NICOTINE BIOSYNTHESIS IN *NICOTIANA*: A METABOLIC OVERVIEW

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Alkaloids are important compounds found in *Nicotiana* plants, essential in plant defense against herbivores. The main alkaloid of *Nicotiana tabacum*, nicotine, is produced in roots and translocated to the leaves. Nicotine is formed by a pyrrolidine and a pyridine ring in a process involving several enzymes. The pyridine ring of nicotine is derived from nicotinic acid, whereas the pyrrolidine ring originates from polyamine putrescine metabolism. After synthesis in root cortical cells, a set of transporters is known to transport nicotine upward to the aerial part and store it in leaf vacuoles. Moreover, nicotine can be metabolized in leaves, giving rise to nornicotine through the *N*-demethylation process. Some *Nicotiana* wild species produce acyltransferase enzymes, which allow the plant to make *N*-acyl-nornicotine, an alkaloid with more potent insecticidal properties than nicotine. However, although we can find a wealth of information about the alkaloid production in *Nicotiana* spp., our understanding about nicotine biosynthesis, transport, and metabolism is still incomplete. This review will summarize these pathways on the basis on recent literature, as well as highlighting questions that need further investigation.

Additional key words: *Nicotiana*, nicotine, nornicotine, *N*-acyl-nornicotine, alkaloid biosynthesis

INTRODUCTION

Plants produce a diverse array of secondary metabolites, among which are some important compounds, such as alkaloids, that are mainly used in defense against pathogens and herbivores (48, 79). Alkaloid-containing plant extracts have been used as medicines, drugs, and poisons since the beginning of human civilization (39). The Solanaceae (nightshade family) is a botanical family with several species producing alkaloids (55). Tobacco (*Nicotiana tabacum*) is one of those species, and it has become an economically important crop plant because of alkaloid production, although the entire *Nicotiana* genus is recognized for producing these type of metabolites (39, 80).

The alkaloids found in *Nicotiana* plants are toxic compounds that play a major role in defense against generalist herbivores (64). The root represents the main site of alkaloid synthesis in *Nicotiana* species (64, 78). After their production, the alkaloids are transported through the xylem and accumulated in leaves, which are the areas more susceptible to herbivore attacks (64).

Alkaloid synthesis is elicited upon herbivore attacks via the canonical jasmonate-signaling pathway (73, 98). In undamaged *Nicotiana sylvestris* plants grown in greenhouses, nicotine represents 0.1–1% of the dry mass, whereas in wounded plants or plants attacked by herbivores, the nicotine concentration increases to 1–4% (2, 3). Thus, a basal level of alkaloids is constantly maintained in plant tissues, and increases after wounding stimuli (98).

Regarding its ecological importance, alkaloid biosynthesis is a complex process that involves several enzymatic steps (Figure 1). Nicotine has two ring moieties, a pyrrolidine ring and a pyridine ring, derived from two branch pathways (18, 27). The pyridine ring of nicotine is derived from nicotinic acid, whereas the pyrrolidine ring originates from polyamine putrescine metabolism, which is gradually modified to *N*-methylpyrrolinium (23, 27). The condensation of a nicotinic acid derivative and *N*-methylpyrrolinium forms nicotine (20, 41, 71; Figure 1). Other *Nicotiana* alkaloids, such as anatabine, are synthesized solely through the pyridine ring pathway, whereas nornicotine is mainly produced via a nicotine *N*-demethylation process (43, 67, 76).

Although a wealth of information is available about alkaloid production in *Nicotiana* spp., some parts of the process are not fully understood. Therefore, this review will present a reconstruction of nicotine biosynthesis, transport, and metabolism pathways based on the current literature, and highlight questions that would merit further investigation. Moreover, we will discuss the ecological importance of nicotine and nicotine-derived alkaloids, nornicotine and *N*-acyl-nornicotine, to different *Nicotiana* species.

