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# Desiccation and topping induced silencing of *putrescine N-methyl transferase2* regulate nicotine biosynthesis in *Nicotiana tabacum* cv. Petite Havana

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**Abstract**

The current study was an attempt to understand the regulation of nicotine biosynthesis due to desiccation and meristem topping in *Nicotiana tabacum* cv. Petite Havana. Our results showed that desiccation induced silencing of Putrescine N-Methyl Transferase 2 (PMT2) (EC 2.1.1.53), a rate limiting enzyme in the nicotine biosynthetic pathway, regulates nicotine levels in tobacco. A hairpin siRNA (hpsiRNA) was designed and expressed against *pmt* using a stress inducible promoter rd29A from *Arabidopsis thaliana*. Stable transgenic RNAi lines, developed by Agrobacterium mediated transformation, showed reduced accumulation of nicotine due to desiccation and topping. The increase in the nicotine level in wild type was 115% while in RNAi lines, RNAi1, RNAi2 and RNAi3 the increase was 19.9%, 30% and 21.62%, respectively, due to desiccation. Furthermore, meristem topping of desiccated plants increased nicotine accumulation by 38% in wild type and only 7–9% in RNAi lines. No secondary surge in any related secondary metabolite was detected. No detectable change in the phenotype was observed in RNAi lines. This study revealed the critical role of *pmt* in nicotine biosynthesis under desiccation and topping and the inducible regulation of endogenous genes for crop improvement.

**Keywords:** RNAi, *Putrescine- N-Methyl Transferase*, *rd29*A, gene silencing, siRNA, low nicotine.
**Abbreviations:** siRNA_small interfering RNA, hpsiRNA_hairpin siRNA, *pmt_Putrescine N-methyl transferase*, *nptII_Neomycin phosphotransferase II*, *Ntpmt2_Nicotiana tabacum Putrescine N-methyl transferase* , *Ns_Nicotiana sylvestris*, NoS_Nopaline synthase, p*pmt2 iRNAi*_plasmid *pmt2* inducible RNAi, ADC_Arginine decarboxylase, ODC_Ornithine decarboxylase.

## Introduction

Nicotine, the primary psychoactive substance in tobacco is synthesized by the condensation of pyridine ring from nicotinic acid and pyrrolidine ring. The pyrrolidine ring comes from methylpyrrolinium cation, which is derived from putrescine via the sequential action of *S*-adenosyl-L-methionine-dependent *putrescine-N-methyltransferase* (*pmt*), a diamine oxidase and a spontaneous chemical rearrangement. Putrescine N-methyl transferase (PMT, EC 2.1.1.53) catalyzes the S-adenosylmethionine dependent N-methylation of putrescine to N-methyl putrescine, the first committed step in nicotine biosynthesis in tobacco (Fig. 1) (Hashimoto and Yamada, 1994; Kutchan, 1995).

The nicotine biosynthetic pathway in solanales has evolved from polyamine pathway diverting the flow of putrescine from polyamine biosynthesis towards the production of nicotine and other alkaloids like tropane, scopolamine, cocaine, and calystegines. *Putrescine N-methyl transferase* genes are known only from solanaceae and convolvulaceae, first detected in the roots (Baldwin and Zhang 1997; Shoji et al., 2000) and in wounded leaves of *Nicotiana tabacum* (Sachan and Falcone, 2002). The *pmt* was also detected in tuber sprouts of *Solanum tuberosum*, a plant that does not produce tropane alkaloids but produce nortropanes named calystegines (Stenzel et al., 2006).

In tobacco, *putrescine N-methyltransferase* is a five member multigene family (Gen number, AF126809; AF126810; AAF126811; AF126812), of which *Ntpmt2* is predominant and highly expressed in roots 24 hours after topping (Dean Riechers and Timko 1999). The synthesis and accumulation of nicotine are controlled by various developmental and environmental cues. Nicotine biosynthesis in tobacco is induced by biotic stress like fungal infection, herbivore feeding (Hashimoto and Yamada, 1994; Hibi et al., 1994; Facchini, 2001) and abiotic stresses like mechanical wounding, drought and pH imbalance (Hashimoto and Yamada, 1994; Kutchan, 1995).

It is highly induced by apical meristem topping, a physiological stress due to auxin depletion (Baldwin, 1989; Baldwin and Prestin, 1999) and respond through jasmonic acid signal transduction pathway (Shoji et al., 2008; Baldwin et al., 1997; Ohnmeiss et al., 1997; Imanishi et al., 1998; Baldwin et al., 1994).

Selective plant breeding techniques fail in producing tobacco plants with low nicotine content as tobacco species have narrow genetic base. Attempts were made to regulate nicotine biosynthesis using antisense for *ADC* and *pmt* (Chintapakorn and Hamill, 2003; Chintapakorn and Hamill, 2007), RNAi for *ODC* (DeBoer et al., 2011) and *pmt* (Wang et al., 2008) genes. Gene silencing by RNA interference is routinely used in crop improvement and value addition (Sinha, 2010; Regina et al., 2010; Sun et al., 2012; Manavalan et al., 2012). Such genetic manipulations are not without limitations, as plants constitutively express genes to regulate bio-synthetic pathways, showing variations either in phenotype or in secondary metabolite profile. Hence, in regulating a metabolic network, the vitality of time, degree and tissue of siRNA expression is critical. This would ensure minimal modification to the genetic makeup of the host as plants

