A model ADDENDUM

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

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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 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, \textit{N}-methylputrescine oxidase; NAD, nicotinamide adenine dinucleotide; NIC2, NICOTINE2; ODC, ornithine decarboxylase; PMT, putrescine \textit{N}-methyltransferase; QPT, quinolinate phosphoribosyltransferase; QS, quinoline synthase

Background

Nicotine, a nitrogen-containing specialized metabolite with potent insecticidal activity, is produced in the roots and accumulates in the leaves of \textit{Nicotiana} species (family Solanaceae), including cultivated tobacco (\textit{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.\textsuperscript{1,5} Elucidation of the complete tobacco genome sequence\textsuperscript{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\textsuperscript{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 \textit{N}-methyltransferase (PMT) from spermidine synthase (SPDS) and \textit{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 \textit{Nicotiana} lineage to satisfy the increased metabolic demands related to downstream nicotine production.\textsuperscript{5} Accordingly, the formation of rings from branches of the alkaloid pathways may

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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.\textsuperscript{5,6} The frequent occurrence of the ERF189/ERF199-binding P-box and MYC2-binding G-box elements in the promoter regions of structural genes involved in the nicotine biosynthesis regulon 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,\textsuperscript{7–10} implying the relatively ancient generation of such clusters through gene duplication and possible functional differentiation among the clustered genes.\textsuperscript{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.\textsuperscript{12}
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