Brief Communication

**NIC1 cloning and gene editing generates low-nicotine tobacco plants**

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Nicotine is the predominant alkaloid in tobacco plants, accounting for ~90% of their total alkaloid content. It is the main addictive substance in cigarettes. Reducing nicotine content in tobacco leaves will aid the development of low-nicotine tobacco products. Prior work has shown that the manipulation of genes involved in nicotine biosynthesis can achieve this purpose (Hidalgo et al., 2020). Here, we focussed on the long-sought major regulator of nicotine biosynthesis, NIC1 (A).

The NIC1 gene, together with a minor locus NIC2 (B), have been identified through genetic analysis of a low-nicotine trait originating from natural mutants of cigar tobacco (Legg and Collins, 1971). Introggression of the low-nicotine trait into Burley 21 (B21) generated near-isogenic lines with different alkaloid levels: high alkaloid (HA, AABB), high intermediate (HI, AAbb), low intermediate (LI, aABB) and low alkaloid (LA, aabb) (Legg and Collins, 1971). The genes coding for nicotine biosynthetic enzymes, such as the rate-limiting PMT and QPT, are downregulated in LA, suggesting that NIC genes are transcriptional regulators orchestrating nicotine biosynthesis (Saunders and Bush, 1979). Transcriptome-based cloning of NIC2 revealed that this locus is clustered with transcription factors from the ethylene response factor (ERF) subfamily. Of these NIC2-ERFs, ERF189 is the most effective and directly targets the GC-rich P-box element in promoters of nicotine biosynthetic genes (Shoji and Hashimoto, 2012; Shoji et al., 2010). Suppression of NIC2-ERFs reduced nicotine content in tobacco, but a significant amount of nicotine remained due to the major NIC1 locus (Kajikawa et al., 2017).

To isolate the NIC1 gene, we conducted map-based cloning using 600 field-growing F₂S derived from a cross between HI and LA. The segregating population was first genotyped with a custom tobacco 30K Infinium Select HD BeadChip. Additional markers were designed through SNP identification based on RNA-seq of the B21 NILs. NIC1 congregated with SNP4 and was flanked by SNP3 and SNP5 on chromosome 7 (Figure 1a). The delimited NIC1 region was bordered by K326 scaffolds Nitab4.5_0003553 and Nitab4.5_0007027. Reciprocal BLAST comparisons were conducted between K326 and TN90 to fill gaps. Gene annotation identified at least seven full-length single-exon ERFS (URES1, ERF199, ERF91, ERF210, ERF29, ERF16 and ERF130) and two truncated ERFS (ERF110 and ERF172) in this region (Figure 1a).

BLAST analysis showed that NIC1 and NIC2 regions were syntenic and originated, respectively, from N. sylvestris (S-genome) and N. tomentosiformis (T-genome). Notably, ERF199 (Nitab4.5_0003090g0030) is homologous to ERF189, sharing an identical binding domain (Figure 1b). Driven by their native promoters, the seven complete NIC1-ERFs were transferred to LA plants using Agrobacterium tumefaciens strain GV3101. Complementation tests showed that transfer of ERF199 (n = 28) significantly increased nicotine levels in potted T0 plants growing in greenhouse. No significant phenotype changes resulted from the remaining six NIC1-ERFs (n > 20) (Figure 1c). The ERF199-mediated nicotine increase was confirmed with T1 plants (n = 35 from 7 T0s) (Figure 1d). We, therefore, concluded that ERF199 is the NIC1 gene.

To determine subcellular localization, we fused ERF199 with a red fluorescent protein (RFP) under the CaMV 35S promoter. Constitutive expression of ERF199-RFP in LA significantly increased nicotine content in both T0 (n = 15) and T1 (n = 28) plants (Figure 1e, f). Co-localization of the fusion protein with 4′, 6-diamidino-2-phenylindole (DAPI)-stained nuclei demonstrated that ERF199 is localized in the nucleus, consistent with its role as a transcriptional regulator (Figure 1g). Direct binding of ERF199 to the P box in the PMT2 promoter was verified by electrophoretic mobility shift assay (EMSA). The resolved mobility shift was eliminated by the addition of excess non-labelled probe in the competition experiment, confirming the specificity of this DNA–protein interaction (Figure 1h). Furthermore, a transient gene expression assay using tobacco protoplasts revealed that ERF199 regulates both PMT2 and QPT, the two key enzymes for nicotine biosynthesis (Figure 1i, j).