**Table 1.** List of media, composition and antibiotics used in the study

| Media | Composition | Purpose |
|---|---|---|
| Germination media (GM) | half MS salts + MS vitamins + 3% sucrose + 0.8% agar | Germination of wild type tobacco seeds *in vitro* |
| Shooting Media (SM) | MS salts + MS vitamins + 3% sucrose + NAA 0.1mg/L + BAP 1mg/L + 0.8% agar | Regeneration of shoot primordia from tobacco leaf disc |
| Co-cultivation media (CM) | MS basal, Vitamins, 3% Sucrose, Naphthalene acetic acid (NAA-0.1mg/L), 6- Benzylaminopurine (BAP-1mg/L) and 0.8% Agar | Co-cultivation of *Agrobacterium tumefaciens* and tobacco leaf disc |
| MS infection broth (MSI) | MS salts + MS vitamins + 3% sucrose | *Agrobacterium tumefaciens* resuspension broth for infection |
| MS wash buffer | MS basal salts | Preparation of antibiotic free *Agrobacterium tumefaciens* for tobacco leaf disc infection |
| Regeneration Media (ReM) | MS basal, Vitamins, 3% Sucrose, Naphthalene acetic acid (NAA-0.1mg/L), 6- Benzylaminopurine (BAP-1mg/L) and 0.8% Agar with kanamycin 50 µg / ml | Regeneration media for *Agrobacterium tumefaciens* infected leaf discs |
| Rooting Media (RoM) | ½ MS basal, B5 Vitamins, 3% Sucrose and 0.8% Agar with kanamycin 50 µg / ml | Root initiation media for putative transgenic plantlets |
| LB | 10 g of tryptone, 5 g of yeast extract, 10 g NaCl, 1.5% agar, pH 7.0 | *E coli* growth media |
| YEP | 10 g of yeast extract powder, 10 g of peptone, 5 g of NaCl, 1.5% agar, pH 7.0 | *Agrobacterium tumefaciens* growth media |
| Rifampicin 10mg/l | rifampicin 10mg/ml in methanol | Antibiotic marker to select *Agrobacterium tumefaciens* |
| Kanamycin 50mg/l | kanamycin 10mg/ml in sterile water | Antibiotic to select recombinant *E coli*, *Agrobacterium tumefaciens* and transgenic tobacco |
| Streptomycin 50mg/l | streptomycin 10mg/ml in sterile water | Antibiotic to select *E coli* competent cells |
| Ampicilin 100mg/l | ampicilin 10mg/ml sterile water | Antibiotic to select recombinant *E coli* with pUC18 |
| Carbenicilin 250mg/l | carbenicilin 10mg/ml in sterile water | Antibiotic to arrest *Agrobacterium tumefaciens* overgrowth in leaf disc |

are very much part of human lifestyle. Here, we attempt to investigate the significance and role of induced expression of siRNA for *pmt2* in regulation of nicotine synthesis and its influence on secondary metabolite profile. In the current study, the *rd29A* from *Arabidopsis thaliana*, ***r***esponsive to ***d***esiccation gene promoter expressed in roots, induced by salinity, desiccation and freezing stress was used.

**Results**

***Design and construction of pmt2 RNAi vector and tobacco transformation***

*Putresine N-methyl transferase* is a well conserved gene family among solanales. Identity analysis for PMT2 protein sequences showed 83 – 91% identity with members of other solanales species such as *Physalis divaricata*, *Datura*, *Solanum tuberosum*, *Atropa belladonna*, and *Anisodus acuntangulus* (Fig 2B). Among the *Nicotiana tabacum* isogenes, PMT2 shares 96% identity with PMT1 and PMT3. Phylogenetic analysis predicted a common ancestor for gene members of genus *Nicotiana*, *Nt*PMT2, being closely related to *Ns*PMT while other members of the solanales form a distinct node (Fig 2A).

BLAST analysis of the putative 162bp siRNA designed against *pmt2*, showed no cross target with *Nicotiana tabacum* gene database. The identities of *pmt2* presiRNA sequence with *pmt1* and *pmt3* isogenes were 65.8 and 59.3%, respectively. The *pmt2* presiRNA sequence, *Arabidopsis thaliana* rd29A promoter and NoS terminator were assembled in pUC18 and then cloned into pCAMBIA 2300 (Fig. 3). The sequence confirmed RNAi vector p*pmt2 iRNAi* did not show any divergence for the *pmt2* sequence in database (AF126809). The p*pmt2 iRNAi* transformed tobacco leaf discs were regenerated and transgenic RNAi plants were maintained in greenhouse (Fig. 4A, 4B).

***PCR confirmation of RNAi lines***

Of the twenty five genetically modified $T_o$ RNAi plants regenerated, nine of them were randomly screened for the RNAi cassette. Eight of them were positive and one (R8) was negative hence excluded from further analysis.

The RNAi lines were confirmed for *nptII* plant selectable marker, and *pmt2*siRNA sequence (Fig 5A and 5B). In the transformed RNAi lines, an 800bp fragment of *nptII* was amplified while it was absent in the wild type (Fig 5A). Similarly, the integration of the siRNA sequence in RNAi lines was confirmed by the presence of 541bp hpsiRNA (Fig 5B).

***Reduced levels of nicotine in desiccated and topped RNAi plants***

The retention time for nicotine was 1.01 minute. HPLC analysis of the wild type and RNAi plants show variation in the levels of nicotine accumulated in leaf upon desiccation and apical meristem topping (Fig. 6A).

