A miRNA-eTM regulatory module for nicotine biosynthesis

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Regulation of Nicotine Biosynthesis by an Endogenous Target Mimicry of miRNA in Tobacco

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ABSTRACT

The interaction between non-coding endogenous target mimicry (eTM) and its corresponding miRNA is a newly discovered regulatory mechanism and plays pivotal roles in various biological processes in plants. Tobacco (*Nicotiana tabacum*) is a model plant for studying secondary metabolite alkaloids, of which nicotine accounts for ~90%. In this work, we identified four novel tobacco-specific miRNAs that were predicted to target key genes of the nicotine biosynthesis and catabolism pathways, and an eTM, nta-eTMX27, for nta-miRX27 that targets *QPT2* encoding a quinolinate phosphoribosyltransferase. The expression level of nta-miRX27 was significantly down-regulated while that of *QPT2* and nta-eTMX27 was significantly up-regulated after topping, and consequently nicotine content increased in the topping treated plants. The topping induced down-regulation of nta-miRX27 and up-regulation of *QPT2* were only observed in plants with a functional nta-eTMX27 but not in transgenic plants containing an RNA interference (RNAi) construct targeting nta-eTMX27. Our results demonstrated that enhanced nicotine biosynthesis in the topping treated tobacco plants is achieved by nta-eTMX27-mediated inhibition of the expression and functions of nta-miRX27. To our knowledge, this is the first report about regulation of secondary metabolite biosynthesis by a miRNA-eTM regulatory module in plants.
MicroRNAs (miRNAs) are a class of small non-coding RNAs with a typical length of 20-22 nucleotides (nt) and have been shown to play important roles in development, signal transduction and responses to biotic and abiotic stresses in plants (Phillips et al., 2007; Khraiwesh et al., 2012). MiRNAs are generated from single-strand RNA precursors (pre-miRNAs) with a stem-loop structure (Bartel, 2004). The pre-miRNA stem-loop structures are processed into miRNA/miRNA* duplexes by a protein-complex including Dicer-like 1 (DCL1, an RNase III-like endoribonuclease). The mature miRNA is then loaded onto the Argonaute (AGO)-containing RISC (RNA-induced silencing complex) to cause target mRNA degradation or translational repression through complementary sequence binding (Naqvi et al., 2012). Most plant miRNAs have perfect, or near-perfect complementarity with their targets (Axtell and Bowman, 2008; Mallory and Bouché, 2008); therefore, plant miRNA targets can usually be successfully predicted based on sequence complementarity between miRNAs and their targets (Rhoades et al., 2002; Sunkar and Zhu, 2004). A perfect base-pairing between miRNAs and their targets at the 9th to 11th positions from the 5’ end of miRNAs is important for effective miRNA-mediated cleavage of targets (Jones-Rhoades et al., 2006; Pasquinelli, 2012).

In Arabidopsis, a long non-protein-coding mRNA gene, **INDUCED BY PHOSPHATE STARVATION1 (IPS1)**, was found to be bound by miR399 with a three-nucleotide bulge in the middle of the miR399 binding site. This central mismatch disrupts crucial base-pairing between miR399 and **IPS1** and hence inhibits miR399-mediated cleavage of **IPS1**. This observation leads to the hypothesis that **IPS1** functions as a non-cleavable endogenous target mimicry (eTM) of miR399, which blocks the interaction between miR399 and its authentic targets by sequestering miR399 and arresting its cleavage activity (Franco-Zorrilla et al., 2007). Later, non-coding eTMs for diverse miRNAs have been identified in Arabidopsis, rice and soybean, and some of them have been shown to regulate plant development by repressing miRNA function (Todesco et al., 2010; Ivashuta et al., 2011; Wu et al.,
These studies also suggested that eTMs are widespread in plant species. Recent transcriptome sequencing studies have demonstrated the widespread of long non-coding RNAs in plants (Li et al., 2014b; Zhang et al., 2014); however, functions of the majority of those long non-coding RNAs are largely unknown. It would not be surprising to find a portion of these long non-coding RNAs acting as eTMs. Additionally, short tandem target mimic (STTM) technology have been developed with an aim to investigate functions of small RNAs by blocking small RNA functions in plants and animals (Tang et al., 2012a; Yan et al., 2012). STTM is a powerful technology complementing the target mimic in plants and the miRNA sponge in animals.

Alkaloids are a major type of secondary metabolites. Nicotine accounts for ~90% of the total alkaloid content and serves as defensive compounds against herbivores in tobacco (Saitoh et al., 1985; Baldwin et al., 2001). Meanwhile, nicotine is also the most important component in tobacco products due to its stimulatory and addictive effects. Nicotine is exclusively synthesized in tobacco roots and then transported to leaves through the xylem (Dewey and Xie, 2013). Nicotine alkaloids are accumulated in most *Nicotiana* species (Baldwin, 1999). The nicotine biosynthesis and catabolism pathways have been extensively studied (see a recent review by Dewey and Xie, 2013). Several key genes encoding enzymes of the nicotine biosynthesis pathway, such as quinolinate phosphoribosyltransferase (QPT), putrescine methyltransferase (PMT), N-methylputrescine oxidase and nicotine N-demethylase (CYP82E4) have been cloned and characterized (Conkling et al., 1990; Hibi et al., 1992; Siminszky et al., 2005; Katoh et al., 2006; Heim et al., 2007). QPT converts quinolinic acid to nicotinic acid mononucleotide (NAMN) and serves as the entry point into the pyridine nucleotide cycle that leads to the production of nicotinic acid and consequently nicotine (Dewey and Xie, 2013). QPT was firstly isolated from tobacco roots by Conkling et al. (Conkling et al., 1990) and later characterized by Song et al. (Song, 1997) and Sinclair et al. (Sinclair et al., 2000). *QPT2* is strongly and exclusively expressed in root tissue and involved in nicotine biosynthesis while its homologue *QPT1* is non-responsive to biotic and abiotic stresses and retains its original function.
in nicotinamide adenine dinucleotide (NAD) production (Dewey and Xie, 2013). Transgenic tobacco lines with a low level of nicotine content have been developed by suppression of QPT2 using the antisense-mediated gene silencing approach (Xie et al., 2004).

