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ARTICLE ADDENDUM

A model for evolution and regulation of nicotine biosynthesis regulon in tobacco

Masataka Kajikawa[a,*], Nicolas Sierro[b,*], Takashi Hashimoto[a], and Tsubasa Shoji[a]

[a]Nara Institute of Science and Technology, Graduate School of Biological Sciences, Takayama, Ikoma, Nara, Japan; [b]Philip Morris International R&D, Philip Morris Products, Neuchâtel, Switzerland

ABSTRACT

In tobacco, the defense alkaloid nicotine is produced in roots and accumulates mainly in leaves. Signaling mediated by jasmonates (JAs) induces the formation of nicotine via a series of structural genes that constitute a regulon and are coordinated by JA-responsive transcription factors of the ethylene response factor (ERF) family. Early steps in the pyrrolidine and pyridine biosynthesis pathways likely arose through duplication of the polyamine and nicotinamide adenine dinucleotide (NAD) biosynthetic pathways, respectively, followed by recruitment of duplicated primary metabolic genes into the nicotine biosynthesis regulon. Transcriptional regulation of nicotine biosynthesis by ERF and cooperatively-acting MYC2 transcription factors is implied by the frequency of cognate *cis*-regulatory elements for these factors in the promoter regions of the downstream structural genes. Indeed, a mutant tobacco with low nicotine content was found to have a large chromosomal deletion in a cluster of closely related *ERF* genes at the nicotine-controlling *NICOTINE2* (*NIC2*) locus.

Abbreviations: AO, aspartate oxidase; BBL, berberine bridge enzyme-like protein; DAO, diamine oxidase; ERF, ethylene response factor; JA, jasmonate; MPO, *N*-methylputrescine oxidase; NAD, nicotinamide adenine dinucleotide; NIC2, NICOTINE2; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyl transferase; QS, quinolinate synthase; SPDS, spermidine synthase

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Background

Nicotine, a nitrogen-containing specialized metabolite with potent insecticidal activity, is produced in the roots and accumulates in the leaves of *Nicotiana* species (family Solanaceae), including cultivated tobacco (*Nicotiana tabacum*). Concerted and substantial expression of biosynthetic genes for nicotine guarantees its massive production in the underground organs. An orthologous pair of ethylene response factor (ERF)—family proteins, ERF189 and ERF199, and the basic helix-loop-helix family protein MYC2, regulate the nicotine biosynthesis pathway. The ERF proteins form a cascade with MYC2 – a conserved component in JA signaling – acting upstream of ERF189 and ERF199, which specifically control the nicotine biosynthetic pathway.[1,2] Elucidation of the complete tobacco genome sequence[3] allowed us to analyze phylogenetic, expression, and other properties of the structural and regulatory genes involved in the pathway.

Repeated pathway duplication

The pyrrolidine and pyridine rings that make up nicotine molecule are generated in early steps of the biosynthetic pathway. Ring formation is followed by less well-defined steps required to couple the two rings[4] (Fig. 1). Our phylogenetic and expression profiling analysis of the metabolic genes involved in this ring formation point to the duplication of the polyamine and NAD biosynthetic pathways, and subsequent incorporation of these duplicated genes into the nicotine biosynthesis regulon through sub-functionalization and neo-functionalization (Fig. 1). The characteristic co-expression of nicotine-regulon genes in nicotine-producing tissues like roots and JA-elicited cultured cells is largely directed by ERF189 and ERF199 transcription factors. In addition to being a precursor of nicotine, the pyrrolidine moiety derived from ornithine is also incorporated into tropane (e.g. scopolamine) and nortropane (e.g., calystegine) alkaloids in many species from the Solanaceae and other families (Fig. 1). Based on the presence of the relevant genes, the establishment of the pyrrolidine-forming extension from polyamine metabolism, accompanied by catalytic innovation (i.e. putrescine *N*-methyltransferase (PMT) from spermidine synthase (SPDS) and *N*-methylputrescine oxidase (MPO) from diamine oxidase (DAO)) (Fig. 1), is presumed to have occurred before plants diversified to produce ornithine-derived alkaloids. Additionally, an early part of the NAD pathway may have doubled around the time of diversification of the *Nicotiana* lineage to satisfy the increased metabolic demands related to downstream nicotine production.[5] Accordingly, the formation of rings from branches of the alkaloid pathways may

CONTACT Tsubasa Shoji t-shouji@bs.naist.jp Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Nara 630–0101, Japan.
*These authors contributed equally to this study.
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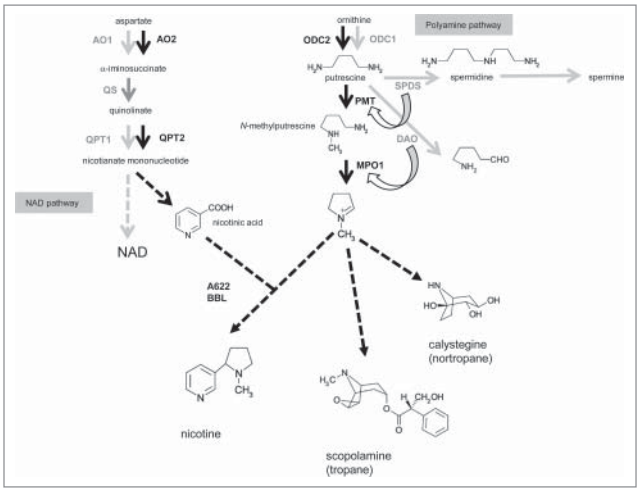