We compared the genomic sequences of ERF199 alleles between HI and LA (~5kb), including 2.3kb upstream of the start codon and 2kb downstream of the stop codon, but no SNPs were
Map-based cloning and gene editing of NIC1

![Diagram of SNP markers and gene locations](image)

- SNP markers: SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP8, SNP9, SNP10, SNP11
- LG7 genes: Nitab4.5_0002555, Nitab4.5_0034622, Nitab4.5_0030072, Nitab4.5_0023103

- Chromosome 7
  - SNP markers: SNP1, SNP2, SNP3
  - Genes: ERF1, ERF11, ERF199, ERF210, ERF130, ERF16

- Chromosome 19
  - SNP markers: SNP1, SNP2, SNP3, SNP4
  - Genes: ERF1, ERF221, ERF104, ERF1, ERF9, ERF11

- Alkaloid levels (%): Nicotine, Nornicotine, Anabasine, Anatabine

- Relative expression level (%): Root, Leaf

- Relative activity: Free probe, ERF199-probe complex, Competitor

- GC-rich motif of PMT promoter

- PMT::Luc, QPT::Luc

- Mutant allele: P, S, T, *

- Wild type allele: P, S, H, E

- Alkaloid contents (%): Nicotine, Nornicotine, Anabasine, Anatabine
detected. Expression analysis indicated that Nic1 was root-specific and significantly downregulated in LA (Figure 1k), suggesting the recessive allele was epigenetically silenced. However, targeted bisulphite sequencing of the same 5 kb did not reveal significantly different DNA methylation in CpG, CHG and CHH sites. A thorough epigenome sequencing may provide insight into the underlying epimutations in the Nic1 locus.

We predicted that lower nicotine than LA could be attained by eliminating ERF199. Using CRISPR technology, we generated a mutated allele caused by an ‘A’ insertion in HI (Figure 1) and evaluated its phenotypic effect at T1. The genotypes of wide-type (WT-T1), heterozygous (Het-T1) and homozygous mutant (Mut-T1) plants were determined by DNA sequencing. Total alkaloid levels in WT-T1 plants (n = 22) were comparable with HI plants, and Het-T1s (n = 29) had intermediate levels. However, alkaloid content was barely discernible in Mut-T1 plants (n = 35); approximately 1/10 of that in LA plants (Figure 1m). Thus, manipulation of the Nic1 gene provides a new strategy for nicotine control. Furthermore, the ultra-low-nicotine levels in Mut-T1 confirmed that ERF199 is the only causal gene for nicotine biosynthesis within the Nic1 locus.

Transcriptional regulation of secondary metabolite production can be controlled by a single ERF. Indeed, GAME9 locates within an ERF cluster and is the only functional regulator of steroidal glycoalcaloid biosynthesis in tomatoes (Cardenas et al., 2016). Although both ERF189 and ERF199 share the same DNA-binding domain and directly bind to P-box elements within the promoters, ERF199 is more effective. A plausible explanation may be the presence of cofactor/coactivator-recruiting activation domains. Further investigation is required to determine whether a unique activation domain or additional transcriptional cofactors play critical roles in the ERF199-regulatory network.

Significant efforts have been made to attenuate tobacco nicotine content (Hidalgo Martinez et al., 2020). Our study genetically and functionally validated ERF199 as the Nic1 gene. The Nic1 locus, originating from the S-genome, is homologous to the T-genome-donated Nic2 locus. Constitutive expression of ERF199 caused increased nicotine levels, and disruption of ERF199 function in the absence of Nic2 dramatically reduced nicotine accumulation in leaves. Thus, genetic regulation of nicotine levels in tobacco plants can be achieved by manipulating the Nic1 gene.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Author contributions
MH, TG, AF, DL and SY conceived and designed the experiments. All authors performed the experiments. SY wrote the first draft of the manuscript. All authors revised the manuscript.

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