STRUCTURAL GENES OF THE NICOTINE PATHWAY

The pathway leading to the formation of a pyrrolidine ring initiates with putrescine (80; Figure 1). Putrescine can be produced via two alternate routes: directly from ornithine or indirectly from arginine. The direct route is catalyzed by ornithine decarboxylase (ODC), whereas the indirect route is catalyzed by arginine decarboxylase (ADC; 7, 12). In the indirect route, the arginine is decarboxylated to

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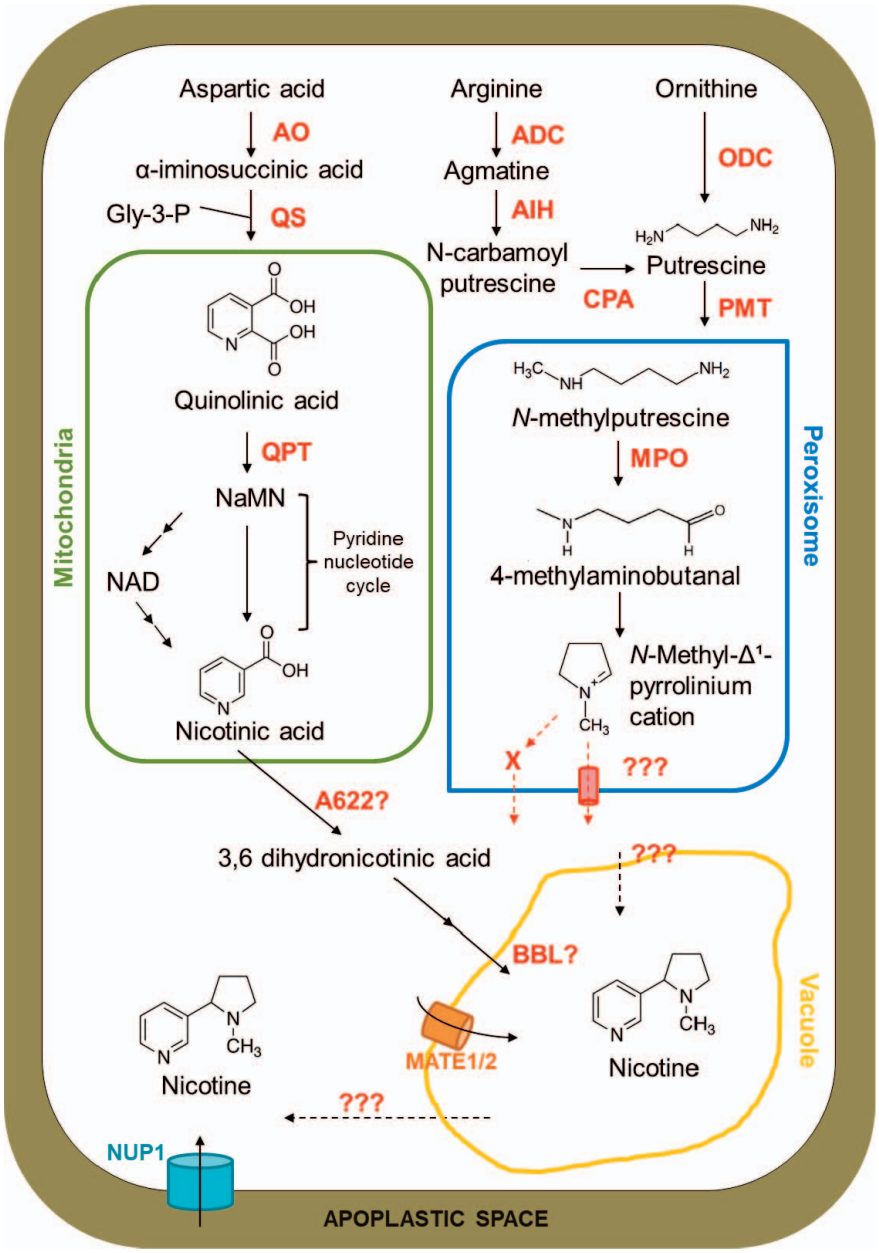