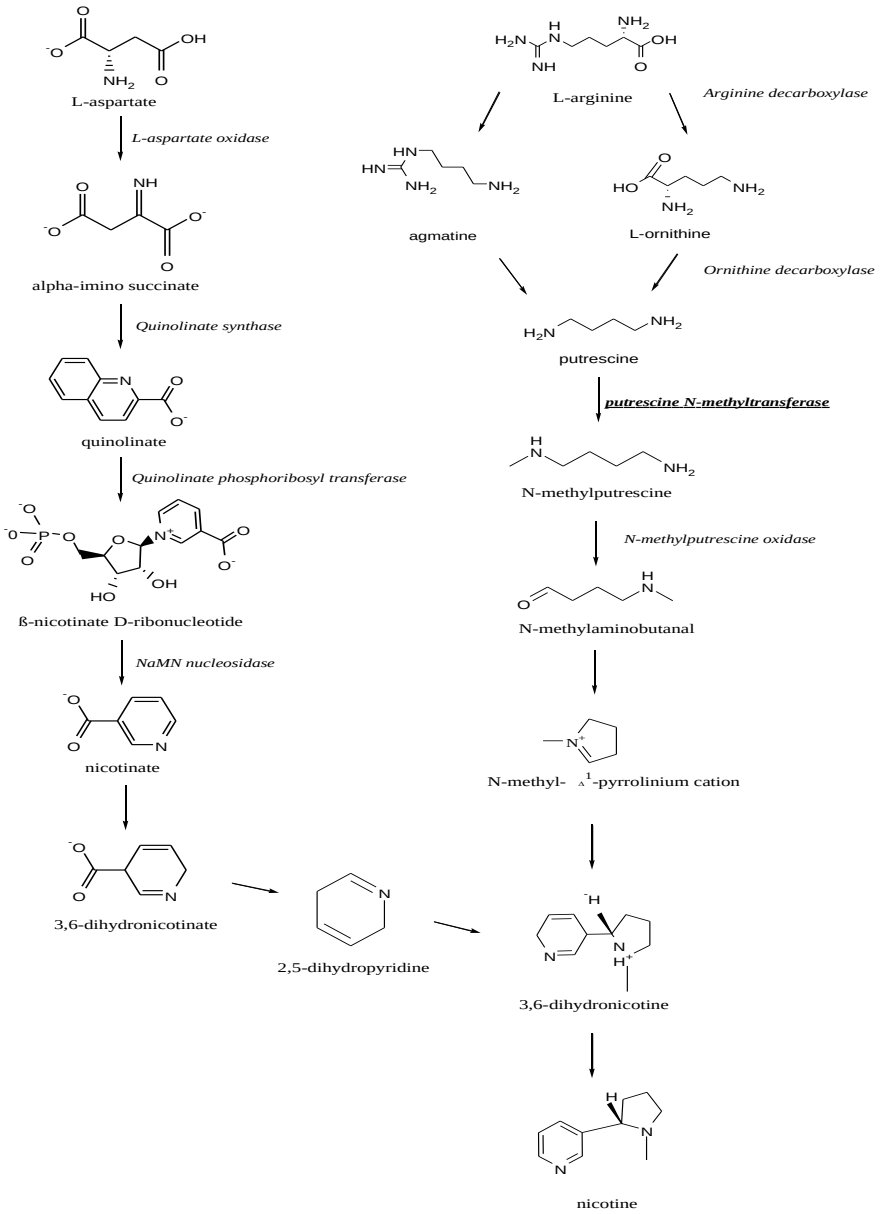



**Fig 1.** Nicotine biosynthetic pathway in *Nicotiana tabacum.* The enzymes catalyzing the reactions are shown in italics. The pyridine ring is derived from L-aspartate by sequential action of aspartate oxidase, quinolinate synthase, quinolinate phosphoribosyl transferase and NaMN nucleosidase. The pyrrolidine ring is formed from arginine via ornithine and putrescine by ADC, ODC, and PMT. The rate limiting enzyme PMT was targeted by RNA interference.

The wild type plant show a 2.15 fold (115%) increase in nicotine content due to desiccation (i) while desiccated and topped (it) plants show 2.96 fold (197%) increase in nicotine. The RNAi lines show drastic reduction in the biosynthesis of nicotine due to desiccation. The nicotine level in RNAi1 line increased 1.15 fold (20%) upon desiccation (i). The variation in the nicotine level in wild type and RNAi lines due to desiccation was statistically significant in one way ANOVA with the *p* value $2.55 \times 10^{-12}$ at alpha 0.05 while insignificant under uninduced state (u) (*p=0.860 at alpha 0.05*). Furthermore, topping in desiccated RNAi1 plant (it) induce an increase in nicotine level by 1.36 fold, while the increase was 2.96 fold (197%) in wild type plants with a p value $6.97 \times 10^{-15}$.

The increase in nicotine level due to topping was 9% in RNAi1 line and 38% in wild type plants. A similar trend was observed with the other two RNAi plants, RNAi2 and RNAi3. This drastic variation in nicotine biosynthesis in wild type and RNAi lines due to desiccation and topping is graphically represented in Fig 6B. Interestingly, a drastic increase in nicotine level of uniduced RNAi plants (wild type) (ut) plants was observed when topped. The increase was 200% in wild type plants and 195% RNAi1 and 190% in RNAi2 and RNAi3 plants, respectively.

