Regulation of nicotine biosynthesis in tobacco

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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. Here I overview enzymes and genes involved in nicotine biosynthesis, and regulatory mechanisms of gene expression involving the *NIC* regulatory loci and jasmonic acid.**

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 Kanpo.

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. 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.

Results and Discussion

1. Site of nicotine formation and transport

Putrescine N-methyltransferase (PMT) is the first committed enzyme in the biosynthetic pathways of nicotine and tropane alkaloids. 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.

2. 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.). 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 is 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 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, 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 SCF, to a nicotine-specific pathway, in which *NIC* genes function. 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.

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