9 Nicotine Biosynthesis, Transport, and Regulation in Tobacco: Insights into the Evolution of a Metabolic Pathway

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Abstract

In *Nicotiana tabacum* (tobacco), nicotine and related pyridine alkaloids are produced in the roots and accumulate mainly in the leaves. Molecular analyses of nicotine biosynthesis, especially of the steps involved in pyrrolidine and pyridine formation, suggest that this specialized pathway evolved through repeated duplication of primary pathways, followed by the recruitment of the metabolic genes into a regulon. In tobacco, jasmonates elicit nicotine formation via a conserved signaling cascade anchored to the downstream nicotine biosynthesis pathway by master transcription factors of the ERF family, particularly ERF189 and its homolog ERF199. ERF transcription factors upregulate metabolic and transport genes directly involved in the pathway by recognizing *cis*-elements in the promoters of target genes. A pair of homologous clusters of related ERF genes, including *ERF189* and *ERF199*, occurs in the tobacco genome. *ERF189* corresponds to the nicotine-controlling *NIC2* locus. A large chromosomal deletion of the cluster that includes *ERF189*, as found in the *nic2* mutant allele, has been exploited to breed low-nicotine tobacco.

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9.1 Introduction

Alkaloids are a large group of nitrogen-containing specialized metabolites, typically with bioactive properties, that are produced by multiple plant species (Shoji 2016). Nicotine and its derivatives, such as nornicotine, anabasine, and anatabine, are pyridine alkaloids found in *Nicotiana* species, including cultivated *Nicotiana tabacum* (tobacco) (Saitoh et al. 1985) (Fig. 9.1). Even though smoking is detrimental to human health, the stimulatory and addictive properties of tobacco alkaloids, which act on nicotinic acetylcholine receptors essential for a range of neuronal activities, account for the widespread consumption of tobacco products.

In tobacco, pyridine alkaloids are synthesized exclusively in underground roots and are largely stored in the leaves as defenses against insects and other predators (Steppuhn et al. 2004). Herbivory can result in increased accumulation of nicotine and other toxic alkaloids (Baldwin 1989). Jasmonates are key signaling molecules that trigger nicotine formation via transcription factors that direct the coordinated expression of genes involved in the nicotine biosynthesis pathway (Shoji et al. 2010). Molecular and genomic analyses have identified genes responsible for nicotine biosynthesis, transport, and regulation (Shoji and Hashimoto 2011a; Dewey and Xie 2013; Shoji and Hashimoto 2013), providing insight into the evolution of this metabolic pathway (Kajikawa et al. 2017a. Shoji 2019).