**Table 2.** List of primers and target used in the study.

| S No | Oligo sequences | Target region |
|---|---|---|
| 1 | *pmt2*F 5'-CCATCTTCAAGAGTGGTG-3'<br>*pmt2*R 5'-AACCAGGCTTAATACAAT-3' | *pmt2* exon 1 in tobacco |
| 2 | *rd29A* F - 5' GGAGCCATAGATGCAAT 3'<br>*rd29A* R - 5' CCAAAGATTTTTTTCTTTCCAATAGAAG 3' | Promoter from *Arabidopsis thaliana* gene *rd29A* |
| 3 | *pmt2*in F - 5'GGCCAGGTTAGTACTGAG 3'<br>*pmt2*in R - 5'CTTCACCTGCAAAATTCG 3' | First intron of the native gene *pmt2* in tobacco |
| 4 | Nos F - 5'–CGTTCAAACATTTGGCAA-3'<br>NoS R - 5'-CCCGATCTAGTAACATAG -3' | *Nopaline synthase* gene terminator in pCAMBIA |
| 5 | *nptII* F - 5' TCAGAAGAACTCGTCAAGAAG 3'<br>*nptII* R - 5' ATGGGGATTGAACAAGATG 3' | *Neomycin phosphotransferase II* gene in pCAMBIA2300 |

***Induced silencing and metabolite profile***

Desiccation and topping induced silencing of *pmt2* showed reduced levels of nicotine but no diversion of the pathway though. This was evident from the 10 minute HPLC chromatogram of RNAi lines (Fig 7). The chromatogram of RNAi lines desiccated (Ri) and desiccated and topped (Rit) were similar to the uninduced RNAi line (Ru) or the wild type plant (Wu). No detectable change in the metabolite profile of the RNAi plant was observed.

**Discussion**

***Induced expression of siRNA and regulation of nicotine levels***

The objective was to study the effect of desiccation and topping induced regulation of *pmt2* in nicotine biosynthesis in *Nicotiana tabacum*. RNAi lines for *putrescine N-methyl transferase2* in *Nicotiana tabacum* showed significant reduction in the biosynthesis of nicotine due to desiccation and apical meristem topping. Nevertheless, the uninduced RNAi lines behaved as normal wild type plants when topped showing a drastic increase in nicotine synthesis after 24 hours of topping. This establishes the imperative role of *putrescine N-methyl transferase* in nicotine biosynthesis in tobacco. This is in concurrence with the finding that nicotine biosynthesis has evolved from polyamine biosynthesis by diverting the flow of putrescine from polyamine biosynthesis towards the production of nicotine (Hashimoto and Yamada, 1994; Kutchan, 1995). Moreover, the nicotine biosynthesis is positively correlated with the cultural practice of apical meristem topping and biotic and abiotic stresses like drought (Hashimoto and Yamada, 1994; Hibi et al., 1994; Dean Riechers and Timko, 1999).

Similarly, reduced levels of nicotine was observed with constructs that are antisense for genes *pmt* and *ADC* (Chintapakorn et al., 2003; Chintapakorn and Hamill, 2007), and RNAi for genes *ODC* (Kathleen DeBoer et al., 2011) and *pmt* (Wang et al., 2008). Such attempts to down-regulate nicotine levels with constitutive expression of either antisense or siRNA resulted in a fivefold increase in anatabine level in *pmt* antisense lines and in *ADC* lines (Chintapakorn et al., 2003; Chintapakorn and Hamill, 2007) and a 2:1 increase in *ODC* RNAi lines (DeBoer et al., 2011). In our study, neither any qualitative change in the related secondary metabolite profile as studied by HPLC nor any phenotypic variation was observed in the RNAi lines. This illustrate, inducible down-regulation of target gene could be a preferred approach over constitutive expression of siRNA to regulate biosynthetic pathways.

The rd29A is a salinity, desiccation and freezing induced promoter, predominantly expressed in roots, whose efficiency is relatively weak during freezing and desiccation compared to saline stress (Yamaguchi-Shinozaki et al., 1992; Yamaguchi-Shinozaki and Shinozaki, 1994). Since *pmt* genes are predominantly root specific (Deluca and St Pierre, 2000; Dean Riechers and Timko 1999; Baldwin and Zhang 1997; Shoji et al., 2000), induced expression of *pmt2* using rd29A may be restricted to roots with limited interference on agronomic characters. This is in accordance with the finding that mis-expression using a constitutive promoter may pose penalty on plant growth and development (Bihmidine et al., 2013). Constitutive expression of antisense or siRNA for pmt or other targets could cause precursor or intermediate redirection in alkaloid biosynthesis pathway with a surge in a related secondary metabolite. Hence, time, degree and tissue of expression of a siRNA needs to be controlled in the plant system.

***siRNA design and gene regulation***

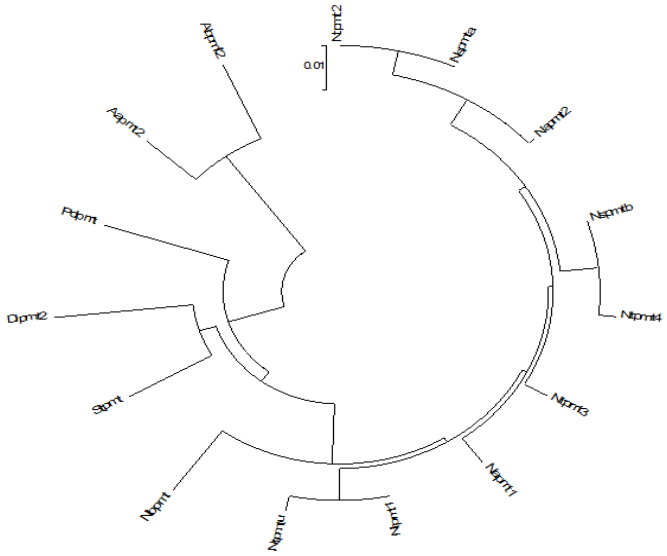
The increase of 20% and 9% of nicotine levels in desiccated and desiccated and topped RNAi plant may not truly reflect the contributions of other *pmt* isogenes. The contributions of isogenes *pmt1* and *pmt3* can be cross silenced by *pmt2* siRNA. In *pmt2* presiRNA, there exist three 22 nucleotide perfect matches for *pmt1* and a single 22 nucleotide perfect match for *pmt3*. In addition, 22 nucleotides with one to three mismatches were also observed in the isogenes. siRNA sequences specific for a gene can cross target if they share a 22–30 nucleotides stretch and can tolerate a few mismatches. Such cross targets have been previously observed with *psbP* multigene family in *Nicotiana tabacum* (Ifuku et al., 2003; Ishihara et al., 2005); starch branching enzyme members in wheat (Regina et al., 2006) and hexokinase in Arabidopsis (Zhai et al., 2009). But, gene specific silencing with contiguous stretches of four to eight identical nucleotides in *OsRac* family members of rice showed no cross targets (Miki et al., 2005). Hence, ingenious design of siRNA for targets would minimize cross targets and off targets.