Recent reports have shown that miRNAs are involved in regulation of secondary-metabolite synthesis in plants. For example, miR163 can change the production profiles of secondary-metabolite in Arabidopsis (Ng et al., 2011); miR393 redirects the secondary-metabolite productions via perturbing auxin signalling (Robert-Seilanianz et al., 2011), and some miRNAs might be involved in biosynthesis of benzylisoquinoline alkaloids in opium poppy (Boke et al., 2015). In tobacco, it has been showed that miR164 and its target NtNAC-R1, a novel NAC transcription factor identified in tobacco, was down- and up-regulated in response to topping, respectively, and resulting in increase of lateral roots and nicotine contents (Fu et al., 2013). However, it is still unclear whether miRNAs are directly involved in regulation of the nicotine biosynthesis pathway in tobacco.

To determine the roles of miRNAs in nicotine biosynthesis, in this study, we first identified nicotine biosynthesis related miRNAs in two small RNA populations generated from topping-treated tobacco roots using the newly available tobacco genome sequence as a reference (Sierro et al., 2014). We found four novel miRNAs that target several key genes of the nicotine biosynthesis and catabolism pathways. Further investigation on nta-miRX27, which targets QPT2, by overexpression and inhibition of the miRNA function confirmed that nta-miRX27 played an important role in regulation of tobacco plants in response to topping and nicotine accumulations. More interestingly, we identified an eTM (nta-eTMX27) for nta-miRX27 and proved that it can effectively inhibit the functions of nta-miRX27 and regulate nicotine biosynthesis in the topping treated tobacco plants.
RESULTS

Identification of Nicotine Biosynthesis Related miRNAs in *N. tabacum*

The recently available genome sequences of tobacco and its two progenitors provided an opportunity to identify miRNAs related to nicotine biosynthesis in tobacco. Based on the small RNA data generated from roots of control and topping-treated tobacco plants by deep sequencing in our previous study (Tang et al., 2012b), we identified a number of novel miRNAs that have not been reported previously, including four that were predicted to target genes (*QPT1*, *QPT2*, *PMT2* and *CYP82E4*) involved in nicotine biosynthesis (Fig. 1A; Supplementary Table 1). These four miRNAs seemed to be tobacco-specific as no homologous sequence was found in the miRBase database.

Previous studies demonstrated that there was a significant increase in the activity of several nicotine biosynthetic enzymes in the tobacco roots at 24–48 hours (h) after topping (Dewey and Xie, 2013). To test the effects of topping on nicotine biosynthesis related miRNAs and their target genes, we measured their expression changes, as well as *NtNAC_148* (targeted by nta-miRX19) that has been shown to be down-regulated upon topping treatment based on our unpublished data, using samples collected at 48 h after topping. As expected, *NAC_148* was down-regulated significantly after topping, which is most likely caused by up-regulation of nta-miRX19 (Fig. 1, B and C). The expression levels of nta-miRX17 and nta-miRX27 were down-regulated upon topping, and consequently their targets *QPT1* and *QPT2* were up-regulated. The negatively correlated relationship was much striking for nta-miRX27 and *QPT2* (Fig. 1, B and C). By contrast, topping seemed to have no effect on the expression of nta-miRX20, nta-miRX13 and their targets *CYP82E4* and *PMT2*, respectively. *CYP82E4*, which is responsible for nicotine to nornicotine demethylation (Dewey and Xie, 2013), has been shown to be not topping responsive. These results suggested that the nta-miRX27-*QPT2* interaction may be important for regulation of nicotine biosynthesis in tobacco.

The reversely correlated expression relationship between nta-miRX27 and its target
QPT2 suggests nta-miRX27-mediated cleavage of QPT2. Two binding sites of nta-miRX27 could be predicted on the QPT2 gene. The first one is located at exon #2 and the second one is located at the intron between exons #8 and #9 (based on AJ748263 deposited in the NCBI) or at exon #8 (based on Fgenesh prediction, www.softberry.com). We used RNA Ligase Mediated Rapid Amplification of 5’ cDNA Ends (RLM-RACE) to confirm nta-miRX27-mediated cleavage of QPT2 but failed for both sites (Supplementary Fig. S1), although the first one was confirmed to be cleaved based on our previous degradome data (Supplementary Fig. S2).
Regulation of nta-miRX27 on Endogenous QPT2 Expression and Nicotine Accumulation

