNA interference; GUS, *β*-glucuronidase; PCR, polymerase chain reaction.

Figure 3. Comparison of silencing results of cosuppression, antisense and RNAi regarding numbers of independent lines at five levels of intervals. The violet bar leftmost represents lines with 80–100% reduction of nicotine level; the purple bar, lines with 60–80% reduction, the yellow bar, lines with 40–60% reduction, the weak blue, lines with 20–40% reduction; and the dark purple bar rightmost, lines with 0–20% reduction.

3.3 *Expression profiling of* PMT *genes revealed a correlation of* PMT *expression levels and nicotine concentrations*

To decide whether the wide difference in reduction of nicotine levels in transgenic lines was caused by different silencing efficiencies of *PMT*, transcript levels of *PMT* were examined. As mentioned previously, *PMTs* are encoded by a multigene family, and it is not suitable to decide their levels by RNA gel blot. So we decided to determine them by RT-QPCR. In comparison with levels of the control lines, transcript levels were correspondingly reduced in RNA-silencing lines, and the data showed a correlation of *PMT* expression levels and nicotine content. However, reduction of nicotine levels was not as distinct as that of *PMT* transcripts (figure 4). For example, in Line A-58, the nicotine level was 15.4% that of the control lines, and the transcript level of *PMT* was only 7% that of control.

4. Discussion

This report provides a successful example of an efficient genetic engineering system using different RNA-silencing approaches to generate plants with widely different, including extremely low, levels of nicotine. Tobacco plants with a variety of nicotine levels have previously been produced by conventional breeding (Valleau 1949) but, because of difficulties in detecting and evaluating the variation in metabolites of the target plants, it was proven to be time-consuming and had the potential of introducing undesirable traits due to its complexity, to remove which several rounds of backcross was commonly needed (Morandini and Salamini 2003). The conventionally bred low-nicotine tobacco has the mutant A and B loci that regulate a series of stress response genes, which have little bearing on nicotine synthesis (Kidd *et al* 2006). It is also more difficult to use conventional breeding to produce low-nicotine plants in different varieties of commercially

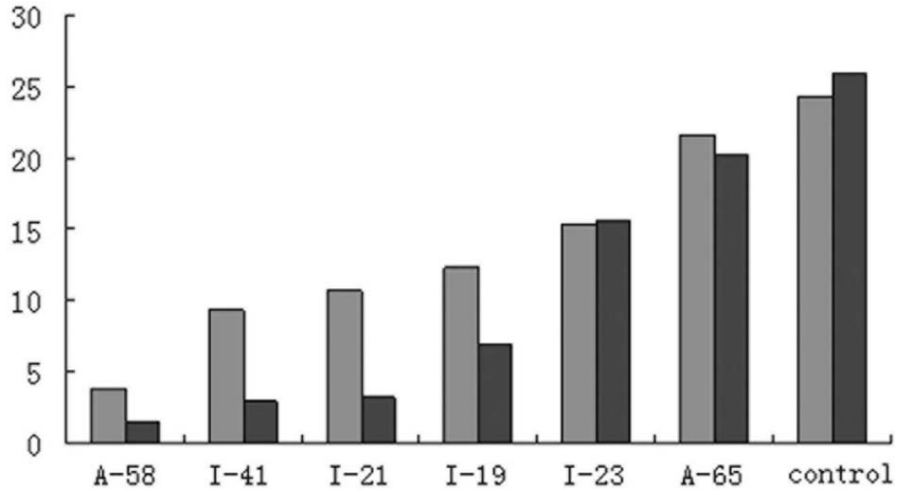

Figure 4. Correlation of nicotine levels and transcript levels of *PMT* in tobacco lines of various silencing grades. ▭ represents nicotine levels (mg/g), and ■ represents expression levels of *PMT* relative to expression of the actin gene. Expression level of *PMT* of the control line is 26.0 mg/g.

important tobacco plants (Morandini and Salamini 2003; Valleau 1949). Efforts have been made to downregulate nicotine biosynthesis through molecular manipulation such as RNA silencing (Nakatani and Malik 1992; Sato *et al* 2001; Voelckel *et al* 2001; Chintapakorn and Hamill 2003; Steppuhn *et al* 2004), but these projects were only partially successful as the reported variations in downregulated nicotine levels were consistently in narrow ranges.