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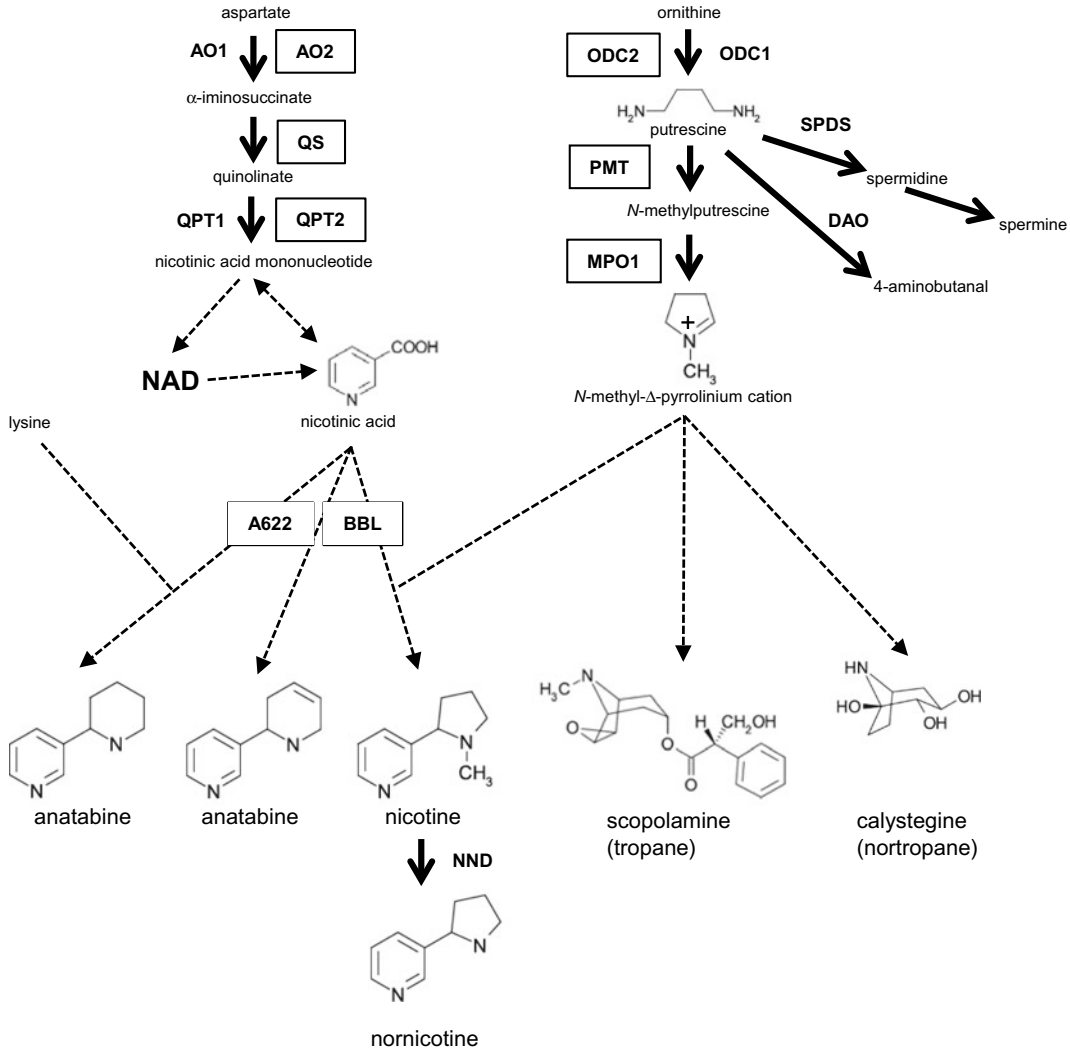

Fig. 9.1 Biosynthesis pathways of nicotine and related alkaloids. Defined steps are shown with arrows and enzyme names; dashed arrows represent undefined or multiple steps. Boxes represent enzymes regulated by ethylene response factor (ERF) transcription factors and thus included in the nicotine biosynthesis regulon. Putrescine *N*-methyltransferase (PMT) and *N*-methylputrescine oxidase (MPO1) have been proposed to evolve from spermidine synthase (SPDS) and diamine oxidase (DAO), respectively (Hibi et al. 1994; Heim et al. 2007; Katoh et al. 2007). AO, aspartate oxidase; BBL, berberine bridge enzyme-like protein; NND, nicotine *N*-demethylase; ODC, ornithine decarboxylase; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthase

9.2 Biosynthesis

Nicotine is composed of heterocyclic pyrrolidine and pyridine rings; these rings are formed early in the pathway and are coupled together in later steps (Fig. 9.1). A five-member pyrrolidine ring is formed from ornithine via a symmetric diamine, putrescine, through three consecutive reactions catalyzed by ornithine decarboxylase (ODC) (Imanishi et al. 1998), putrescine *N*-methyltransferase (PMT) (Hibi et al. 1994), and *N*-methylputrescine oxidase (MPO) (Heim et al. 2007; Katoh et al. 2007) (Fig. 9.1). An alternative