Figure 1. Nicotine biosynthesis in a root cortical cell of *Nicotiana*. Enzymes are indicated in red letters. AO, aspartate oxidase; QS, quinolinic acid synthase; Gly-3-P, glyceraldehyde-3-P; QPT, quinolinate phosphoribosyltransferase; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; PMT, putrescine *N*-methyltransferase; MPO, *N*-methylputrescine oxidase; BBL, berberine bridge enzyme-like; MATE1/2, multidrug and toxic compound extrusion 1 and 2; NUP1, nicotine uptake permease 1; ???: undefined step.

agmatine, which is subsequently hydrolyzed to *N*-carbamoylputrescine by agmatine iminohydrolase and further to putrescine by *N*-carbamoylputrescine amidohydrolase (30; Figure 1). Then, putrescine is converted to *N*-methylputrescine by putrescine *N*-methyltransferase (PMT; 27). Finally, *N*-methylputrescine is deaminated oxidatively by *N*-methylputrescine oxidase (MPO) to 4-methylaminobutanal, which spontaneously cyclizes to form the *N*-methylpyrrolinium cation that contains the pyrrolidine ring (21; Figure 1).

The pyridine ring of nicotine is derived from nicotinic acid, which is formed by the same enzymes involved in the early steps of nicotinamide adenine dinucleotide (NAD) biosynthesis, such as aspartate oxidase, quinolinic acid synthase (QS), and quinolinic acid phosphoribosyl transferase (QPT; 37, 77; Figure 1). The exact metabolite derived from nicotinic acid that is used in nicotine biosynthesis is not known (50). However, it was suggested that nicotinic acid needs to be reduced to 3,6-dihydronicotinic acid before its condensation with *N*-methylpyrrolinium cation to form nicotine (42, 43). Moreover, a phosphatidylinositol phosphate (PIP) family oxidoreductase member, A622, also participates in the last steps of pyridine alkaloid formation in *Nicotiana*,

although it is not clear if it is related to nicotinic acid reduction or not (33). *Berberine bridge enzyme-like* (*BBL*) genes are expressed in the vacuoles of tobacco root cells, and were previously described to be involved in the pyridine pathway (34). All tobacco genes encoding the proteins mentioned above are known as the structural genes of the nicotine pathway (71; Figure 1).

ADC and ODC — Origin of Putrescine. Putrescine may be synthesized from arginine via a three-step enzymatic process initiated by ADC, or directly from ornithine via the action of ODC (7, 12; Figure 1). ADC activity is predominant in the putrescine biosynthesis of plants, whereas ODC is more frequently found in animals and fungi (7). However, in the case of nicotine biosynthesis in tobacco, ODC seems to have a more prominent role (13). The impairment of ODC activity is related to plants with increased capacity of anatabine production (13, 71). Thus, the imbalance in the putrescine supply (pyrrolidine pathway) causes an oversupply of pyridine pathway derivatives, from which anatabine is entirely derived (11, 88).

Putrescine Methyltransferase. PMT is the enzyme responsible for the *N*-methylation of putrescine, generating *N*-methylputrescine (51; Figure 1). PMT is expressed in pericycle tissue, and its activity takes place in the apical portion of roots (74, 81, 93). *Nicotiana tabacum* has five genes encoding functional PMT; three of them are similar to those of *N. sylvestris*, whereas the other two are similar to *PMT* genes present in *Nicotiana tomentosiformis* (60).