Wang and Wagner (2003) compared the efficacy of the RNAi, antisense and cosuppression techniques by silencing the CBT-ol cyclase gene and CYP71D16 gene in tobacco; their results showed that the RNAi approach was more efficient than antisense and cosuppression. But this result was based on comparison of data of the lowest target levels of metabolites. We also found that RNAi is the most efficient among the three kinds of silencing methods in regulating nicotine biosynthesis, based on datasets of the three groups of tobacco lines, which showed wide metabolite spectra besides the lowest metabolite levels.

We suggest here that the variations in nicotine biosynthesis and gene expression were the results of position effects of transferred DNA (T-DNA) insertion in the tobacco genome, rather than environmental variation or stochastic factors. Gene expression involves a series of recruitments of transcription machinery and alterations in chromatin structure (Vignali *et al* 2000; Jenuwein and Allis 2001). Unveiling the complexity of the underlying system is necessary to identify regulatory variants and provide clues to dissect the genetic background and environmental variation of metabolites in tobacco and, probably, other plant systems.

Cigarette smoking is among the major causes of diseases worldwide, and nicotine is the crucial component that results in addiction to smoking. Currently, there are several methods available to treat nicotine addiction, including pharmaceutical approaches such as nicotine replacement therapy (Jimenez-Ruiz *et al* 1998; Fiore *et al* 2000). These methods typically do not produce ideal results, and their levels of efficiency are low. An alternative method is to reduce the level of nicotine in tobacco by fractionation procedures (Fiore *et al* 2000). However, the removal of nicotine in cigarettes is a complex and expensive process, and often results in a change in the flavour and aroma (Nakatani and Malik 1992). In contrast, producing and using transgenic tobacco lines that have various nicotine concentrations would solve these problems. Generating these tobacco lines via the RNAi approach reported in the present study is simple and, once generated, will require no additional processing expenses. A tobacco line with a certain level of nicotine could be selected for a particular period of treatment for nicotine addiction by sequentially using cigarettes with gradually lower levels of nicotine. Moreover, low-nicotine tobacco can be used to produce low-nicotine cigarettes for commercial use. Obviously, the plasmid used here can be transferred into other commercially important tobacco cultivars. The approach used in this report also can be applied to generate different reduced levels of specific compounds such as toxic materials, unfavourable taste, or allergens in different crop plants (Giovannoni 2006). However, assessment of the biosafety of these transgenic plants is needed in a trial release prior to commercial use, because the hygromycin resistance gene used as a selection marker poses a potential risk of gene flow into natural plants, which could create tough hygromycin-resistant strains. In this report, the hygromycin resistance gene was used to screen positive regenerated plants, and the *gusA* gene was

used to remove plants without target transgenic fragments, which would make PCR confirmation of tobacco plants much more effective. Alternatively, the potential problem of gene flow could be solved by gene knockout to create lines without corresponding selection markers and/or reporter genes.

Acknowledgements

The authors thank Dr Peter Waterhouse at the Plant Industry, CSIRO, Australia for providing the plasmid pHANNIBAL, and Jingjing Liang, Ying Shen and Wei Mao for technical help. The authors would like to express their gratitude to the anonymous reviewers for their suggestions. This work was funded by National Basic Research Program of China (973 Program, 2007CB108805), China Ministry of Education and Shanghai Agriculture Committee.

References

Allen R S, Millgate A G, Chitty J A, Thisleton J, Miller J A C, Fist A J, Gerlach W L and Larkin P J 2004 RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy; *Nat. Biotechnol.* **22** 1559–1566

Baldwin I T 1989 Mechanism of damage-induced alkaloid production in wild tobacco; *J. Chem. Ecol.* **15** 1661–1680

Baulcombe D 2004 RNA silencing in plants; *Nature (London)* **431** 356–363

Brodersen P and Voinnet O 2006 The diversity of RNA silencing pathways in plants; *Trends Genet.* **22** 268–280

Chintapakorn Y and Hamill J D 2003 Antisense-mediated down-regulation of putrescine N-methyltransferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anatabine at the expense of nicotine; *Plant Mol. Biol.* **53** 87–105

Dawson R F 1941 Accumulation of nicotine in reciprocal grafts of tomato and tobacco; *Am. J. Bot.* **29** 66–71

Dixon R A 2005 Engineering of plant natural product pathways; *Curr. Opin. Plant Biol.* **8** 329–336

Facchini P J 2001 Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications; *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52** 29–66

Fiore M C, Bailey W C, Cohen S J, Dorfman S F, Goldstein M G, Gritz E R, Heyman R B, Jaen C R, Kottke T E and Lando H A 2000 *Treating tobacco use and dependence: clinical practice guidelines* (Rockville, MA, USA: US Department of Health and Human Services, Public Health Service)