**Materials and Methods**

***Plant and microbial culture***

*Escherichia coli* strain Top 10 and *Agrobacterium tumefaciens* strain LBA4404 were used for bacterial transformation and *Nicotania tabacum* cv. Petite Havana for

**2A**




**2B**

| | *Ntpmt3* | *Nbpmt* | *Napmt1* | *Ntpmt2* | *Nspmta* | *Napmt2* | *Nspmtb* | *Ntpmt4* | *Ntpmt1* | *Ntpmtu* | *Stpmt* | *Dipmt2* | *Pdpmt* | *Aapmt2* | *Abpmt2* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Ntpmt3* | 100.00 | 96.33 | 98.69 | 96.53 | 97.11 | 96.70 | 97.63 | 97.36 | 96.47 | 96.20 | 92.49 | 88.79 | 88.30 | 83.63 | 83.53 |
| *Nbpmt* | | 100.00 | 95.88 | 94.05 | 94.05 | 94.07 | 94.30 | 94.04 | 93.87 | 93.60 | 90.00 | 87.65 | 87.17 | 81.05 | 82.14 |
| *Napmt1* | | | 100.00 | 96.03 | 96.32 | 96.50 | 97.41 | 97.15 | 96.27 | 96.00 | 91.18 | 88.53 | 87.76 | 81.34 | 82.14 |
| *Ntpmt2* | | | | 100.00 | 99.15 | 97.17 | 96.60 | 96.32 | 96.03 | 95.75 | 91.45 | 88.72 | 88.01 | 82.84 | 83.04 |
| *Nspmta* | | | | | 100.00 | 97.45 | 96.88 | 96.60 | 96.32 | 96.03 | 91.45 | 88.72 | 88.01 | 82.54 | 82.74 |
| *Napmt2* | | | | | | 100.00 | 96.50 | 96.23 | 95.69 | 95.42 | 91.15 | 89.05 | 87.13 | 81.82 | 82.44 |
| *Nspmtb* | | | | | | | 100.00 | 99.76 | 96.80 | 96.53 | 91.74 | 89.35 | 87.43 | 81.34 | 82.14 |
| *Ntpmt4* | | | | | | | | 100.00 | 96.53 | 96.27 | 91.45 | 89.05 | 87.13 | 81.05 | 81.85 |
| *Ntpmt1* | | | | | | | | | 100.00 | 99.73 | 91.74 | 89.64 | 88.30 | 81.63 | 83.04 |
| *Ntpmtu* | | | | | | | | | | 100.00 | 91.45 | 89.35 | 88.01 | 81.34 | 82.74 |
| *Stpmt* | | | | | | | | | | | 100.00 | 92.10 | 91.27 | 84.50 | 83.89 |
| *Dipmt2* | | | | | | | | | | | | 100.00 | 87.87 | 83.18 | 83.03 |
| *Pdpmt* | | | | | | | | | | | | | 100.00 | 83.48 | 83.04 |
| *Aapmt2* | | | | | | | | | | | | | | 100.00 | 93.69 |
| *Abpmt2* | | | | | | | | | | | | | | | 100.00 |

**Fig 2.** A. Molecular phylogenetic analysis by Neighbor-Joining method. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.22981388 is shown. The tree is drawn to scale. The evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5. B. Amino acid percent identity matrix generated using Clustal 2.1. This matrix shows the conserved identity of *pmt* genes among solanales. The *pmt* genes and their accession number are *Nicotiana tabacum* (*Ntpmt2* -AF126809, *Ntpmt*-AF126811, *Ntpmt*-AF126812, *Ntpmt*-AF126810, *Ntpmt u*-D28506), *Nicotiana sylvestris* (*Nspmt* a-AB004322, *Nspmt b*- AB004324), *Nicotiana attenuata* (*Napmt2*-AF280403, *Napmt1*-AF280402), *Nicotiana benthamiana* (*Nbpmt* -EU165356), *Atropa belladona* (*Aapmt2* -EU670746, *Abpmt2*-AB018571), *Physalis divaricata* (*Pdpmt* -AM177611), *Datura innoxia* (*Dipmt2*-AM177610), *Solanum tuberosum* (*Stpmt* -AJ605553).

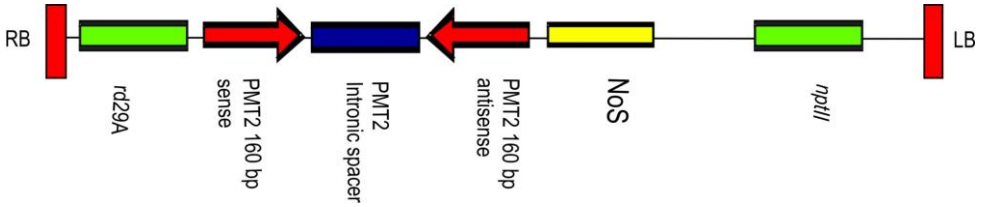



**Fig 3.** Design of RNAi vector p*pmt2 iRNAi*. The vector contains 162 bp of presiRNA in sense and antisense orientation separated by *pmt* intron. The presiRNA is transcribed by a stress responsive promoter rd29A cloned from *Arabidopsis thaliana*. The rd29A is a salinity, desiccation and freezing tolerance promoter predominantly expressed in roots of *Arabidopsis thaliana*.