route to putrescine from arginine exists, which includes a step catalyzed by arginine decarboxylase (ADC). However, this route is not considered to contribute greatly to nicotine biosynthesis, because transgenic suppression of *ADC,* but not of *ODC* (De Boer et al. 2011a), failed to alter nicotine content significantly (Chintapakorn and Hamill 1990). Because of their structural similarities, PMT and MPO are thought to have arisen through catalytic innovation from primary enzymes involved in related polyamine metabolism, namely, spermidine synthase and diamine oxidase, respectively (Junker et al. 2013; Naconsie et al. 2014) (Fig. 9.1). The ornithine-derived moiety is also used to produce tropane alkaloids (e.g., the clinically important hyoscyamine and scopolamine) and nortropane alkaloids (e.g., calystegines, which inhibit glycosidase) in various Solanaceae species (Shoji and Hashimoto 2015) (Fig. 9.1). The shared branch for pyrrolidine formation may have developed through duplication of the polyamine pathway. The resulting doubling of *ODC* genes may have enabled increased metabolic flow into this branch, and the subsequent innovative evolution of *PMT* and *MPO1* (Kajikawa et al. 2017a). The establishment of the ring-forming extension before the Solanaceae species diversified is underlined by the common existence of *PMT* and *MPO1* genes in Solanaceae genomes (Kajikawa et al. 2017b; Xu et al. 2017).

A pyridine ring of nicotine and related alkaloids is derived from nicotinic acid, a primary metabolite in the pathway that supplies nicotinamide adenine dinucleotide (NAD), an important co-factor for oxidation–reduction reactions. In the NAD pathway, aspartate is converted to nicotinic acid mononucleotide via quinolinate via steps catalyzed by aspartate oxidase (AO), quinolinate synthase (QS), and quinolinate phosphoribosyltransferase (QPT) (Sinclair et al. 2000; Katoh et al. 2006) (Fig. 9.1). To meet increased metabolic demands to support massive downstream production of nicotine, *AO* and *QPT* genes have been duplicated in *Nicotiana* species, but not in other lineages (Kajikawa et al. 2017b; Xu et al. 2017). Nicotinic acid is supplied as an intermediate of a cyclic pathway for de novo and salvage production of NAD, which starts with nicotinic acid mononucleotide (Noctor et al. 2006) (Fig. 9.1). It is unclear whether nicotinic acid itself or its derivatives are directly incorporated into alkaloids (Shoji and Hashimoto 2011a). To avoid excess accumulation of toxic nicotinic acid, plants have developed mechanisms to convert nicotinic acid to less-toxic derivatives (Li et al. 2015a, b; Li et al. 2017). Using nicotinic acid for alkaloid production may have originally emerged as one such detoxification reaction.

Little is known about the late steps required to couple the heterocyclic rings. It has been proposed that two oxidoreductases, A622 (De Boer et al. 2009; Kajikawa et al. 2009) and berberine bridge enzyme-like protein (BBL) (Kajikawa et al. 2011), catalyze the late stages of the pathway (Fig. 9.1), though biochemical details of their reactions are yet to be defined. A622 and BBL are required to produce not only nicotine, but also other pyridine alkaloids (De Boer et al. 2009; Kajikawa et al. 2009, 2011), implying that A622- and BBL-dependent steps are shared between the pathways that produce the different alkaloids.

Nornicotine is formed from nicotine via demethylation mediated by nicotine *N*-demethylase (NND) (Fig. 9.1), an enzyme belonging to the CYP82E subfamily of cytochrome P450 monooxygenases. Three genes, *CYP82E4*, *CYP82E5*, and *CYP82E10*, encoding functional NND enzymes have been cloned from tobacco (Siminszky et al. 2005; Gavilano and Siminszky 2007; Lewis et al. 2010). Nornicotine typically accounts for 3–5% of the total alkaloids in mature tobacco leaves. *CYP82E5* and *CYP82E10* contribute to this conventional accumulation of nornicotine (Gavilano and Siminszky 2007; Lewis et al. 2010). However, a small number of plants within tobacco populations, especially in Burley cultivars, are termed converters, as they convert over 90% of their nicotine to nornicotine during leaf senescence and curing (Griffith et al. 1955). *CYP82E4* is responsible for this unstable conversion phenotype, which depends on occasional reactivation of normally silenced *CYP82E4* in converters (Siminszky et al. 2005).

