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Review

Smoking out the masters: transcriptional regulators for nicotine biosynthesis in tobacco

Tsubasa Shoji*, Takashi Hashimoto

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan
*E-mail: t-shouji@bs.naist.jp Tel: +81-743-72-5524 Fax: +81-743-72-5529

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Abstract Nicotine and tropane alkaloids are specialized metabolites produced in certain species of Solanaceae, and some of these alkaloids have been used as pharmacological agents. In tobacco plants, nicotine is a defensive toxin against herbivorous insects, and jasmonate (JA) signaling leads to the induction of nicotine biosynthesis. JA-responsive structural genes of the nicotine pathway have been identified as being down-regulated in a low-nicotine tobacco mutant, which possesses mutant alleles at two loci, *NICOTINE1* and *NICOTINE2* (*NIC1* and *NIC2*). A group of JA-responsive genes that encode homologous ERF transcription factors are clustered at the *NIC2* locus and deleted in the mutant. These *NIC2*-locus ERFs up-regulate the structural genes of the biosynthetic pathway by recognizing GCC-like boxes in their promoters, forming a regulon for nicotine biosynthesis with the downstream targeted genes. The three basic components in JA signaling, COI1, JAZ, and MYC2, are required for JA-induced nicotine formation in tobacco. The bHLH transcription factor MYC2 positively regulates the structural genes, both directly by recognizing G boxes in their promoters and indirectly by up-regulating *NIC2*-locus *ERF* genes. Molecular elucidation of nicotine regulation would lead us to better understand the JA-dependent regulation of a wide range of phytochemicals.

Key words: nicotine, tobacco, ERF transcription factor, jasmonate signaling.

Human use of Solanaceae alkaloids

The family Solanaceae includes a handful of species that accumulate a group of highly toxic pyrrolidine alkaloids: tropane alkaloids in *Atropa*, *Datura*, and *Hyoscyamus*, and biogenetically related nicotine alkaloids in *Nicotiana* (Hashimoto and Yamada 1994) (Figure 1). While intoxication by these alkaloids is sporadically reported, their use by humans can be traced back to ancient times. The Egyptian queen Cleopatra (BC 69 to 30) is said to have used extract of *Atropa belladonna*, a representative medicinal plant which contains tropane alkaloids such as hyoscyamine and scopolamine, to dilate her pupils and so make herself more attractive as a political tactic. In the eighteenth century, a mixture including a tropane-containing extract of *Datura metel* was used as a general anesthetic for the first time by the Japanese surgeon Seishu Hanaoka (1760 to 1835) to treat his wife's breast cancer. Atropine, a racemic mixture of two hyoscyamine forms, and scopolamine, are used as anticholinergic medicines that generally lower the parasympathetic activity of muscles and glands. Even today, due to their complex chemistry, these alkaloids are extracted from tissues of medicinal plants that are mainly cultivated in tropical plantations.

When Columbus explored the New World, its native inhabitants were already smoking the leaves of wild *Nicotiana* species indigenous to the region. Because of its addictive nature, smoking has become widespread around the world and tobacco (*Nicotiana tabacum*) is still one of the major crops cultivated worldwide (Goodman 1993). Of course, smoking is no longer recommended because of its markedly harmful effects on human health (Hecht 2003), and regulations on smoking are becoming increasingly restrictive nowadays. In tobacco products, nicotine is the major determinant of addiction to smoking, and reducing nicotine level is thus an important goal in tobacco breeding.

Biosynthetic pathway

Classical metabolic labeling experiments followed by enzymatic studies defined the biosynthetic pathways of the alkaloids (Figure 1; Hashimoto and Yamada 1994; Leete 1983). In particular, the use of cultured tissues and cells selected for high alkaloid production

Abbreviations: AO, aspartate oxidase; AP, APETALA; BBL, berberine bridge enzyme-like protein; bHLH, basic helix-loop-helix; COI, coronatine insensitive; ERF, ethylene response factor; JA, jasmonate; JAZ, jasmonate ZIM-domain; MATE, multidrug and toxic compound extrusion transporter; MPO, *N*-methylputrescine oxidase; NIC, NICOTINE; NUP, nicotine uptake permease; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthase.