To investigate whether nta-miRX27 functions to repress expression of its target QPT2 and plays any role in nicotine production, we generated transgenic tobacco plants overexpressing or silencing nta-miRX27. The silencing transgenic plants (nta-STTMX27) were generated using the STTM (short tandem target mimic) strategy (Fig. 2A). The expression levels of nta-miRX27 in the overexpressing and silencing transgenic plants were confirmed to be significantly up- and down-regulated, respectively (Fig. 2C). The expression changes of nta-miRX27 in all transgenic plants did not cause observable phenotypic changes (Fig. 2B), consistent with the expected effects of nta-miRX27. The expression level of QPT2 was decreased by ~50% in the nta-miRX27 transgenic plants compared to the control plants, but a significantly increased level of QPT2 was observed in the nta-STTMX27 transgenic plants (Fig. 2C). Consistent to these expression changes of QPT2, decreased and increased nicotine contents were observed in the nta-miRX27 and nta-STTMX27 transgenic plants, respectively. The nicotine content in the leaves of the nta-STTMX27 and nta-miRX27 transgenic plants was 9.17±1.29 mg g⁻¹ and 2.17±1.15 mg g⁻¹, respectively (Fig. 2D). These results strongly suggested that nta-miRX27-mediated regulation of its target QPT2 plays an important role in nicotine biosynthesis in tobacco.

Identification and Expression Analysis of eTMs for nta-miRX27

To determine potentially additional regulators related to the nat-miRX27-QPT2 module involved in regulation of nicotine biosynthesis, we performed eTM identification using various available transcripts, including those in-house assembled using available tobacco RNA-Seq data, and identified an eTM for nta-miRX27, which was named as nta-eTMX27. The putative transcript of nta-eTMX27 was 1,213 base pair (bp) long with the largest predicted open reading frame being 144-bp in length. It was covered by several ESTs (FS399281, FS416548 and FG201217) and at least one
EST (FG201217) covered the target mimic site of nta-eTMX27 although a few single
nucleotide polymorphisms were found between nta-eTMX27 and FG201217. The
binding site of nta-miRX27 in nta-eTMX27 contains a three-nucleotide bulge between

Figure 2. Nta-miRX27 repressed QPT2 expressions and reduced nicotine contents. A. Diagram of nta-miRX27 and nta-STTMX27 structures showing the design strategy. Nta-miRX27 gene and two tandem target mimics (STTMX27) spaced by a 48-nucleotide (nt) imperfect stem-loop linker were cloned between a cauliflower mosaic virus 35S promoter (P-35S) and NOS terminator (T-NOS) to produce nta-miRX27 and nta-STTMX27 construct, respectively. Red indicates the nta-miRX27 gene; green indicates the spacer region and the spacer sequence, blue indicates the mature nta-miRX27 sequences and orange indicates the bulge sequences in the miRNA binding sites. B. Phenotypes of 30-day-old transgenic plants containing nta-miRX27 or nta-STTMX27 compared with that of plants transformed with an empty vector (Vec). Two independent and representative transgenic lines overexpressing nta-miRX27 (nta-miRX27-11# and nta-miRX27-16#) and silencing nta-miRX27 (nta-STTMX27-6# and nta-STTMX27-23#) were shown. Vec represents the transgenic plant transformed with an empty vector. C. Relative expression levels of nta-miRX27 and its target gene QPT2 in nta-miRX27 and nta-STTMX27 transgenic plants compared with that of the Vec plants. Three individual plants in per genotype were used in RT-qPCR, and bars represent SD of three replicates. D. Average nicotine contents in leaves sampled from the Vec, nta-miRX27 and nta-STTMX27 plants. Each mean value was derived from three independent experiments (n=9). Error bars indicate the SD of three replicates. Significance tests were performed by the Student’s t test. A single asterisk indicates a significant difference (P<0.05) and double asterisks indicate a highly significant difference (P<0.01) between the two paired samples.
Moreover, according to the degradome data generated previously (Tang et al. 2012b), no degradomic read was mapped to the target mimic site of nta-eTMX27, supporting the notion that nta-eTMX27 may act as a decoy for nta-miRX27. Nta-eTMX27 was found to be expressed in root, stem and leaf tissues but not in flowers (Fig. 3B).

To determine whether the predicted nta-eTMX27 plays a role in repressing miRNA function, its expression changes together with that of nta-miRX27 and its target QPT2 were analyzed in roots of the tobacco plants sampled at 3-48 h after the topping treatment (Fig. 3C). Apparently, the expression level of nta-eTMX27 increased steadily after the topping treatment and was ~9-folds higher than that of the control (0 h after topping) at 48 h after the topping treatment (Fig. 3C, the first column). Similarly, the expression level of QPT2 was also up-regulated after topping (Fig. 3C, the second column), while the expression level of nta-miRX27 was down-regulated during the same time period after the topping treatment (Fig. 3C, the third column). These results indicate that topping treatment induces nta-eTMX27 expression, which leads to down-regulation of nta-miRX27 and increase of QPT2 expression.
**Nta-eTMX27 Induced miRNA Degradation and Repressed miRNA Function**

To confirm the function of nta-eTMX27, *i.e.* sequestration of nta-miRX27 away from its target *QPT2*, we generated transgenic tobacco plants overexpressing nta-eTMX27 or harboring a RNA interference (RNAi) construct (ds-nta-eTMX27) targeting nta-eTMX27. Under the greenhouse conditions, all transgenic plants grew normally and did not show obvious altered phenotypes compared to the plants transformed with the empty vector (Vec, Fig. 4A). RT-qPCR assay showed that the expression level of nta-eTMX27 was approximately 3-folds higher in the nta-eTMX27 overexpressing lines than in the vector-only (Vec) lines, while the expression levels of nta-miRX27 and *QPT2* were reduced by ~50% and increased by more than two folds, respectively (Fig. 4B). In the nta-eTMX27 RNA interference (RNAi) transgenic plants (ds-nta-eTMX27), the expression level of nta-eTMX27 was reduced to approximately 23% of the level in the Vec plants. As a result, the expression levels of nta-miRX27 and *QPT2* were increased and decreased, respectively (Fig. 4B). In addition, the nicotine contents in the nta-eTMX27 and ds-nta-eTMX27 transgenic lines were significantly increased and decreased, respectively (Fig. 4C). These results not only confirmed the regulatory module involving nta-miRX27, *QPT2* and nta-eTMX27 but indicate that the nicotine contents in tobacco plants can be manipulated by overexpression or silencing of nta-eTMX27.