Nornicotine is reduced to nominal levels by knockout mutations of all three *CYP82E* genes (Lewis et al. 2010). Because *N*′-nitrosonornicotine is a carcinogen that is more harmful than other tobacco-specific nitrosamines and is readily formed from nornicotine during the curing process (Bush et al. 2001), reducing nornicotine levels is a desirable breeding goal.

9.3 Transport

Many plant metabolites, both end products and intermediates, move within and between cells. Various membrane-localized transporters, such as those belonging to the ATP-binding cassette, multidrug and toxic compound extrusion (MATE), and purine permease families, mediate the active transport of low-molecular-weight compounds across biological membranes, including alkaloids, which are often positively charged and thus membrane-impermeable (Shoji 2014; Shitan et al. 2014a).

To prevent cytotoxicity when accumulated at high concentrations, nicotine is sequestered into storage vacuoles in tobacco cells. Tonoplast-localized MATE transporters, *N. tabacum* jasmonate-inducible alkaloid transporter 1 (Nt-JAT1) (Morita et al. 2009), Nt-JAT2 (Shitan et al. 2014b), NtMATE1, and NtMATE2 (Shoji et al. 2009), are proton antiporters that sequester nicotine in the vacuole. These transporters couple proton gradients across the membrane with the energy-consuming uptake of alkaloids into organelles. The Nt-JAT1 and Nt-JAT2 homologs, which are phylogenetically related to xenobiotic-transporting DTX1 from *Arabidopsis*, mediate the vacuolar sequestration of nicotine in tobacco leaves (Morita et al. 2009; Shitan et al. 2014b). *NtMATE1* and *NtMATE2*, which encode homologs of flavonoid transporters, are co-expressed with nicotine biosynthesis genes, and the encoded proteins are thereby involved in the uptake of nicotine into the vacuoles in alkaloid-producing roots (Shoji et al. 2009). It is noteworthy that *NtMATE2* resides near a gene encoding a late-step enzyme A622 (see "Biosynthesis" section) on chromosome (chr) 12 in the tobacco genome; this is the only example of non-homologous clustering among nicotine pathway genes reported to date (Kajikawa et al. 2017b).

Nicotine uptake permease 1 (NUP1) is a plasma membrane-localized purine permease-family transporter of the pyridine ring-bearing molecules nicotine and vitamin B6 (e.g., pyridoxine) (Hildreth et al. 2011; Kato et al. 2014, 2015). Unlike other nicotine pathway genes expressed in inner cell layers (Shoji et al. 2000, 2002, 2009; Shoji and Hashimoto 2011b; Kajikawa et al. 2017b), *NUP1* is mainly expressed in root epidermal cells (Kato et al. 2014). In addition to its role in metabolite transport, *NUP1* is involved in the genetic regulation of nicotine biosynthesis by way of master transcription factors (see "Regulation" section) and in the regulation of root growth (Hildreth et al. 2011; Kato et al. 2014). The mechanisms underlying this regulation, however, are unclear.

Dawson (1942) conducted a classic grafting experiment that clearly demonstrated root-to-shoot transport of tobacco alkaloids between tobacco rootstock and *Solanum lycopersicum* (tomato) scion. It is known that nicotine moves up through the xylem along the transpiration stream, and that xylem loading and unloading depend on nicotine efflux from root cells and influx into leaf cells, respectively, although the transporters responsible for these processes have yet to be defined. Unlike most species in the *Nicotiana* genus, the flowering tobacco *N. alata* is devoid of alkaloids in aboveground shoots, because it lacks long-distance translocation ability (Pakdeechanuan et al. 2012). Elucidating the genetic basis of this natural variation may provide mechanistic insights into alkaloid transport.