Giovannoni J J 2006 Breeding new life into plant metabolism; *Nat. Biotechnol.* **24** 418–419

Hall J 2005 Application of RNAi in biomedical research; *Chimia* **59** 803–807

Hashimoto T and Yamada Y 1994 Alkaloid biogenesis – molecular aspects; *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45** 257–285

Ito M, Kawano K, Miyagishi M and Taira K 2005 Genome-wide application of RNAi to the discovery of potential drug targets; *FEBS Lett.* **579** 5988–5995

Jefferson R A, Kavanagh T A and Bevan M A 1987 *β*-glucuronidase as a sensitive and versatile marker in higher plants; *EMBO J.* **6** 3301–3307

Jenuwein T and Allis C D 2001 Translating the histone code; *Science* **293** 1074–1080

Jimenez-Ruiz C, Kunze M and Fagerstrom K O 1998 Nicotine replacement: a new approach to reducing tobacco-related harm; *Eur. Respir. J.* **11** 473–479

Kidd S K, Melillo A A, Lu R H, Reed D G, Kuno N, Uchida K, Furuya M and Jelesko J G 2006 The A and B loci in tobacco regulate a network of stress response genes, few of which are associated with nicotine biosynthesis; *Plant Mol. Biol.* **60** 699–716

Laviolette S R and van der Kooy D 2004 The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour; *Nat. Rev. Neurosci.* **5** 55–65

Morandini P and Salamini F 2003 Plant biotechnology and breeding: allied for years to come; *Trends Plant Sci.* **8** 70–75

Nakatani H and Malik V S 1992 Putrescine *N*-methyltransferase, recombinant DNA molecules encoding putrescine *N*-methyltrasferase, and transgenic tobacco plants with altered nicotine content; *European Patent Application Number* 91310283.6

Nugroho L H and Verpoorte R 2002 Secondary metabolism in tobacco; *Plant Cell Tiss. Org. Cult.* **68** 105–125

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N and Sano H 2003 RNA interference – producing decaffeinated coffee plants; *Nature (London)* **423** 823

Powledge T M 2004 Nicotine as therapy; *PLoS Biol.* **2** 1707–1710

Riechers D E and Timko M P 1999 Structure and expression of the gene family encoding putrescine *N*-methyltransferase in *Nicotiana tabacum*: new clues to the evolutionary origin of cultivated tobacco; *Plant Mol .Biol.* **41** 387–401

Saitoh F, Noma M and Kawashima N 1985 The alkaloid contents of sixty *Nicotiana* species; *Phytochemistry* **24** 477–480

Sato F, Hashimoto T, Hachiya A, Tamura K, Choi K B, Morishige T, Fujimoto H and Yamada Y 2001 Metabolic engineering of plant alkaloid biosynthesis; *Proc. Natl. Acad. Sci. USA* **98** 367–372

Saunders J A and Blume D E 1981 Quantitation of major tobacco alkaloids by high-performance liquid chromatography; *J. Chromatogr.* **205** 147–154

Shi Z and Fu L W 2004 RNAi and its application in tumor study; *Prog. Biochem. Biophys*. **31** 492–499

Steppuhn A, Gase K, Krock B, Halitschke R and Baldwin I T 2004 Nicotine's defensive function in nature; *PLoS Biol.* **2** 1074–1080

Tang G L and Galili G 2004 Using RNAi to improve plant nutritional value: from mechanism to application; *Trends Biotechnol.* **22** 463–469

Valleau W D 1949 Breeding low-nicotine tobacco; *J. Agric. Res.* **78** 171–181

Vignali M, Hassan A H, Neely K E and Workman J L 2000 ATP-dependent chromatin-remodeling complexes; *Mol. Cell. Biol.* **20** 1899–1910

Voelckel C, Krugel T, Gase K, Heidrich N, van Dam N M, Winz R and Baldwin I T 2001 Anti-sense expression of putrescine N-methyltransferase confirms defensive role of nicotine in *Nicotiana sylvestris* against *Manduca sexta*; *Chemoecology* **11** 121–126

Wang E M and Wagner G J 2003 Elucidation of the functions of genes central to diterpene metabolism in tobacco trichomes using posttranscriptional gene silencing; *Planta* **216** 686–691

Wingard S A 1928 Hosts and symptoms of ring spot, a virus disease of plants; *J. Agric. Res.* **37** 127–153

MS received 27 November 2007; accepted 17 March 2008

*e*Publication: 3 April 2008

Corresponding editor: JITENDRA P KHURANA