The opposite functions of nta-miRX27 and nta-eTMX27 in regulating *QPT2* transcript levels and nicotine biosynthesis led us to postulate that nta-eTMX27 may induce nta-miRX27 degradation and repress miRNA function in response to external stimuli, such as topping, during plant growth and development. To test this possibility, we compared the expression changes of nta-miRX27 and *QPT2* in the transgenic plants containing the Vec or ds-nta-eTMX27 construct after the topping treatment. As expected, expression of nta-miRX27 was down-regulated while expression of *QPT2* and nta-eTMX27 was up-regulated in the Vec plants (Fig. 4, D, E and F); however, these changes were not observed in the ds-nta-eTMX27 transgenic plants (Fig. 4, D, E and F). These results indicate that nta-eTMX27 seems to be essential for...
topping-induced down-regulation of nta-miRX27 and that nta-eTMX27 may play a role in repressing the function of nta-miRX27 by degradation under stress conditions.

Evolution of nta-miRX27 and nta-eTMX27

No ortholog of the primary transcript of nta-miRX27 or the nta-miRX27 mature sequence could be found in the current tomato genome (http://solgenomics.net/) and
miRBase, suggesting that nta-miRX27 may be Nicotiana-specific. A near identical sequence of nta-miRX27 was found in *N. sylvestris* but not in *N. tomentosiformis* (Fig. 5A), suggesting that nta-miRX27 was most likely derived from the progenitor *N. sylvestris*. Meanwhile, *QPT2* of common tobacco also showed more sequence similarity, including the presence of the nta-miRX27 binding site, with its ortholog in *N. sylvestris*. In contrast, no miRNA and miRNA target site could be predicted in the ortholog of *QPT2* and its surrounding region in *N. tomentosiformis* because of nucleotide mutations, implying that the nta-miRX27-*QPT2* regulatory module might have been only evolved in the progenitor *N. sylvestris* but not in *N. tomentosiformis*. Interestingly, an almost identical sequence of nta-eTMX27 was found in *N. tomentosiformis* but not in *N. sylvestris* (Fig. 5A). Compared to common tobacco and *N. tomentosiformis*, *N. sylvestris* had one nucleotide substitution and an indel (insertion/deletion) in the target mimic site of the sequence corresponding to nta-eTMX27 (data not shown). Consequently the nta-miRX27-nta-eTMX27 interaction could not be predicted in *N. sylvestris* genome based on our current bioinformatic pipeline.
**DISCUSSION**

Role of miRNAs in secondary metabolite biosynthesis has been demonstrated in plants (Ng et al., 2011; Robert- Seilaniantz et al., 2011), and potential regulation of alkaloid biosynthesis by miRNA in opium poppy has also been suggested (Boke et al., 2015). Target mimicry has emerged as a new mechanism regulating function of miRNAs and a number of long non-coding RNAs acting as eTMs to block the biological function of miRNAs have been identified in plants (Franco-Zorrilla et al., 2007; Todesco et al., 2010; Ivashuta et al., 2011; Meng et al., 2012; Wu et al., 2013; Ye et al., 2014). In Arabidopsis, besides of IPS1, the first validated functional eTM in plants, eTMs for miR160 and miR166 have also been shown to play an important role in regulation of plant development, which was possibly achieved by eTM-induced miRNA degradation (Wu et al., 2013). In this study, we showed evidence that topping could induce the expression changes of nicotine-related miRNAs and their targets. For example, the expression levels of nta-miRX27 and its target QPT2, one of the key genes of the nicotine biosynthesis pathway, were down- and up-regulated in the topping treated tobacco plants, respectively. Using transgenic plants overexpressing or silencing nta-miRX27, we demonstrated that nta-miRX27 directly regulates expression of QPT2, and consequently the accumulation of nicotine. Furthermore, we identified a long non-coding RNA, i.e. nta-eTMX27 that contains a non-cleavable nta-miRX27 binding site. Similar to QPT2, the expression of nta-eTMX27 was induced in response to topping. As reported previously in other plant species (Wu et al., 2013), topping-induced down-regulation of nta-miRX27 and up-regulation of QPT2 in tobacco observed in this study (Fig. 3C) might be a result of nta-miRX27 degradation caused by up-regulated nta-eTMX27. This notion was confirmed using transgenic plants containing a hairpin construct targeting nta-eTMX27, in which no topping-induced down-regulation of nta-miRX27 and up-regulation of QPT2 were observed. Our results demonstrated a crucial role of nta-eTMX27 in regulation of nicotine biosynthesis by acting as a decoy of nta-miRX27 to sequester and degrade nta-miRX27. To our knowledge, this is the first report showing that eTM played a role
in biosynthesis of secondary metabolites in plants.

