ABSTRACT

Tobacco epidemic is one of the major threats to human health killing nearly 6 million people each year with an estimated 8 million deaths in the year 2030. More than 2500 chemicals are found in tobacco and its smoke, among which terpenoids and alkaloids are major groups. Though there is clinical uncertainty about the carcinogenic property of nicotine, evidences have proved nicotine as the main psychoactive agent responsible for the development of tobacco dependence. It is estimated that 3-5 mg/day is the threshold level that readily establishes and sustains addiction among smokers. The LD$_{50}$ of nicotine for adult and children has been estimated to be 30-60 mg and 10 mg, respectively. There exist multi-pronged strategies to reduce the consumption of tobacco products. A couple of nicotine replacement therapy is commercially available. The use of FDA approved nicotine patch, gum, nasal spray, inhalers and lozenges have their own shortcomings in the form of sleeplessness, nausea, skin irritations, withdrawal symptoms, and more. Similarly, the nicotine acetylcholine receptor agonist (chantix ®) has been reported to cause depression, paranoia, psychosis, hallucinations, delusions and other neuropsychiatric events in patients.

Reduction of the nicotine content in tobacco products can bring down addiction rate and other ill effects of tobacco consumption. Achieving this by physical and chemical processing of tobacco is not cost effective. Therefore, developing low nicotine tobacco varieties would be a viable strategy to bring down nicotine level in tobacco products. Being a non-edible crop, genetically engineered low nicotine tobacco would be more acceptable than transgenic crop plants. Previous studies have reported significant reduction in nicotine content by using cosuppression and RNAi methods. PMT is a rate limiting enzyme in the nicotine biosynthesis encoded by a multigene family consisting of five genes ($PMT1$, $PMT2$, $PMT3$, $PMT4$, and $PMT5$) of which $PMT2$ is the major one which is expressed in the roots of tobacco Here we
report that RNAi mediated silencing of PMT genes under a constitutive promoter could reduce the nicotine content by 98.0% with concomitant increase in chlorogenic acid by 3 to 6 fold.

The sequence characteristics of the RNAi trigger sequence used for silencing plays major role in both. An RNAi trigger was designed for PMT2/4 based on pairwise alignment of the 5-member gene family. The 162bp RNAi trigger sequence used in the present study was derived from the PMT2 gene which is the major gene (highly expressed) in the 5-member PMT gene family. The trigger sequence was derived from the first exon which showed the maximum divergence primarily due to the insertions of a variable number of 33 nucleotide repeats. It was designed to target the major PMT 2 gene but cross silencing of the minor PMT4 genes was expected due to 76% nucleotide identity. PMT1, PMT3 and PMT5 may also be cross silenced to some extent due to the sharing of 22 nucleotide perfect match sequences with the RNAi trigger. The 162bp RNAi trigger sequence contained guide siRNA sequences with preferred antisense thermodynamic properties for efficient silencing. The spacer sequence used to generate hairpin dsRNA of the trigger sequence also play a crucial role in the silencing of the target genes. RNAi trigger with intronic spacers provides dsRNA template for efficient dicer processing and thereby, increases the efficiency of the RNAi vectors. We have used native intron including the conserved 5’ and 3’ splice sites as a self-cleaving spacer. This may be one of the reasons that we have observed the highest level of silencing as evident from the lowest nicotine content found in the RNAi plants.