PMT genes are not exclusive to *Nicotiana* spp. and have been identified in many species that produce pyridine and tropane alkaloids (24, 26, 54, 86). The sequence similarities indicate that tobacco *PMT* has evolved from *spermidine synthase* (*SPDS*) during the diversification of Solanaceae (22, 27). SPDS can be found in various organisms, e.g., *Escherichia coli* (8), *Bos taurus* (57), and soybean, *Glycine max* (95). In contrast, PMT activity was purified only from plants (6). Therefore, the concept of gene duplication and neofunctionalization may explain *PMT* evolution, since current PMT and SPDS catalyze different reactions with high specificities (82).

***N*-Methylputrescine Oxidase.** MPO is an amine oxidase that requires copper as a cofactor (25, 36). MPO catalyzes the oxidative deamination of *N*-methylputrescine in the second step of nicotine biosynthesis (54; Figure 1). *N*-Methylputrescine is the preferred substrate of MPO, although this enzyme can also use other polyamines such as putrescine and cadaverine (53). It was demonstrated in vitro that when putrescine is used as substrate, MPO produces an unmethylated pyrrolinium salt, resulting in the direct biosynthesis of nornicotine, whereas when cadaverine is used, anabasine is synthesized (25). It has been suggested that *N. tabacum* MPO preference for *N*-methylputrescine instead of other substrates may have contributed to the alkaloid pool characteristic of tobacco plants, with nicotine being the main alkaloid, followed by nornicotine and anabasine (25, 62).

Nicotiana tabacum MPO1 (*NtabMPO1*) possibly evolved from diamine oxidase (*NtabDAO1*) after gene duplication and subsequent neofunctionalization (54). Throughout this process, the cis elements that placed *MPO* under the control of the *NIC2* locus emerged in its promoter region (54, 68). Moreover, during the neofunctionalization process, the substrate specificity of MPO1 was altered to *N*-methylputrescine (54). *MPO1* homologs were found in *Solanum lycopersicum* and *Solanum tuberosum* genomes, indicating that molecular evolution of *MPO1* occurred early in the diversification of the Solanaceae family (54). In fact, in other Solanaceae species MPO is involved in the biosynthesis of several alkaloids, such as calystegines, hyoscyamine, and scopolamine (5, 15).

Previously, MPO1 was described as acting in the peroxisome, the same subcellular localization of DAO1 (54). This added a new cellular compartment to the nicotine biosynthesis pathway, contributing to a better understanding of the route (58). The product derived from the MPO1 reaction, 4-methyl-aminobutanal, spontaneously undergoes intramolecular cyclization to form the *N*-methylpyrrolinium cation (36). As a cation, it is not permeable to biological membranes, and a transporter should be required to move it to the cytoplasm (54; Figure 1). Another possibility is that an unknown peroxisomal enzyme may condense the cation with another compound, generating a membrane-permeable product (54; Figure 1).

Quinolinate Phosphoribosyltransferase. QPT is an enzyme that converts quinolate to nicotinate ribonucleotide (Figure 1). The activity of QPT is required for both primary and secondary metabolism in *Nicotiana* spp. (77). As a primary metabolic enzyme, QPT is essential for NAD synthesis and in secondary metabolism it is involved in the formation of several alkaloids (47, 83–85). QPT is encoded by duplicate genes (*QPT1* and *QPT2*) in *N. tabacum*, *N. sylvestris*, *N. tomentosiformis*, and *Nicotiana glauca* (61). *QPT2* activity increases several fold after wounding of aerial tissues, and it was correlated with wound-induced nicotine production (77).

A622 and BBL — Late Steps in Nicotine Biosynthesis. A622 is a member of the PIP family of NAD phosphate (reduced)-dependent reductases, with homology to phenylcoumaran benzylic ether reductase (PCBER), pterocarpan reductase, and isoflavone reductase (49, 89). However, considering that pterocarpans and isoflavonoids are phytoalexins not produced by wild-type tobacco, and A622 did not demonstrate PCBER activity, the specific substrate of A622 remains unclear (33, 74, 96). It was suggested that because of the high similarity, these genes may have diverged recently, and possibly the substrate for A622 may be structurally similar to phytoalexins (27).