Two binding or cleavage sites of nta-miRX27 were predicted on the *QPT2* gene with a predicted targeting score of 4.5 and 2.5, respectively. The first predicted binding site at exon #2 was confirmed by degradome data (Supplementary Fig. S1 and S2); however, our further RLM-RACE experiments failed to confirm nta-miRX27-mediated cleavage at both sites. We repeated the RLM-RACE experiment a couple of times with different primers. For the second binding site, we used primers based on both the cDNA and the intron sequence down-stream of the predicted binding site which is located at an intron based on the *QPT2* gene (AJ748263) deposited in the NCBI. For both sites, the RLM-RACE products were mapped to the flanking regions of the predicted cleavage sites of nta-miRX27 but not at the predicted cleavage sites (Supplementary Fig. S1). The distribution pattern of the 5’ ends of the RACE products suggests that the clones we sequenced are randomly degraded products, although similar distribution pattern of 5’ RACE products has been observed for some conserved but lowly expressed miRNAs (Shen et al., 2014; Chen et al., 2015). We cannot rule out the possibility that we failed to amplify and clone the right cleavage products due to technical issue, but it is most likely that the fragmented *QPT2* resulted from nta-miRX27-mediated cleavage is very susceptible to degradation induced by RNA decay proteins, such as nuclear exoribonucleases (5’-3’) and exosome complex (3’-5’). In addition, we cannot exclude the possibility that nta-miRX27 targets un-processed *QPT2* in the nucleus because the second predicted nta-miRX27 binding site is located at intron of alternatively spliced *QPT2*. If that is case, nta-miRX27 could be involved in regulation of *QPT2* by interfering its splicing and/or mRNA maturation. Whatever the possible reason for the negative RACE result, the results observed in transgenic plants clearly suggest the transcript level of *QPT2* is regulated by nta-miRX27. Nevertheless, it is of our interest to perform further experiment to find out the mechanism underlying nta-miRX27-mediated regulation of *QPT2*.

Among various approaches for functional characterization of miRNAs, STTM technology has received more attention due to its high efficiency in suppressing
miRNA functions using the target mimicry mechanism (Yan et al., 2012). In our work, overexpression of nta-STTMX27 could reduce the nta-miRX27 levels to approximately 20% of the control levels and produced transgenic lines with the highest contents of nicotine in this study. This result confirmed the efficiency of the technology and also suggested that nta-miRX27 indeed played an important role in nicotine biosynthesis. Transgenic plants overexpressing and silencing nta-cTMX27 showed increased and reduced accumulation of nicotine, respectively, which was consistent with its function in repressing nta-miRX27 and facilitating expression of QPT2.

Common tobacco is a model plant organism for studying diverse fundamental biological processes, such as disease susceptibility and secondary metabolites. Tobacco has rich secondary metabolites (>4,000 chemical components) and prompted numerous studies on biologically active metabolic substances (Sierro et al., 2014). For example, the alkaloid biosynthesis pathway received much attentions of scientists and has been well studies in the past thirty years (Dewey and Xie, 2013). A gene network including over ten nodes or protein-coding genes has been characterized for the pathway. Recent studies indicated that some transcription factors, such as NAC, ERF and MYC, might also be indirectly involved in alkaloid biosynthesis via regulating plant hormones (Fu et al., 2013; Zhang et al., 2011). In this study, additional regulator, i.e. non-coding RNA acting as miRNA or miRNA decoy, was demonstrated to be directly involved in regulation of the key genes of the alkaloid biosynthesis pathway. We believe that more such non-coding genes will be identified for the pathway in the future. In this sense, our study opened new channel for investigation of the genetic network related to alkaloid biosynthesis in plants.

Modern tobacco is a natural amphidiploid whose genomes originated from the hybridization of two wild progenitors N. tomentosiformis and N. sylvestris (Dewey and Xie, 2013). It is a relatively new species, but experienced a dramatically domestication selection after the hybridization of two progenitors. For example, over time, loss-of-function mutations in the major nicotine demethylase genes have been selected that enabled modern tobacco to accumulate nicotine rather than nornicotine.
(Gavilano et al., 2007). By contrast, the normal demethylase genes in the two progenitors made them to accumulate a higher level of nornicotine content but not nicotine. According to our phylogenetic analysis results, we believe that the regulatory module of nta-miRX27-\textit{QPT2}-nta-eTMX27 was only evolved in \textit{N. tabacum} after the hybridization of its two progenitors, \textit{N. tomentosiformis} and \textit{N. sylvestris}, but not in the progenitors themselves (Fig. 5B). \textit{QPT2} plays a key role in biosynthesis of nicotine, one of the main agronomic traits in tobacco under artificial selection. Evolution of the nta-eTMX27-nta-miRX27-\textit{QPT2} regulatory module in modern tobacco could be driven by domestication and subsequently intensive genetic improvement that aimed to increase the nicotine content.
MATERIALS AND METHODS

Plant Materials and Treatment

All root samples were collected from tobacco (*N. tabacum*) cultivar Hicks Broad. Tobacco plants were grown at 25°C and 65% humidity in a growth chamber with 16 h of light and 8 h of dark. At least three 60-day-old (days-after-seeding) plants were used for topping treatment, and plants with a similar size without topping treatment were used as control. After topping treatment, the plants were kept for another 3-48 hours in the growth chamber before sample collection.