Figure 1. Biosynthetic pathways for ornithine-derived alkaloids, polyamine, and nicotinamide adenine dinucleotide (NAD). Each defined step of the pathway is represented by an arrow and named enzyme, while undefined parts dependent on single or multiple steps are shown with broken arrows. Steps in black are hypothesized to be involved predominantly in alkaloid biosynthesis, while those predominantly associated with related primary metabolic pathways are shown in gray. Quinolinate synthase (QS) is shown in dark gray, as it contributes to both nicotine and NAD pathways. It has been proposed that putrescine *N*-methyltransferase (PMT) and *N*-methylputrescine oxidase (MPO) have evolved from spermidine synthase (SPDS) and diamine oxidase (DAO), respectively. AO; aspartate oxidase, QPT; quinolinate phosphoribosyl transferase, ODC; ornithine decarboxylase.

have been preceded by independent and repeated duplications of primary pathways.

Recruitment of structural genes into the regulon

How did the duplicated metabolic genes and other genes for late steps, such as those encoding A622 and berberine bridge enzyme-like (BBL) oxidoreductases (Fig. 1), become regulated by master transcription factors and thereby recruited into the nicotine-biosynthesis regulon? To be involved in the regulon, structural genes may have acquired novel expression patterns through acquisition of cognate *cis*-regulatory elements within their promoters, which are recognized by *trans*-acting transcription factors.[5,6] The frequent occurrence of the ERF189/ERF199-binding P-box and MYC2-binding G-box elements in the regulon promoters supports such a scenario (Fig. 2), and is complemented by our identification, through non-targeted analysis, of sequences related to P-boxes and G-boxes as motifs conserved among the promoters.

Clusters of transcription factor genes

We found a pair of homologous clusters of closely related *ERF* genes, including *ERF189* and *ERF199*, in the genome of allotetraploid tobacco. Similar genomic clustering has been reported for related *ERF* genes from other plants,[7-10] implying the relatively ancient generation of such clusters through gene duplication and possible functional differentiation among the clustered genes.[11] Further, we found that a large chromosomal region (about 650 kb), encompassing 10 genes, including *ERF189*, out of 12 clustered genes at the *NIC2* locus, was deleted in a mutant allele used to breed tobacco cultivars with low nicotine levels.[12]

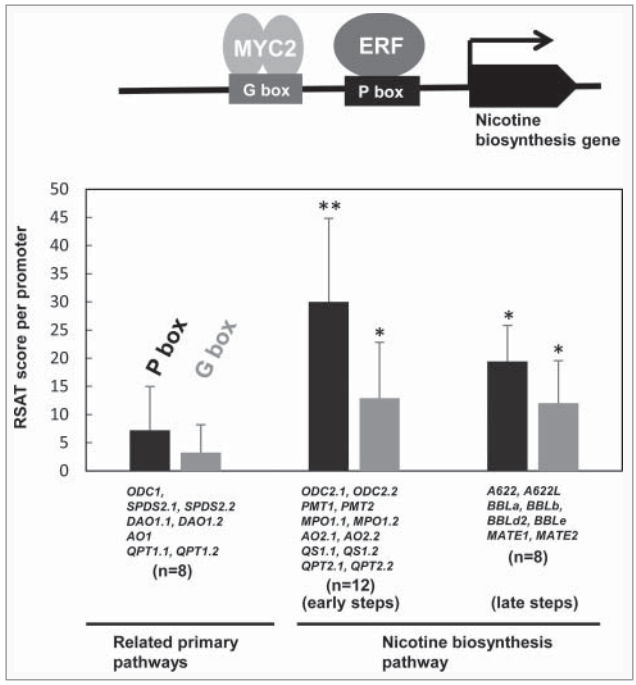

Figure 2. Enrichment of ERF189/ERF199-binding P-box and MYC2-binding G-box elements in the promoter regions of structural genes involved in the nicotine biosynthesis pathway. *Cis*-regulatory P-box and G-box elements bound with cognate transcription factors in the promoter regions of nicotine biosynthesis genes are shown schematically at the top. As detailed in Kajikawa and Sierro et al. (2017), elements within 5′-flanking regions from −1,500 to −1 (numbered from the first ATG) were searched and scored using RSAT software (http://rsat.ulb.ac.be/rsat); elements with scores over 5.5 for P-boxes and 5.0 for G-boxes were adapted. Scores for each predicted element were summed for each gene promoter. Average values of the scores for each gene set (indicated below) are represented as black and gray bars with SDs for P-box and G-box elements, respectively. Student's t-test was used to calculate significant differences between values marked and those for genes involved in related primary pathways (left). $^{**}P < 0.01$, $^{*}P < 0.05$, n; numbers of genes involved in indicated gene sets.

Concluding remarks

Based on molecular and genomic information, we recently inferred the evolution of a metabolic pathway specific to plants in the *Nicotiana* lineage. Our model invokes repeated duplication of primary pathways and of structural genes in the ERF-controlled regulon. Our work revealed the structure of the *ERF* gene clusters, and that there is a molecular lesion in one of the clusters at the *NIC2* locus that causes a low-nicotine phenotype. Insights into tobacco's nicotine biosynthesis regulon will provide valuable clues for understanding JA-dependent defense metabolic pathways regulated by transcription factors related to ERF189 and ERF199.

Disclosure of potential conflicts of interest

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