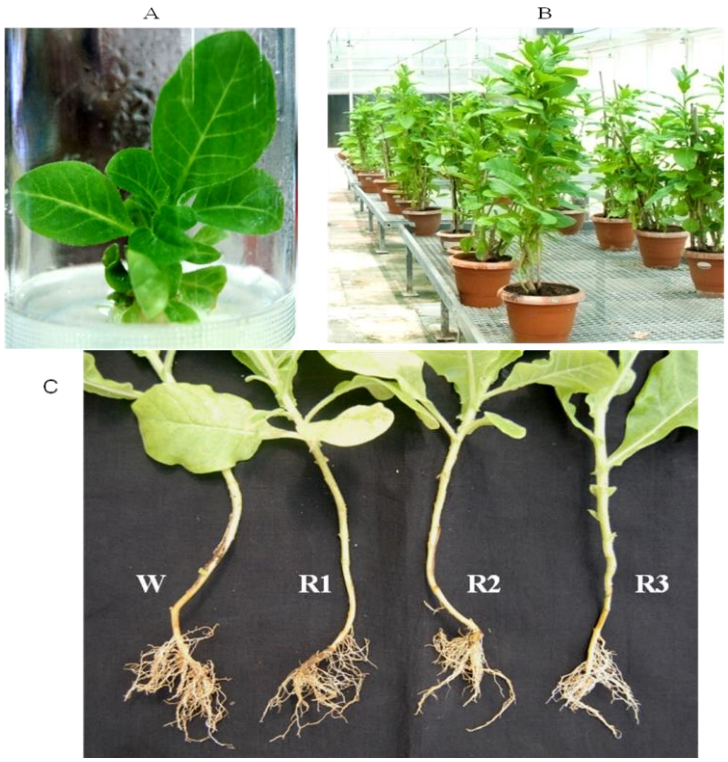



**Fig 4. (A)** Genetically modified RNAi plant in rooting media. (**B)** RNAi and wild type plants maintained in green house. **(C)** Induction of desiccation in wild type (W), and RNAi lines R1, R2 and R3. Uprooted plants were desiccated at room temperature for 48 hours and after 24 hours of desiccation the apical meristem was topped. Samples were collected from uninduced, induced and topped plants for analysis.

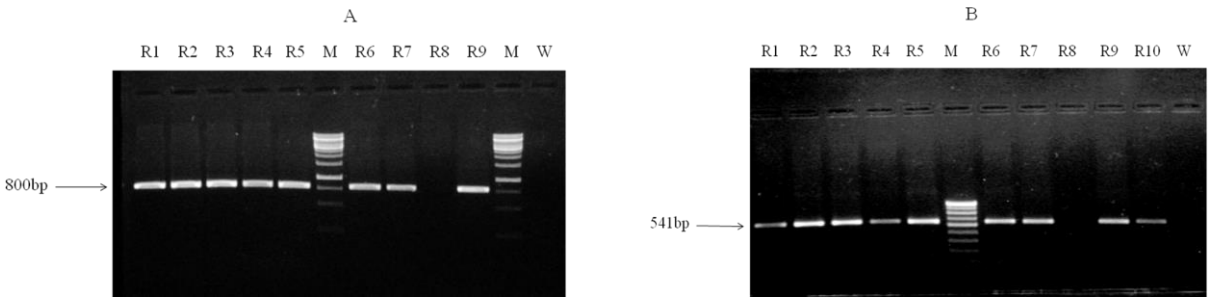


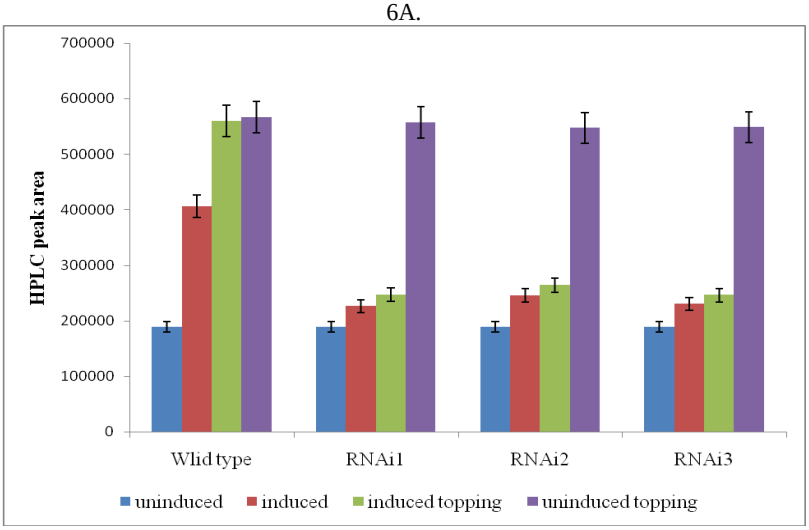

**Fig 5.** PCR confirmation of transgenic RNAi lines. Analysis of genetically modified $T_0$ and wild type plants for (A) plant transformation marker neomycin phosphotransferase (*nptII)* gene (B) presiRNA comprising of sense–intron–antisense (541bp). R– genetically modified RNAi plants, W–wild type tobacco plants, M– marker.

plant transformation. Plasmid pUC18 and pCAMBIA 2300 were used for cloning and construction of RNAi vector. The media and antibiotics used for bacterial cloning, plant transformation and plant culture are listed in Table 1.