Identification of miRNAs, their Targets and eTMs

The tobacco small RNA dataset and the approach reported in our previous study (Tang et al., 2012b) were used to identify miRNAs based on the newly available tobacco reference genome (Sierro et al., 2014). Targets of miRNAs were predicted using the web-based tool psRNATarget with the default settings (Dai and Zhao, 2011) and the tobacco degradomic dataset were further used to confirm the predictions using the method in our previous study (Tang et al., 2012b). Genes, *i.e.* *QPT1* (AJ748262), *QPT2* (AJ748263) and *CYP82E4* (KC120817), involved in nicotine biosynthesis were based on previous studies (Dewey and Xie 2013; Sierro et al. 2014). *NAC_148* (http://compsysbio.achs.virginia.edu/tobfac/; XM_009626731; Rushton et al. 2008), a NAC transcription factor that was responsive to topping in tobacco based on our unpublished results, was used as a positive control for the topping treatment in this study. Based on the publicly available EST and Plant Genome Database (PlantGDB)-assembled unique transcript databases and transcriptome (RNA-Seq) data of tobacco, we identified an eTM for nta-miRX27 in intergenic region, which was named as nta-eTMX27. The pipeline developed by our previous study (Ye et al., 2014) was used to predict eTMs for the miRNAs.

Phylogenetic Tree

Sequences of nta-miRX27 and its target *QPT2* and eTM (nta-eTMX27) were used
as queries to search for their orthologs in the available genome sequences of two
progenitors \textit{N. tomentosiformis} and \textit{N. sylvestris} as well as other two \textit{Nicotiana}
species \textit{N. benthamiana} and \textit{N. otophora} (Bombarely et al., 2012; Sierro et al., 2013;
Sierro et al., 2014). Phylogenetic trees were constructed using NJ method in MEGA6
with bootstrap 1000 (Tamura et al., 2013).

\textbf{Plasmid Constructs and Plant Transformation}

The precursor sequence of nta-miRX27 was amplified from tobacco root DNA
using a pair of primers containing \textit{Bam}H I and \textit{Sal} I site, respectively. To generate the
nta-STTMX27-containing construct, a pair of back-to-back primers each containing a
nta-miRX27 binding site with a central bulge, the 48-nt oligonucleotide spacer
sequence and a \textit{Bam}H I or \textit{Sal} I restriction site (Supplemental Table S2) were used in
PCR to generate a fragment containing two nta-miRX27 binding sites with the 48-nt
spacer in between. The fragment harboring nta-miRX27 or nta-STTMX27 was then
sub-cloned into the binary vector pCHF3 between the \textit{Bam}H I and \textit{Sal} I sites, and
downstream of the Cauliflower mosaic virus (CaMV) 35S promoter to produce
pCHF3-35S-nta-miRX27 and pCHF3-35S-nta-STTMX27, respectively.

The nta-eTMX27 was amplified from tobacco root cDNA using a pair of primer
eTMX27-Full-F/eTMX27-Full-R, and the PCR products were sub-cloned into the
binary vector pCHF3 to produce pCHF3-35S-nta-eTMX27. An RNAi construct
containing an nta-eTMX27 inverted repeat sequences that were spaced by a soybean
intron was produced by overlapping PCR. The fragment of the nta-eTMX27 sense
sequence was amplified with eTMX27-A-F/eTMX27-intron-A-R primer pair and
overlapped with intron sequence amplified by eTMX27-intron-B-F/Intron-B-R
primers. The overlapping product was cloned into pCHF3 between the \textit{Sac} I and
\textit{Bam}H I sites to produce pCHF3-35S-nta-eTMX27-intron. The corresponding
antisense nta-eTMX27 fragment was amplified with the primer pair
eTMX27-C-F/eTMX27-C-R and subsequently cloned into
pCHF3-35S-nta-eTMX27-intron between \textit{Bam}H I and \textit{Sal} I sites to produce the RNAi
construct pCHF3-35S-dsNta-eTMX27. The \textit{Agrobacterium}-mediated \textit{N. tabacum} leaf
disc transformation method was used to generate transgenic tobacco plants. Selection
(on 200 μg/ml kanamycin media) of transformants and positive transgenic plants were
performed as described previously (Li et al., 2014a). Primers used in PCR and
real-time quantitative PCR (RT-qPCR) analyses were listed in Supplemental Table S2.

**Real-Time Quantitative PCR (RT-qPCR) Analysis**

Total RNA was isolated from root samples of the control and topping-treated plants
as well as various plant organ tissues using the Trizol reagent (Invitrogen, America). Three independent experiments, each experiment consists of at least three control and
topping-treated plants, were used in quantification analyses. Expression levels of
miRNA in root tissues were analyzed by RT-qPCR as described (Shi and Chiang,
2005). Briefly, total RNA (1 μg) treated with RNase-free DNase I (Fermentas, EU, St.
Leon-Rot, Germany) was polyadenylated using *Escherichia coli* poly(A) polymerase
(NEB, Ipswich, MA). After phenol-chloroform extraction and ethanol precipitation,
the RNAs were dissolved in RNase-free water. Reverse transcription (RT) was
performed at 42°C for 30 min using a poly(T) adapter and Quantscript reverse
transcriptase according to the manufacturer’s instructions (Tiangen). RT-qPCR
reactions were performed using a LightCycler 480 real-time PCR instrument (Roche,
Rotkreuz, Switzerland) and SYBR Green I Master kit using a forward primer
complementary to the miRNA and a universal reverse primer (miRNA-qPCR-R)
complementary to the poly(T) adapter. Mitochondrial 5S RNA was used as an internal
control for data normalization.