9.4 Regulation

While primary pathways are nearly constitutive, pathways for specialized metabolites are subject to dynamic regulation in developmental and environmental contexts. Metabolic flow through a long, multistep pathway relies on the

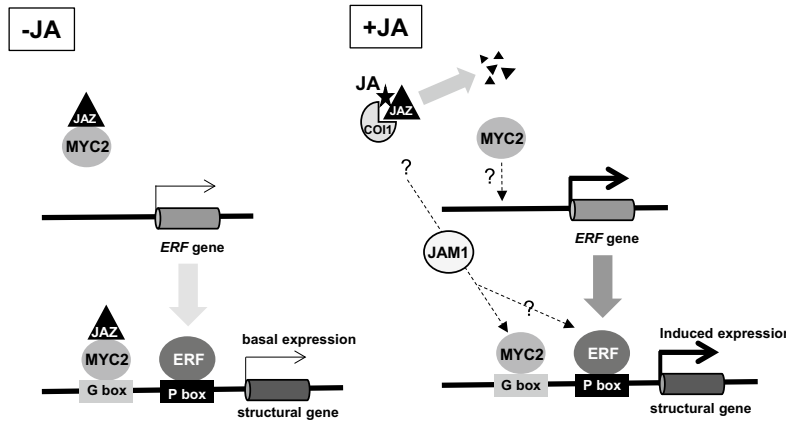

Fig. 9.2 A model for jasmonate (JA)-dependent regulation of nicotine biosynthesis in tobacco. Ethylene response factor (ERF) transcription factors regulate the structural genes of the nicotine pathway by binding at P box elements in their promoters. JA-induced transcription of the *ERF* genes is mediated by a basic helix-loop-helix (bHLH)-family MYC2 transcription factor, a direct target of upstream jasmonate ZIM-domain (JAZ) repressors. MYC2 regulates structural genes along with ERF by directly binding to G box elements. JAZ proteins are degraded when a co-receptor complex comprised of coronatine-insensitive 1 (COI1) and JAZ proteins perceives a JA signal. ERF and MYC2 transcription factors are stimulated by a phosphorylation cascade that includes the JA factor-stimulating MAPKK (JAM1). Other details of the regulation of *ERF* by MYC2 and the phosphorylation cascade are unclear

coordinated expression of metabolic and transport genes, or structural genes. Transcription factors typically control this coordination at the transcriptional level, often forming a multigene network, or regulon, with downstream structural genes (Shoji 2019).

A few transcription factors in the ethylene response factor (ERF) family, particularly ERF189 and its closest homolog ERF199, are involved in the master transcriptional regulation of nicotine pathway genes in tobacco (Dewey and Xie 2013; Shoji and Hashimoto 2013a) (Fig. 9.2). *ERF189* and *ERF199* are expressed strongly, but not exclusively, in the roots and are induced by jasmonates along with structural genes at the transcript level (Shoji et al. 2010; Kajikawa et al. 2017b). ERF transcription factors upregulate nearly the entire series of nicotine metabolic and transport genes, including *ODC2*, *PMT*, *MPO1*, *AO2*, *QS*, *QPT2*, *A622*, *BBL*, *NtMATE1*, and *NtMATE2* (Fig. 9.1), but not *NUP1*, *NtJAT1*, or *NtJAT2*, by directly recognizing specific *cis*-regulatory elements, termed P boxes, in their promoter regions (Fig. 9.2) (Todd et al. 2010; Shoji et al. 2010; De Boer et al. 2011b; Shoji and Hashimoto 2011b; Kajikawa et al. 2017b). The GC-rich P box resembles a canonical GCC box, and a few amino acid residues critical for its recognition have been identified within the DNA-binding domain of the ERF transcription factors (Shoji et al. 2013).