The suppression of *A622* in tobacco roots results in the inhibition of condensation reactions to form several pyridine alkaloids and the accumulation of nicotinic acid *N*-glucoside (NaNG). Since nicotinic acid itself is not a substrate for A622, and NaNG is a nicotinic acid metabolite, it is speculated whether a derivative of nicotinic acid may be used as A622 substrate (33; Figure 1).

Like other structural genes of the nicotine pathway, *A622* expression occurs in roots, more strongly in the cortex of the root tip and in the outer cortex layer and endodermis in the differentiated region (33). In *N. sylvestris*,

the expression of *A622* is strongly up-regulated by methyl jasmonate (MeJA), and ethylene may work as an antagonist, suppressing the MeJA-induced expression of *A622* (72, 74). At a subcellular level, A622 protein was detected mainly in the cytoplasm of *N. sylvestris* (74).

Recently, a *BBL* gene family was identified for *N. tabacum* and was found to act downstream in the nicotine biosynthetic pathway (45). *BBL*s are expressed in vacuoles of tobacco root cells, and their suppression inhibited anatabine biosynthesis, demonstrating that these genes participate in the pyridine pathway (34).

NICOTINE TRANSLOCATION: FROM THE ROOT TO THE SHOOT OF TOBACCO PLANTS

Nicotine is synthesized in root cortical cells and needs to be transported to the leaves, where it is accumulated and used as antiherbivory defense (79). The nicotine path from roots to leaves implies long-distance transport through the xylem, and should include several transporters (65).

Although most nicotine produced in roots is transported and accumulates in aboveground tissues, a portion of nicotine may stay in root cells (28). High levels of nicotine in roots might trigger feedback inhibition of genes involved in its synthesis (87). Therefore, the maintenance of low nicotine concentrations in the cytoplasm of root cells may be important to ensure its continuous production (70).

The multidrug and toxic compound extrusion (MATE)-type transporters, NtMATE1 and NtMATE2 (collectively called NtMATE1/2), were identified in alkaloid-synthesizing root cells. These transporters are located in the tonoplast and use an antiport of protons from the vacuole to move cytoplasmic nicotine through the tonoplast of root cells (Figure 1). The vacuolar sequestration of nicotine may be necessary to protect the nicotine-synthesizing cells from a potential cytotoxicity caused by this alkaloid (70).

Another transporter from the MATE family, *N. tabacum* jasmonate-inducible alkaloid transporter 1 (Nt-JAT1), is involved in vacuolar accumulation of nicotine in aerial parts of tobacco plants. The expression of *Nt-JAT1* was detected in roots, stems, and leaves, but its subcellular localization in tonoplast was shown just in green leaves (52). However, further investigation is required to clarify the role of *Nt-JAT1* and its location in root cells.

A similar transporter, Nt-JAT2, was found in the tonoplast of tobacco leaves. Nt-JAT2 acts in nicotine transport to the vacuolar lumen specifically in the leaves. It was suggested that Nt-JAT1 is responsible for steady-state alkaloid transport in various tissues, whereas Nt-JAT2 works on the nicotine accumulation in leaves upon herbivore attack (66).

After synthesis, the nicotine produced in roots may move to the apoplast. At this point, it is energetically important for the plant to prevent this nicotine from being secreted into the rhizosphere (28). Nicotine uptake permease 1 (NUP1; Figure 1) is a plasma membrane-localized transporter of purine uptake permease family, presenting a high degree of substrate specificity for nicotine (28, 35). NUP1 uses proton symport for uptake of nicotine (28). *NUP1* expression occurs primarily in epidermal cells of root tips, although low expression was also detected in leaves and stems of tobacco plants. NUP1 may prevent the loss of apoplastic nicotine, or it may even retrieve secreted nicotine back into the root tissues (35).