Quantification of the target genes and eTM was also carried out using RT-qPCR
using a LightCycler 480 real-time PCR instrument. One μg of DNase I-treated total
RNA was used in generating the first strand cDNA using an oligo-dT primer and a
reverse transcription kit with gDNase (Tiangen, KR106). The expression levels of
nta-eTMX27 in different plant tissues were analyzed by semi-quantitative PCR.
*NtGAPDH* was used as a control in both qPCR and semi-qPCR.

**RNA ligase-mediated rapid amplification of 5’ cDNA ends (RLM-5’ RACE)**
Total RNA was extracted from topping-treated plants at 48 h using a Trizol reagent (Invitrogen, America) as recommended by the manufacturer followed by further purification with an RNeasy Plant Mini Kit (QIAGEN, Germany). The purified RNA was treated by DNase I (Thermo Scientific, America) to eliminate possible DNA contamination, and extracted using a standard phenol-chloroform method followed by ethanol precipitation. Amplification of 5’ cDNA ends (5’-RACE) was performed using the DNase-treated RNA and the SMARTer™ RACE cDNA Amplification Kit (Clontech, America). Reverse primers QPT2-1R-new or QPT2-2R-new (for the first nta-miRX27 target site) and QPT2-3R or QPT2-4R (for the second nta-miRX27 target site) were used in the respective reverse transcription reactions. Reverse transcribed cDNAs were then used in the first round PCR reactions using the corresponding primer used in reverse transcription (i.e. QPT2-2R-new for the first nta-miRX27 target site, and QPT2-4R for the second nta-miRX27 target site) in combination with the Universal Primer A Mix (UPA) that anneals to the 5’ adaptor. The nested PCR was performed according to the manufacturer’s instructions (Clontech, America) using a nested primer (i.e. QPT2-1R-new for the first nta-miRX27 target site, and QPT2-3R for the second nta-miRX27 target site) in combination with the 5’-Nested Universal Primer (5’-NUP). PCR products with expected size were gel purified and cloned to pMD-18T vector (TakaRa, Japan). Clones with insert were sequenced using M13 primer. Sequencing results were analyzed using CLUSTALW (Thompsonet et al., 1994). Primers used in 5’ RACE were listed in Supplemental Table S2.

Nicotine Measurement

Fresh leaves were collected from the same positions of the topping-treated and control tobacco plants, and dried at 105°C for 30 minutes and then 60°C for three days. Nicotine contents of the transgenic and control lines were measured in three biological samples each with three technical replicates following the standard continuous flow protocol (YC/T160-2002) described by the State Tobacco Monopoly Administration of China.
Figure 1. Genes involved in nicotine biosynthesis in tobacco and expression changes of miRNAs and their nicotine-related targets at 48 hours (h) after topping. A, A schematic diagram of the nicotine biosynthesis pathway in *N. tabacum* (adapted from Dewey and Xie, 2013). A622: isoflavone reductase-like protein; BBL: berberine bridge enzyme-like; MPO: N-methylputrescine oxidase; CYP82E4: nicotine N-demethylase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate phosphoribosyltransferase. B, Alignment of nta-miRNAs and their targets. Base pairing between miRNA and its target are shown, in which a vertical line means a Watson-Crick pair, two dots represent a G-U pair and 0 means a mismatch. C, RT-qPCR analyses of nta-miRX17, nta-miRX27, nta-miRX20 and nta-miRX19 at 48 h after topping. Mitochondrial 5S RNA was served as an internal standard for expression normalization. D, RT-qPCR analyses of miRNA target genes (*QPT1, QPT2, CYP83E4* and *NAC_148*) at 48 h after topping. *GAPDH* mRNA was served as an internal standard for expression normalization. Three independent experiments, each consisting of three control and topping-treated plants, were carried out for quantification analyses, and representative results in one time are presented. The expression levels of miRNAs and their targets in plants without topping treatment (control) are arbitrarily set as 1. Error bars indicate standard deviation (SD). Student’s *t* tests were performed to compare differences of miRNAs and their targets between the control and the topping treatment. A single asterisk indicates a significant difference (*P*<0.05) and double asterisks indicate a highly significant difference (*P*<0.01) between the two paired samples.

Figure 2. Nta-miRX27 repressed *QPT2* expressions and reduced nicotine contents. A, Diagram of nta-miRX27 and nta-STTMX27 structures showing the design strategy. Nta-miRX27 gene and two tandem target mimics (STTMX27) spaced by a 48-nucleotide (nt) imperfect stem-loop linker were cloned between a cauliflower mosaic virus 35S promoter (P-35S) and NOS terminator (T-NOS) to produce
nta-miRX27 and nta-STTMX27 construct, respectively. Red indicates the nta-miRX27 gene, green indicates the spacer region and the spacer sequence, blue indicates the mature nta-miRX27 sequences and orange indicates the bulge sequences in the miRNA binding sites. B, Phenotypes of 30-day-old transgenic plants containing nta-miRX27 or nta-STTMX27 compared with that of plants transformed with an empty vector (Vec). Two independent and representative transgenic lines overexpressing nta-miRX27 (nta-miRX27-11# and nta-miRX27-16#) and silencing nta-miRX27 (nta-STTMX27-6# and nta-STTMX27-23#) were shown. Vec represents the transgenic plant transformed with an empty vector. C, Relative expression levels of nta-miRX27 and its target gene QPT2 in nta-miRX27 and nta-STTMX27 transgenic plants compared with that of the Vec plants. Three individual plants in per genotype were used in RT-qPCR, and bars represent SD of three replicates. D, Average nicotine contents in leaves sampled from the Vec, nta-miRX27 and nta-STTMX27 plants. Each mean value was derived from three independent experiments (n=9). Error bars indicate the SD of three replicates. Significance tests were performed by the Student’s t test. A single asterisk indicates a significant difference ($P<$0.05) and double asterisks indicate a highly significant difference ($P<$0.01) between the two paired samples.