In the allotetraploid *N. tabacum* genome, there are two homologous clusters of related *ERF* genes, including *ERF189* and *ERF 199*. In one cluster, *ERF189* is located on chr 19, and in the other, *ERF199* is located on chr 7 (Fig. 9.3). These clusters may have originated from the diploid ancestral parents of *N. tabacum*, *N. tomentosiformis,* and *N. sylvestris*, respectively (Shoji et al. 2010; Kajikawa et al. 2017b). The *ERF189*-containing cluster corresponds to the *NIC2* locus, one of two genetic loci controlling nicotine content in tobacco (Shoji et al. 2010). A substantial chromosomal deletion (ca. 650 kb) encompassing a large portion of the cluster, including *ERF189* (Fig. 9.3), was found in a *nic2*

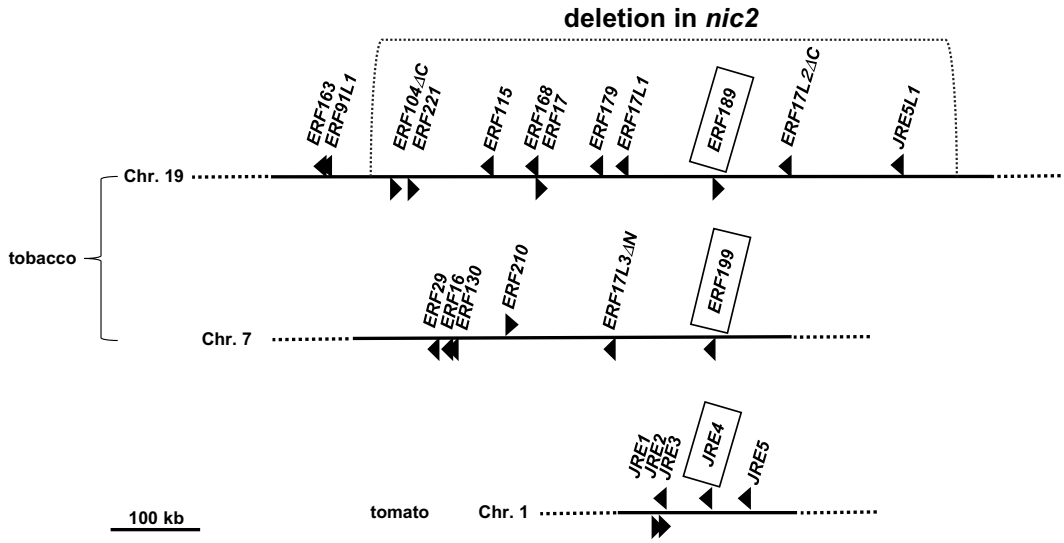

Fig. 9.3 Clustered ethylene response factor (ERF) transcription factor genes in tobacco and tomato. Arrowheads indicate positions and orientations of predicted open reading frames of *ERF* genes. A chromosomal region of tobacco chr. 19 deleted in *nic2* mutant is indicated. Boxes indicate *ERF189* and *ERF199* from tobacco, which are involved in nicotine regulation, and *JRE4* from tomato, which is involved in steroidal glycoalkaloid regulation. Tobacco *ERF* genes, denoted by Δ, possibly encode non-functional transcription factors that lack full-length DNA-binding domains

mutant allele (Kajikawa et al. 2017b) used to breed low-nicotine tobacco cultivars (Legg and Collins 1971).

Given that *ERF* genes exhibit low basal levels of expression and are induced by salt stress (Shoji and Hashimoto 2015; Kajikawa et al. 2017b), and considering the limited effects of overexpression on nicotine biosynthesis (Shoji et al. 2010), the contribution to nicotine regulation of clustered *ERF* genes other than *ERF189* and *ERF199* is believed to be limited. Experimental evidence contradicting this view, however, exists (De Boer et al. 2011b; Sears et al. 2014). Further work is required to functionally differentiate related and mostly clustered *ERF* genes in the regulation of nicotine and other pathways.