Transporters responsible for loading nicotine into the xylem in roots, as well as unloading of nicotine from xylem into leaf cells, remain unknown (87; Figure 2). Alkaloids are protonated in apoplastic space because of the acidic condition, becoming more hydrophilic and consequently harder to transpose the plasma membrane (52). Thus, it was speculated that a plasma membrane-localized transporter, like NUP1, may import the nicotine from xylem to leaf cells (31). Therefore, further studies are needed to unravel the whole translocation mechanism of nicotine.

GENETIC AND HORMONAL REGULATION OF NICOTINE STRUCTURAL-GENE EXPRESSION

There are two regulatory loci specifically controlling the expression of nicotine-related structural genes in tobacco, *NIC1* and *NIC2*. Studies using mutants showed that *nic1* and *nic2* mutations are semidominant or show dose-dependent effects on nicotine levels, but the effect of *nic1* was 2.4 times stronger than *nic2* (27, 71). The downregulation of nicotine biosynthesis genes using *nic* mutants has been confirmed in several studies (10, 27, 44, 59).

When compared with wild type, *nic1* and *nic2* double mutants (*nic1nic2*) present the lowest *N*-methylputrescine content, whereas the total content of putrescine, spermidine, and spermine were the highest (27). This suggests that the metabolism of putrescine is blocked through the impairment of *PMT*, and this polyamine accumulates in roots (27). Moreover, *A622*, *QPT*, *QS*, and *BBL* were also shown to be regulated by *NIC* loci (33, 38, 68, 74).

Although the *NIC1* locus remains uncharacterized, the impact of *NIC2* locus on leaf nicotine content became considerably stronger in the presence of *nic1* mutation (44). The *NIC2* locus was shown to encode for a group of transcription factor genes from the IXa ethylene response factor (ERF) subfamily. At least seven *NIC2*-locus *ERF* genes were found as deleted in *nic2* mutants: *ERF189, ERF115, ERF221, ERF104, ERF179, ERF17*, and *ERF168* (71).

Regarding hormonal regulation, it was already shown that most of the genes participating in nicotine biosynthesis and transport, such as *PMT*, *QPT*, *A622*, *MATE1/2*, *Nt-JAT1*, and *NtJAT-2*, are up-regulated by jasmonates (27). Tobacco uses canonical jasmonate signaling components to activate structural genes involved in nicotine biosynthesis (56, 73). Coronative insensitive 1 acts in the perception of jasmonate, degrading jasmonate zim domain (JAZ) proteins, which allows the release of transcription factors to activate the transcription of jasmonate-responsive genes (90).

One of the transcription factors released after JAZ degradation is the highly conserved basic helix-loop-helix MYC2. MYC2 was shown to control the expression of