Figure 3. Topping-induced expression of endogenous nta-eTMX27 in tobacco root. A, The predicted base-pairing pattern between nta-miRX27 and its eTM nta-eTMX27. B, Semi-quantitative RT-PCR analysis of expressions of nta-eTMX27 in four tissues. NiGAPDH was used as control. RT- represents RT negative control, i.e. RT without reverse transcriptase. C, The relative transcription levels of nta-eTMX27, QPT2 and nta-miRX27 in N. tabacum root tissues measured at 3, 6, 12, 24 and 48 h after topping. Three independent experiments, each consisting of three control and topping-treated plants, were carried out for quantification analyses, and representative results in one time are presented. The expression level at the 0 h time point (control) was arbitrarily set as 1. Error bars indicate the SD.
Figure 4. Functional analyses of nta-eTMX27. A, Phenotypes of nta-eTMX27 overexpression and silencing plants. Two independent and representative transgenic lines overexpressing nta-eTMX27 (nta-eTMX27-2# and nta-eTMX27-5#) and silencing nta-eTMX27 (ds-nta-eTMX27-4# and ds-nta-eTMX27-9#) were shown. B, Relative expression levels of nta-eTMX27, nta-miRX27 and QPT2 in nta-eTMX27 overexpressing and silencing transgenic plants compared with that of the Vec plants. Three individual plants per genotype were used in RT-qPCR. Bars show SD. C. Average leaf nicotine contents of the Vec, nta-miRX27 and nta-STTMX27 lines. A single asterisk indicates a significant difference ($p<0.05$) between the nta-miRX27 or nta-STTMX27 line and the Vec control. Error bars indicate the SD. D, Expression levels of nta-eTMX27 in roots of transgenic plants transformed with the Vector (Vec) or ds-nta-eTMX27 construct in response to topping. Samples were collected at 48 h after topping treatment (without topping as control). The expression level in the Vec plants without topping treatment was arbitrarily set as 1. Error bars indicate SD. Double asterisks indicate a significant difference (Student’s $t$ test, $p<0.01$) between the two samples. E, Relative expression levels of nta-eTMX27 in roots of transgenic plants transformed with the Vec or ds-nta-eTMX27 after topping treatment at 48 h. The expression level of the Vec plants with control treatment was arbitrarily set as 1. Error bars indicate the SD. F, Relative expression levels of QPT2 in the Vec and ds-nta-eTMX27 transgenic plants after topping treatment at 48 h. Error bars indicate SD. The expression level of the Vec plants with control treatment was arbitrarily set as 1. A single asterisk indicates a significant difference (Student’s $t$ test, $p<0.05$) and double asterisks indicate a highly significant difference (Student’s $t$ test, $p<0.01$) between the two paired samples.

Figure 5. Phylogenetic tree (A) and evolution model (B) of nta-miRX27, its target QPT2 and target mimic nta-eTMX27 in the Nicotiana genus. The nucleotide sequences of nta-miRX27, QPT2 and nta-eTMX27 from N. tabacum, N. sylvestris, N. benthamiana, N. otophora and N. tomentosiformis were aligned using the neighbor-joining method with 1000 replications.
Supplemental Data

**Supplemental Table S1.** Summary of predicted and identified novel miRNAs targeting nicotine pathway genes. *RPM: Repeat normalized readSummary of predicted and identified novel miRNAs targeting nicotine pathway genes.*

**Supplemental Table S2.** Primers used in plasmid construction and other experiments in this study. a, MiRNAequence is underlined; b, Restriction site used for cloning is underlined; c, STTM mimic sequence is underlined.

**Supplemental Figure S1**

**Figure S1.** QPT2 gene annotation and validation of nta-miRX27-mediated cleavage of QPT2 by RLM-RACE. Gene annotation in NCBI (the first track) and the predicted gene structures of QPT2 using Fgenesh (the second track), and assembled transcripts based on RNA-seq data from roots (SRX495526-7,9, Sierro et al., 2014) were shown. Two nta-miRX27 targeting sites were predicted (indicated by red lines) and the first targeting site located at exon #2 of QPT2 was confirmed to be cleaved by nta-miRX27 based on our degradome data (Fig. S2) although none of the RLM-RACE products was mapped the expected cleavage position at both predicted targeting sites. The 5’ end positions of the RLM-RACE products were indicated by dark yellow lines, their distances relative to the predicted cleavage sites and the corresponding number of clones at each position were shown at the bottom of the graph.

**Supplemental Figure S2**

**Figure S2.** Validating nta-miRX27-mediated cleavage of the predicted target site (the first one at exon #2 in Fig. S1) using degradome data from roots of topping-treated tobacco plants. Base pairing between nta-miRX27 and its target QPT2 is shown, in which a vertical line means a Watson-Crick pair, two dots represent a G-U pair and 0 represents mismatch. The numbers below the alignment indicate the numbers of
degradomic reads at the positions indicated. The expected cleavage site is indicated by a red arrow.


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