Clusters of transcription factor genes homologous to tobacco *ERF*s are present in the genomes of other species (Cárdenas et al. 2016; Thagun et al. 2016; Paul et al. 2017). For instance, *JRE4* (also known as *GAME9*) from tomato (Fig. 9.3) and potato (*Solanum tuberosum*) (Cárdenas et al. 2016; Thagun et al. 2016, Nakayasu et al. 2018) and *ORCA*s from *Catharanthus roseus* (van der Fits and Memelink 2000; Paul et al. 2017) regulate the jasmonate-induced production of specialized metabolites, suggesting that these related factors are functionally similar (Shoji and Hashimoto 2013b). Consistent with such notion, cell type-specific and jasmonate-inducible expression of a promoter reporter of tobacco *QPT2* regulated by ERF189 was found to be mediated by JRE4 in tomato (Shoji and Hashimoto 2019).

In plants, jasmonate signals are perceived by a co-receptor complex comprised of coronatine-insensitive 1 (COI1) and jasmonate ZIM-domain (JAZ) proteins. This triggers proteasome-dependent degradation of JAZs and subsequent transcriptional activation mediated by a basic helix-loop-helix (bHLH)-family MYC2 transcription factor liberated from repression by JAZs (Wasternack and Hause 2013) (Fig. 9.2). Jasmonate-induced formation of nicotine depends on COI1, JAZ, and MYC2 proteins in tobacco (Paschold et al. 2007; Shoji et al. 2008; Todd et al. 2010; De Boer et al. 2011b; Shoji and

Hashimoto 2011c; Zhang et al. 2012) (Fig. 9.2). In cooperation with ERF factors, tobacco MYC2 factors regulate a series of nicotine pathway genes directly by recognizing G box elements in their promoters (Shoji and Hashimoto 2011c; Zhang et al. 2012). These factors also act indirectly through transcriptional activation of the *ERF* genes (Shoji and Hashimoto 2011c) (Fig. 9.2). Similar schemes linking MYC2, a central player in general jasmonate signaling, to downstream ERFs and defense metabolism have been demonstrated in other plants (Zhang et al. 2011; Cárdenas et al. 2016; Paul et al. 2017). In addition to transcriptional regulation, ERF proteins are postulated to be regulated by protein phosphorylation in *N. tabacum* (De Boer et al. 2011b; Paul et al. 2017). In tobacco, a protein phosphorylation cascade involving a mitogen-activated protein kinase kinase (MAPKK), jasmonate factor-stimulating MAPKK (JAM1), has been proposed to stimulate nicotine biosynthesis via the ERF and MYC2 transcription factors (De Boer et al. 2011b) (Fig. 9.2).

A long non-coding RNA acting as an endogenous target mimicry and its corresponding microRNA predicted to target *QPT2* gene were identified and found to be involved in topping-triggered induction of nicotine accumulation (Li et al. 2015a, b), presenting an example of regulation of specialized metabolism by a module consisting of non-coding RNAs.

9.5 Perspectives

Molecular and genomic studies have greatly advanced the understanding of the nicotine biosynthesis pathway in tobacco. Complementing gene cloning efforts based mainly on homologies and expression profiles, the genome sequences of tobacco (Sierro et al. 2014) and two wild *Nicotiana* species (Xu et al. 2017) have revealed properties of the entire suite of genes involved in nicotine biosynthesis and regulation (Kajikawa et al. 2017b; Xu et al. 2017). With several structural and regulatory genes now known, it is easier to genetically manipulate the biosynthesis of the toxic alkaloids (Sato et al. 2001; Lewis et al. 2010; De Boer et al. 2011a). Recent advances have identified a critical role for transcription factors in nicotine regulation, demonstrating conserved regulatory circuits centering on jasmonate-responsive ERF transcription factors. Molecular studies of the nicotine biosynthesis pathway have elucidated how such coordinated systems were established during plant evolution (Kajikawa et al. 2017b; Shoji 2019).

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