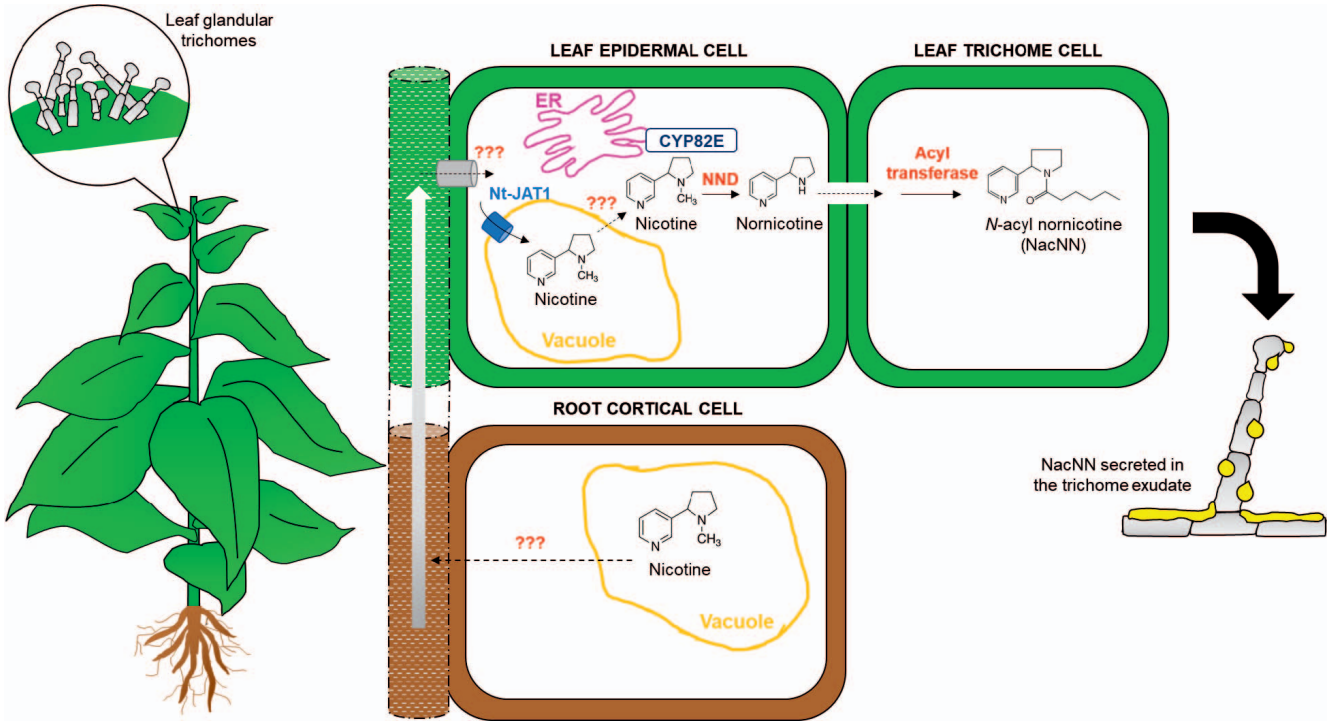

Figure 2. Nicotine produced in the *Nicotiana* root cells is translocated through the xylem to the leaves, where it can be demethylated to nornicotine. Nornicotine, by the action of an acyltransferase, is acylated to *N*-acylnornicotine (NacNN) in the trichomes. NacNN is secreted by the glandular trichome, and acts in the antiherbivory defense. ER, endoplasmic reticulum.

nicotine biosynthetic genes by directly binding its cis elements to the target promoters, some of them (GCC and G-box) derived from transposable element insertions (94). MYC2 also up-regulates *NIC2*-locus *ERF* genes (69). MYC2 also induces the expression of *NUP1*, which in turn positively controls the expression of *ERF189*, one of the major regulators of the nicotine biosynthetic pathway (35).

Another phytohormone involved in the nicotine pathway is auxin, which downregulates nicotine biosynthesis (27). A very common practice among tobacco growers is the removal of the flower head and several young leaves of tobacco plants, also called "topping" (27). This practice switches the plant from its reproductive to its vegetative phase, eliminating apical dominance and favoring root growth (46). After topping, the nicotine concentration increases in tobacco leaves, and it is highly dependent on the removal of apical meristems, the primary source of auxin in the plant (64, 92). Indeed, it was suggested that a reduction of auxin supply to roots might result in activation or derepression of *PMT* (27).

Nicotine biosynthesis must be tightly regulated, since it is highly demanding for the plant (3). An example of this fine regulation is observed when nicotine-tolerant herbivores fed on tobacco leaves, triggering an ethylene burst that suppressed the jasmonate-mediated activation of nicotine biosynthesis genes (32). In this case, the ethylene signaling may prevent the plant from wasting resources on an ineffective nicotine defense (91).

The cross-talk between different signaling pathways, mainly jasmonates and ethylene in nicotine biosynthesis in tobacco, may be critical to managing the destination of the plant energy sources (72). In a herbivore attack scenario, most of the nitrogen available is redirected to alkaloid biosynthesis, targeting plant defense (4). However, since it is not possible to recover alkaloid nitrogen and reinvest it in other metabolic processes, its biosynthesis requires optimum regulation (39).