***Design of pmt2 siRNA and construction of ppmt2 iRNAi vector***

Phylogenetic analysis was performed with *Nt*PMT2 protein sequence using MEGA5.2 (Saitou and Nei, 1987) and percent identity matrix was created with Clustal 2.1. Pairwise alignments were used to study sequence identity between *pmt* isogenes. The presiRNA sequence for *pmt2* was validated using IDT RNAi target finder (http://www.idtdna.com/scitools/applications/rnai/rnai.aspx) and cross targets if any was determined by BLAST. The primers used for the cloning of *pmt2* presiRNA and construction of iRNAi vector are given in Table 2. The gene specific presiRNA of 162 bp from *pmt2* exon1 was PCR amplified with primer set 1 and placed downstream of the promoter in the sense and antisense orientations between *Xba*I, *Bam*H1 and *Kpn*I, *Sac*I restriction sites respectively.

6A.




6B.

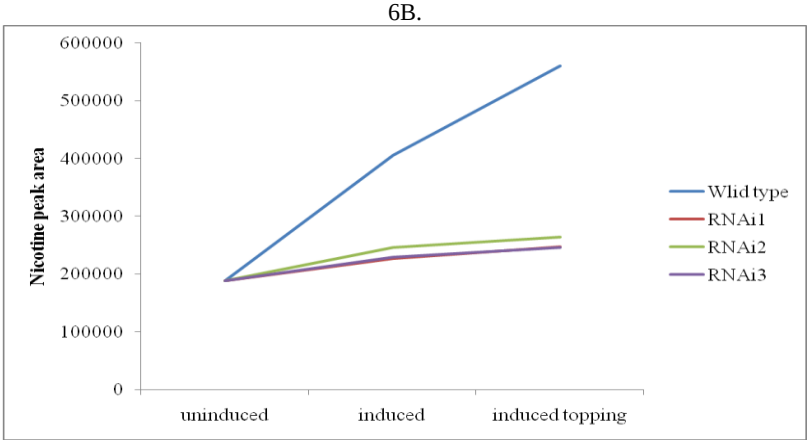



**Fig 6.** Accumulation of nicotine in wild type and genetically modified RNAi lines as determined by HPLC. (**A**) Nicotine levels were determined before desiccation (u), after desiccation (i), after desiccation and topping (it), and uninduced topping (ut). The values are means of triplicates. (**B**) Graphical representation of the significant variation in the biosynthesis of nicotine in tobacco among wild type and RNAi lines RNAi1, RNAi2, and RNAi3 due to desiccation stress and apical meristem topping.

Stress inducible rd29A promoter was PCR amplified using primer set 2 and cloned into *Hind*III and *Xba*I restriction site in pUC18. The *pmt*2 first intron of length 192 bp was cloned as intronic spacer using primer set 3 between *Bam*H1 and *Kpn*I. The NoS terminator was cloned between *Sac*I and *Eco*RI with primer set 4. This entire RNAi cassette in pUC18, *ppmt2* was moved in to pCAMBIA 2300, to make the RNAi vector p*pmt2 iRNAi* (Fig. 3). The p*pmt2 iRNAi* was sequenced in ABI 3130 XL genetic analyzer (Applied Biosystems) for sequence confirmation.

***Agrobacterium mediated tobacco transformation***

Agrobacterium culture in YEP broth was harvested at OD 0.5 at $A_{600}$ and was washed with MS wash buffer twice to remove antibiotics and re-suspended in 10ml of MSI broth. Tobacco leaf discs previously incubated in SM were infected with *Agrobacterium tumefaciens* harboring p*pmt2 iRNAi*. The infected leaf discs were co-cultivated in CM for 48 hours in dark. The co-cultivated leaf discs were washed with carbenicilin 250mg/l and regenerated in ReM. The regenerated transgenic plants were rooted in RoM. The RNAi lines were potted and maintained in transgenic greenhouse at standard growth conditions until further analysis.

***PCR confirmation of RNAi lines***

Young leaf tissue of wild type and RNAi tobacco (100mg) ground with preheated (1ml) CTAB buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was incubated at $60^0$C for 30 minutes with occasional shaking. This was extracted with equal volume of chloroform-isoamyl alcohol (24:1), and the supernatant precipitated with 0.7 volume of ice-cold isopropanol at room temperature for 20 minutes. The precipitated DNA pellet was air dried and re-suspended in 50µl of sterile water. The plants were confirmed for the presence of the RNA interference construct by PCR using gene specific primers (Table 2). Appropriate controls were maintained. The general PCR conditions used were $94^0$C for five minutes as initial denaturation, 35 cycles of $94^0$C for 30 seconds, $58^0$C for 30 seconds, $72^0$C for 30 seconds and a final extension of three minutes.

***Induction of desiccation stress in wild type and RNAi lines***

Three transgenic RNAi lines and wild type plants were desiccated by uprooting the plants.

