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Minireview

Molecular regulation of nicotine biosynthesis

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Abstract Nicotine is most familiar to us as a principal pharmacologically active component of cigarettes. This alkaloid is synthesized in the root in response to insect damage and then transported to the aerial parts of tobacco plants. In this short review, we summarize enzymes and genes involved in nicotine biosynthesis, regulatory mechanisms of gene expression involving the *NIC* regulatory loci and jasmonic acid, and finally metabolic engineering of nicotine formation.

Key words: Alkaloid, biosynthesis, nicotine, tobacco.

Alkaloids are low-molecular-weight nitrogen-containing basic compounds. Currently more than 12,000 chemical structures of alkaloids are known and constitute the second most diversified compound family in plants, only exceeded by terpenoids (Croteau et al. 2000). Many alkaloids act on animal nervous systems, and are used in prescriptions of modern medicine and as ingredients of eastern folk medicine Kampo. We here review nicotine biosynthesis and its molecular regulation.

Putrescine, a symmetrical diamine, is formed from basic amino acids, ornithine and/or arginine, and is metabolized to higher polyamines in all organisms and to particular alkaloids in restricted plant species (Hashimoto and Yamada 1994). Putrescine is metabolized to nicotine in tobacco and other *Nicotiana* and related species, and to pharmacologically active tropane alkaloids, such as hyoscyamine and scopolamine, in some medical solanaceous plants (Figure 1). Since nicotine and tropane alkaloids are expected to share the same evolutionary origin during the diversification of the Solanaceae, basic principles and molecular components revealed in the nicotine regulation may well be applied to tropane alkaloid biosynthesis.

Biosynthetic pathway and structural genes

Nicotine is composed of the pyrrolidine ring and the pyridine ring. The pyrrolidine moiety is derived from *N*-methylpyrrolinum cation, a spontaneous cyclization product of the oxidative deamination reaction from *N*-methylputrescine. Diamine oxidase (DAO) catalyzes the deamination of *N*-methylputrescine, which is formed from putrescine by putrescine *N*-methyltransferase (PMT) (Hashimoto and Yamada 1994). The pyridine moiety of nicotine is supplied from the NAD biosynthesis pathway (Dawson et al. 1958; Dawson et al. 1956; Yang et al. 1956). Although labeled nicotinic acid was incorporated into the pyridine ring of nicotine when administered to tobacco (Leete 1983; Leete 1979; Leete and Liu 1973), it is not known whether nicotinic acid itself or a metabolite derived from it is the direct precursor of nicotine.

The amino acid sequence of PMT is highly homologous to the sequence of spermidine synthase (SPDS), which transfers the amino-propyl moiety of decarboxylated *S*-adenosylmethionine (dSAM) to

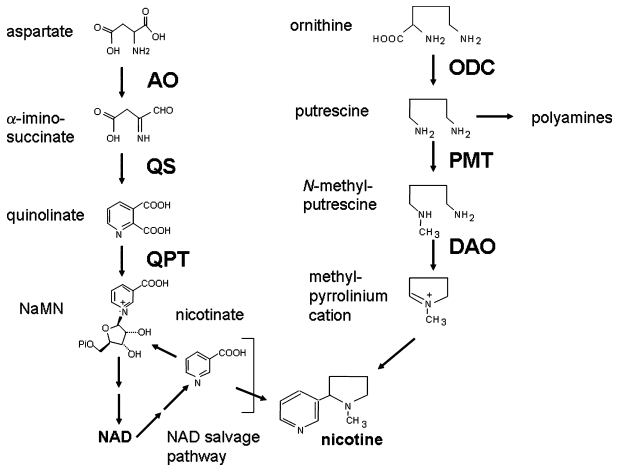

Figure 1. Biosynthetic pathway of nicotine. Nicotine is synthesized by condensation of an intermediate in the NAD salvage pathway and the methylpyrrolinium cation derived from ornithine via putrescine. This cation is also used for biosynthesis of tropane alkaloids, such as hyoscyamine and scopolamine. Enzymes involved in nicotine synthesis are indicated: ODC; ornithine decarboxylase, PMT; putrescine *N*-methyltransferase, DAO; diamine oxidase, AO; aspartate oxidase, QS; quinolinate synthase, and QPT; quinolinate phospho-ribosyltransferase.

putrescine (Hibi et al. 1994). PMT catalyzes a transfer of the methyl moiety of S-adenosylmethionine (SAM) to putrescine. It is proposed that PMT evolved from SPDS after restricted alterations of critical dSAM binding amino acid residues (Hashimoto et al. 1998b). Although tobacco PMT differs from SPDS by the addition of tandem repeats of eleven amino acid residues at the *N*-terminus, the repeat element is not required for the enzymatic activity. Five PMT genes of tobacco possess variable repeat numbers, whereas the tandem repeats are absent in PMTs from *Solanaceae* plants producing tropane alkaloids (Hashimoto et al. 1998a).