Fourty-five putative transgenic plants were screened by PCR amplification of NPTII gene and RNAi trigger. Twelve PCR positive RNAi plants (T1 – T12) were transferred to pots and grown to maturity. Southern hybridization was carried out for four putative transgenic plants for the confirmation of transgene integration and estimation of the copy number. Differential expression of PMT gene family in genetically engineered low nicotine tobacco plants was not studied before. In the present study, PMT2/4 genes were found to be silenced to the highest level in all the low nicotine plants of the T0 generation. However, cross silencing of PMT1/5 and PMT3 was also observed which may be due to the sharing of 22 nucleotide perfect match sequences. Additionally, 22 nucleotide sequences with one to three mismatches were also observed between predicted guide siRNA sequence and the members of the PMT gene family.
Previous attempts made to reduce the nicotine content in tobacco targeting different genes had varied levels of success. Antisense for arginine decarboxylase gene (ADC) and RNAi for ornithine decarboxylase gene (ODC) reduced the nicotine content only to 20% and 50%, respectively. However, silencing of PMT genes in the diploid in *Nicotiana sylvestris* also reduced nicotine content to 2.0% with attendant morphological deformities such as fused leaves, branched fluorescence and small seed set. In a preliminary report, Wang et al. have successfully used conserved RNAi trigger sequence against the PMT genes to reduce the nicotine content to 3.3% in *Nicotiana tabacum*. Detailed information on any morphological abnormalities, differential expression of PMT genes, and compensating metabolites were not reported. We have gene-specific trigger sequence targeting the major PMT2 gene and observed that nicotine content was reduced to as low as 1.6% compared with the wild type. The low nicotine RNAi plants showed normal phenotypes with no detectable morphological variations. The morphological phenotypes were normal in the subsequent generations but the nicotine content was marginally increased. The difference in the nicotine content may be attributed to the segregation, differences in the plant culture conditions or natural adaptation.

Accumulation or diversion of precursors and intermediate metabolites is expected in case of metabolic engineering of the biosynthetic pathways wherein expression of one or more enzymes is manipulated. In the present study, constitutive silencing of PMT genes was targeted to block or reduce the expression of putrescine methyl transferase that catalyzes the conversion of putrescine to methyl putrescine. It was reported earlier that silencing of PMT genes resulted in an increased level of putrescine and spermidine in *Nicotiana sylvestris* and anatabine in *Nicotiana tabacum*. Silencing of ADC which converts arginine to putrescine and ODC which converts ornithine to putrescine also produced low nicotine plants with increased levels of anatabine in *Nicotiana tabacum*. Anatabine is a cholinergic agonist toxin alkaloid in tobacco derived from nicotinic acid. We have not observed accumulation of this anatabine in the low-nicotine RNAi plants. Instead, we report for the first time that reduction of nicotine content results in concomitant increase in the content of chlorogenic acid. Chlorogenic acid is an ester formed between caffeic acid and quinic acid with proven antioxidant properties. Accumulation of putrescine and nicotinic acid is expected when the nicotine biosynthesis is blocked by silencing the PMT genes. Nicotininc acid is an intermediate in the formation of anatabine and anabasine in tobacco. MS ESI spectra in positive and negative mode did not show accumulation of such
compounds. On the other hand, catabolism of putrescine produces gamma aminobutyric acid that can conjugate with caffeic acid to form N-caffeoyl 4-amino-\(n\)-butyric acid. Putrescine can also combine with hydroxycinnamates to produce hydroxy cinnamate-putrescine conjugates such as caffeoyl putrescine, coumaryl putrescine, ferulyl putrescine or cinnamoyl putrescine. We propose that enrichment of chlorogenic acid as one of the plausible metabolic compensations for the reduction in nicotine content.

Tobacco use is a global issue, very prominent among middle and less income countries. Quitting on tobacco use is not easy due to the addictive properties of the psychoactive alkaloid nicotine. Low nicotine content will reduce the addictive properties and minimize tobacco dependence. Here, we show that down regulation of \(\text{PMT}\) gene drastically reduce the nicotine content in tobacco. Reduction in the nicotine content was compensated by an increase in a nontoxic compound chlorogenic acid. The international standard for the amount of nicotine per cigarette is 1 mg. However, the nicotine content in the uncured leaves of commercial varieties used in cigarette contains 15-20 mg/g. The tobacco leaves undergo curing process to meet the international standards. A daily dose of nicotine between 3.0 and 5.0 mg can lead to addiction. With 1 mg nicotine per cigarette at 25% bioavailability it would require 15 to 20 cigarettes per day to create and maintain addiction. The nicotine content in the best RNAi plant was only 1.6% of the wild type tobacco. Curing process would further reduce the nicotine content in the leaves of these plants when used in cigarettes and the possibility of reaching the addiction threshold is remote. At the same time, it may provide enough nicotine for taste and sensory stimulation. Such low nicotine tobacco can be used in tobacco weaning programs, as alternate to nicotine patch, gum, and nasal spray. Further, tobacco is the most preferred host for expressing human therapeutic proteins in plants but its alkaloid content is an obstacle in animal studies and clinical trials. The low nicotine tobacco plants would provide a new platform for the production of human recombinant therapeutic proteins.