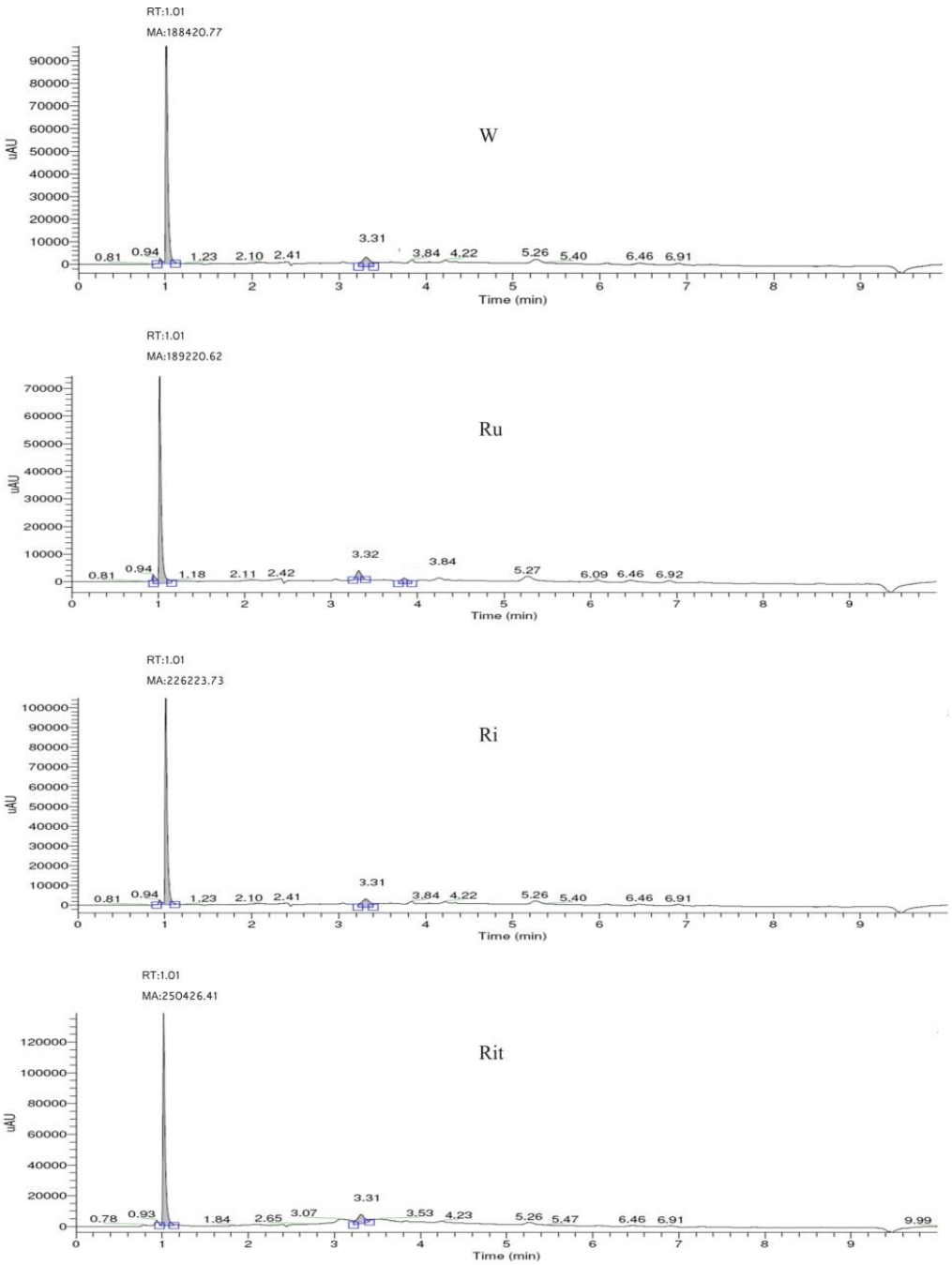



**Fig 7.** HPLC analytical profile for nicotine in wild type (W), RNAi line uninduced (Ru), desiccation induced (Ri), desiccated and apical meristem topped (Rit) tobacco plants. The profile show variation in the accumulation of nicotine but no detectable change in any other related secondary metabolite.

After 30 days of transplanting, wild type and RNAi lines were uprooted from the soil and left desiccated at room temperature for 48 hours (Fig. 4C). After 24hours of desiccation, leaf samples were collected from fourth or fifth leaf from the apex for nicotine analysis by HPLC. Later the apical meristem was topped in wild type and RNAi lines. To study the effect of topping on induced plants, samples were collected from the topped plants as before. Leaf samples were also collected from uninduced controls and uninduced topped controls.

***Quantitative analysis of nicotine levels in wild type and RNAi lines using HPLC***

The leaf samples dried to constant dry weight were powdered and 100 mg of the sample was extracted with 40% methanol in 20mM phosphate buffer pH 7.0 (James and Blume, 1981) and incubated in a shaker at $28^0$C overnight. The extract was filter sterilized in 0.22 µm filter for analysis. The analysis of nicotine was conducted using high performance liquid chromatography (HPLC, Prominence Shimadzu system with PDA detector) equipped with Zorbax XDB 75 mm × 4.6 mm, 3.5µm column.

The gradient mobile phase used was formic acid in water (0.1% v/v) and acetonitrile. The flow rate was 0.8 mL/min with column temperature $25^0$C and injection volume of 10 µl. Peak area was calculated according to the spectral characteristics at 254nm. Quantifications of nicotine were performed based on the concentration of the standard nicotine (N3876, Sigma Inc. USA).

***Statistical analysis***

The nicotine quantification experiments were carried out in triplicates. The mean, standard deviation and standard error for the concentration of nicotine was performed using SPSS. One way ANOVA was used to compare the means of the nicotine quantification experiments. The ANOVA was used to test the null hypothesis, that there exists no deviation in the means of the nicotine levels under various treatments among wild type and RNAi lines at alpha 0.05.

**Conclusion**

The genetically modified plants were as normal as the wild type in morphology and other biometric parameters without any detectable deleterious effects. Their nicotine levels are regulated on induction. No variation in secondary metabolite profile was observed in this study. RNA interference can be used to silence specific gene targets or a family of genes by designing appropriate siRNA. This attempt to regulate the level of nicotine in smoking tobacco opens new avenues to reduce toxins and anti-nutritional substances in palatable food upon induction.

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