ALKALOID CONTENT IN DIFFERENT *NICOTIANA* SPECIES

The alkaloid composition within *Nicotiana* is species specific and extremely variable (62, 78). The major alkaloids in *Nicotiana* are nicotine, nornicotine, anatabine, and anabasine, but a single alkaloid usually predominates in each species (62, 80). *Nicotiana tabacum* typically produces nicotine as the most abundant alkaloid (87).

However, despite being a natural allotetraploid derived from interspecific hybridization between ancestral *N. sylvestris* and *N. tomentosiformis, N. tabacum* remarkably presents an alkaloid profile different from both of its progenitor species (19). Dewey and Xie (14) proposed a model of molecular evolution of *N. tabacum* alkaloid content to try to explain these differences. *Nicotiana tomentosiformis* primarily accumulates nornicotine, whereas nicotine and nornicotine are the predominant alkaloids in *N. sylvestris* green and senescing leaves, respectively (78).

Nicotine and nornicotine are the major alkaloids in *Nicotiana* spp., and the insecticidal activity of these alkaloids varies widely depending on the insect species (17). Additionally, in some *Nicotiana* wild species,

nornicotine has an important role as a precursor of *N*-acyl-nornicotine (NacNN), an alkaloid that exhibits 1,000-fold higher activity against nicotine-resistant *Manduca sexta* (tobacco hornworm) than nicotine (29, 40).

NacNN was found in the trichome exudate produced at the epidermis of *Nicotiana repanda*, *Nicotiana stocktonii*, and *Nicotiana nesophila* aerial parts (29, 40, 97). It was suggested as a route by which nicotine is *N*-demethylated to nornicotine in leaves, followed by its mobilization to the trichomes upon herbivory in these species (97; Figure 2). Then, nornicotine is acylated with straight-chain fatty acids, forming hydrophobic NacNN, which is secreted from the gland to the coat of leaf surface (63, 97; Figure 2).

CONCLUDING REMARKS

Alkaloids, especially nicotine, have an essential role in antiherbivory defense of *Nicotiana* (39). The site of nicotine production is the root cortical tissue, after which the alkaloid is translocated via xylem to the leaves, where it is stored in vacuoles (80). Depending on *Nicotiana* species, nicotine can be metabolized in leaves, resulting in different alkaloids, such as nornicotine and NacNN (9, 62, 97; Figure 2). Nicotine biosynthesis, transport to the leaves, and metabolism are multienzymatic and complex processes that have not been completely elucidated. This review presented the known nicotine biosynthesis, transport, and metabolism pathways, and highlighted the points that need further investigation: A) the late steps of nicotine biosynthesis and B) the involvement of the long-distance transport to leaves.

The suppression of both *A622* and *BBL* in tobacco roots inhibited the pyridine pathway, demonstrating the importance of these enzymes to pyridine alkaloid formation (33, 34). Although there is a consensus that *A622* and *BBL* act downstream in this route, it is not clear what the substrate is of each enzyme (45). Moreover, the translocation of nicotine upward to the aerial part of *Nicotiana* involves several transporters. There are currently five transporters known to conduct nicotine translocation in *Nicotiana*: NtMATE1, NtMATE2, NUP1, NtJAT1, and NtJAT2 (28, 35, 52, 66, 70). However, the transporters responsible for loading nicotine into xylem in the root and for unloading nicotine from xylem into leaf cells remain unknown (87).

A recent study reported an improved tobacco genome assembly, increasing the percentage of the tobacco genome anchored to chromosomal locations when compared with the previous report (16, 75). The new genetic road map will be very important for tobacco and plant science research and may also help to elucidate the alkaloid biosynthetic pathway in tobacco. Thus, further studies are needed to unravel the whole biosynthesis and translocation pathways of nicotine in *Nicotiana*.

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