Tobacco DAO may have been evolved from a DAO widespread in nature by optimization of substrate specificity. The DAOs involved in nicotine and tropane alkaloid biosynthesis have higher affinity for *N*-methylputrescine than for putresine and other symmetrical diamines (Haslam and Young 1992; Hashimoto et al. 1990; Walton and McLauchlan 1990). In contrast, pea and pig DAOs bind *N*-methylputrescine with low affinity.

Site of nicotine formation and transport

PMT and A622 oxidoreductase genes are specifically expressed in the root of tobacco plants (Hibi et al. 1994). The A622 gene is coordinately regulated with the PMT gene, and thus is postulated to encode an enzyme in nicotine pathway. Analysis by immunohistochemistry and promoter::GUS fusion reporters showed that both enzymes are localized in the same cell types in the root (Shoji et al. 2000; Shoji et al. 2002). High expression was observed at epidermis and cortex cells in the root tips, whereas in the differentiated region of the root, the outermost layer of the cortex and parenchyma cells surrounding xylem in vascular bundle were stained.

The cortex cells of the root tip have not differentiated the Casparian band and the apoplastic flow of metabolites from the cortex to the stele is presumably not restricted. Nicotine transported to the xylem is subsequently translocated to the aerial parts. In the root differentiation zone, the suberin layer in the Casparian band prevents free apoplastic flow. In this region, nicotine synthesis in the parenchyma cells surrounding the xylem may facilitate nicotine loading into the xylem for translocation.

Nicotine translocated to the leaf and other aerial tissues finally accumulates in the vacuole. It is not known whether a specific transporter is required to unload nicotine from the xylem and take it up to the vacuole from the cytoplasm. Nicotine might pass through tonoplast membrane spontaneously and might be trapped inside the vacuole after forming ion-pairs with organic acids.

Transcriptional control

Some plant alkaloids can function as direct chemical defenses against herbivores. Tobacco plants that have much reduced nicotine contents, either by the *nic* mutations or by transgenic suppression of the PMT genes, are much more susceptible to insect herbivory than control plants with wild-type nicotine contents (Steppuhn et al. 2004; Legg et al. 1970). Herbivore damage induces jasmonic acid formation and activates wound signaling pathway (Halitschke and Baldwin 2003.). The *CORONATINE INSENSITIVE 1* (*COI1*) gene was initially identified in a genetic screen of Arabidopsis jasmonate-insensitive mutants (Devoto et al. 2005), and is now believed to play a central role in jasmonate signaling in other plant species, including tobacco (Liu et al. 2004). Wound signal generated in the leaf spreads systematically and also travels down to the root where root-specific genes, such as those involved in nicotine accumulation, are activated. The signal that transmits from the leaf to the root is not established but may be jasmonic acid itself (Li et al. 2002). PMT and other genes involved in nicotine formation show basal low expression in the root of undamaged tobacco plants, but wounding and jasmonate treatment to the leaf increase the gene expression levels 3–4 folds (Shoji et al. 2000; Sinclair et al. 2000). Functional analysis of the tobacco PMT promoter revealed that jasmonate-induced expression requires G-box and GCC-motif elements in the proximal region of the PMT promoter, which are often found in jasmonate-responsive promoters (Oki and Hashimoto 2004; Xu and Timko 2004). Ethylene supplied simultaneously with jasmonic acid effectively abrogated jasmonate activation of the PMT and A622 promoters. In nicotine biosynthesis, ethylene signals can antagonize jasmonate signals.

Genetic loci affecting nicotine contents have been utilized to reduce nicotine contents in the present tobacco varieties. The original mutant was discovered in a Cuban cigar variety in the early 1930s in Germany and the low-nicotine genes were subsequently incorporated into cigarette varieties through a series of backcrosses to meet the expected demand for low-nicotine cigarettes in the United States (Valleau 1949). Thorough genetic studies demonstrated that the low-nicotine phenotype is caused by synergistic effects of two non-linked loci, which we named *nic1* and *nic2*. The *nic1nic2* double mutant has highly reduced nicotine content (about 5% of wild type) but is otherwise not different from parental lines. Molecular studies revealed that expression levels of nicotine-biosynthetic genes are remarkably decreased in the mutant roots (Hibi et al. 1994; Cane et al. 2005), and PMT and A622 oxidoreductase promoters are specifically down-regulated in the *nic* mutant background (Shoji et al., unpublished). Thus, *NIC* loci

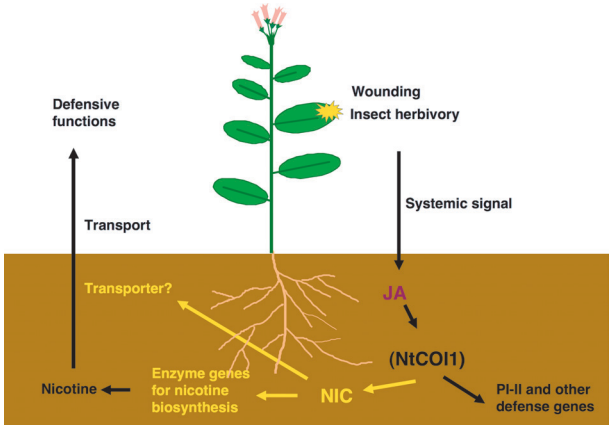

Figure 2. Activation of nicotine biosynthesis by jasmonic acid. Insect hervibory and wounding on the leaf generate systemic signal(s) that moves to the root where it activates a signaling cascade of jsamonic acid (JA). The JA pathway induces expression of general defense genes, such as PI-II, but, in the tobacco root, also enhances expression of enzyme genes for nicotine biosynthesis and putative nicotine transporters, which requires the regulatory locus *NIC*. Nicotine produced in the root is transported to the aerial parts where it functions as a chemical defense compound against insects.

specifically regulate expression of nicotine-biosynthetic genes.

We generated partially normalized complementary DNA (cDNA) libraries from a diploid tobacco *N. sylvestris*, and prepared cDNA micro-array sets for comparative transcriptome analysis between wild-type tobacco and the *nic* mutants (Katoh et al. 2003). Extensive micro-array analysis (Katoh et al., unpublished) as well as careful differential display analysis (Inai et al., unpublished) showed that although wounding and jasmonic acid induce hundreds of tobacco genes, including *PROTEINASE INHIBITOR-II* (*PI-II*; Choi et al. 2000), only about a dozen of the wound- and jasmonate-inducible genes are controlled by *NIC* regulatory loci. Two simple regulatory models are possible. In one model, the general jasmonate signaling pathway branches off, possibly at or after the tobacco *COI1*, to a nicotine-specific pathway, in which *NIC* genes function (Figure 2). Alternatively, the jasmonate signaling pathway and the independent *NIC* signaling pathway converge at the nicotine biosynthetic genes, and simultaneous signaling inputs from the two pathways are required to activate target nicotine genes, possibly by activating specific transcriptional factors. To distinguish these two models, we need to molecularly clone the *NIC* genes and to study the biochemical functions of NIC proteins.

Metabolic engineering of nicotine biosynthesis

Previously, overexpression of yeast ornithine decarboxylase gene was shown to moderately increase leaf nicotine levels in tobacco (Hamill et al. 1990). We overexpressed PMT under the control of constitutive CaMV35S promoter in tobacco plants. The transgenic tobacco lines accumulated *N*-methyl putrescine, the direct product of the PMT reaction, in whole plants, whereas accumulation of nicotine increased by 40% compared to wild-type plants (Sato et al. 2001). This moderate increase of the final metabolite indicates that overexpression of one enzyme in the pathway made subsequent reactions more rate-limiting. To achieve further increase of nicotine accumulation, multiple steps in the pathway should be fortified.

A co-suppression line, in which PMT expression level was decreased to 16% of the wild-type level, accumulated nicotine at the level only 2% of wild type. The low-nicotine line accumulated high amounts of putrescine and spermidine, indicating that the efficient inhibition of PMT activity shifted the nitrogen flow from nicotine synthesis to polyamine formation. The co-suppression line also showed several distinct morphological phenotypes: neighboring leaves were fused at their bases, forming a continuous spiral sheet along the stem, inflorescent stems often were branched, and self-pollinated flower produced only a small seed set (less than 10% of wild type). These abnormalities may be caused by increased accumulation of polyamines, which possess hormonal functions for plant development (Galston and Kaur-Sawhney 1995).

Conclusion

Biosynthesis of many alkaloids may have evolved as chemical defense systems against insect pest, and is often induced or further activated after insect hervibory by signal transduction pathways involving jasmonic acid. It is interesting to know how distinct regulation of genus-specific alkaloid metabolism has evolved from general jasmonate signaling pathways. The *nic* regulatory mutants of nicotine biosynthesis should be important as a model system to study evolution of regulatory aspects of secondary products. Molecular studies of *NIC* genes may reveal specificity and generality of alkaloid regulation.

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