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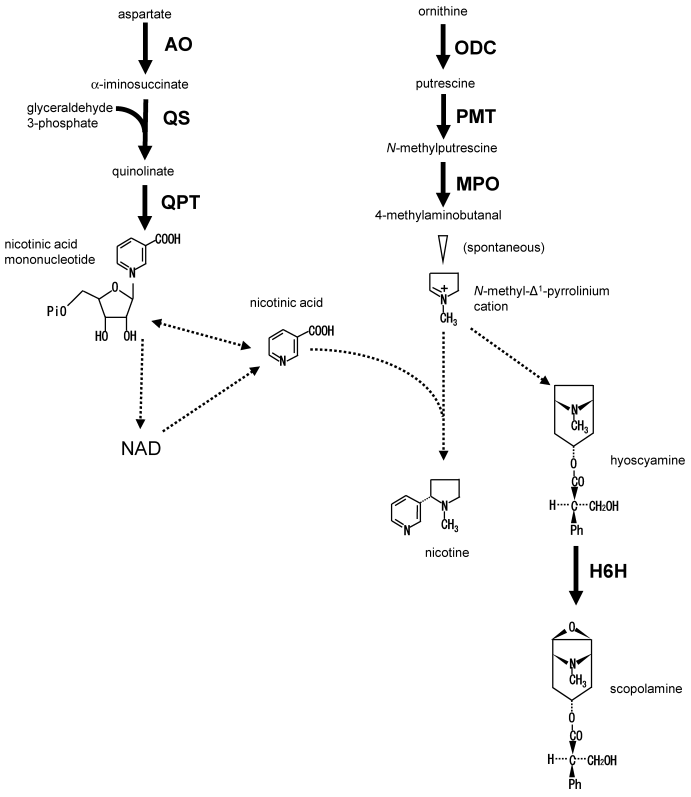

Figure 1. Biosynthesis pathways of nicotine and tropane alkaloids. Solid lines indicate defined steps, while broken lines indicate undefined steps or steps including multiple reactions. Abbreviations are as follows: ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; MPO, *N*-methyltransferase oxidase; AO, aspartate oxidase; QS, quinolinate synthase; QPT, quinolinate phosphoribosyltransferase; H6H, hyoscyamine 6β-hydroxylase; Pi, inorganic phosphate; Ph, phenyl group.

facilitated detailed biochemical studies, such as the purification of enzymes that allowed the molecular cloning of corresponding genes. Efforts to clone alkaloid biosynthesis genes began in the early 1990s (Hibi et al. 1994; Matsuda et al. 1991) and became widespread with the advent of molecular biology (Facchini 2001; Hashimoto and Yamada 1994, 2003; Ziegler and Facchini 2008).

A pyrrolidine ring, which is incorporated into nicotine or further converted to a bi-cyclic tropane ring, is generated from ornithine through three consecutive reactions catalyzed by ornithine decarboxylase (ODC), putrescine *N*-methyltransferase (PMT), and *N*-methylputrescine oxidase (MPO), and this part of the pathway is shared among all species producing pyrrolidine alkaloids (Hashimoto and Yamada 1994; Hibi et al. 1994; Shoji and Hashimoto 2011a; Shoji and Hashimoto 2013) (Figure 1). A pyridine ring, the other ring of nicotine, is derived from nicotinic acid, a primary metabolite in NAD metabolism, which occurs in every organism to supply the essential co-factor. In dicotyledonous plants, including tobacco, the pathway that generates NAD and nicotinic acid starts from aspartate, which is converted to nicotinic acid mononucleotide through reactions catalyzed by aspartate oxidase (AO), quinolinate synthase (QS), and quinolinate phosphoribosyltransferase (QPT) (Katoh and Hashimoto 2004; Katoh et al. 2006) (Figure 1). A cyclic pathway that starts from nicotinic acid mononucleotide performs de novo and salvage production of NAD, supplying nicotinic acid as an intermediate (Katoh and Hashimoto 2004). In contrast to the early steps that form the rings, little is known about the late steps responsible for ring coupling. Two orphan reductases, A622 of the PIP family (named after its founding members, pinoresinol-lariciresinol reductase, isoflavone reductase, and phenylcoumaran benzylic ether reductase) and berberine bridge enzyme-like protein (BBL) (belonging to a protein family including berberine bridge enzymes, carbohydrate oxidases, cannabinoid synthases, and 6-hydroxynicotine oxidases), are postulated to be involved in the late steps, but details of the reactions that they catalyze have yet to be defined (De Boer et al. 2009; Kajikawa et al. 2009; Kajikawa et al. 2011).

Once formed, nicotine moves dynamically through biological membranes via tonoplast-localized multidrug and toxic compound extrusion transporters (MATEs), MATE1, MATE2, and jasmonate-inducible alkaloid transporter 1 (JAT1) (Morita et al. 2009; Shoji et al. 2009), and plasma membrane-localized purine permease family member nicotine uptake permease 1 (NUP1) (Hildreth et al. 2011). There are likely to be

further, unknown transporters for nicotine and pathway intermediates.

ERF transcription factor genes cluster at the nicotine-controlling NICOTINE2 locus

Mutants displaying altered nicotine levels are valuable resources for biosynthesis studies. A naturally occurring low-nicotine tobacco mutant was discovered in Europe in the early 1930s, and the trait was introduced through repeated introgressions into commercial tobacco cultivars to meet a demand for low-nicotine cigarettes. Through the breeding process, the genetic basis of this mutant became clear: mutant alleles at two distinct loci, *NICOTINE1* and *NICOTINE2* (*NIC1* and *NIC2*, originally called the *A* and *B* loci), are responsible for the low-nicotine trait. Only a slight reduction in nicotine level is observed in either single mutant, *nic1* having a stronger effect than *nic2* (Legg and Collins 1971). Biochemical and molecular characterization of the low-nicotine mutant revealed that multiple biosynthesis steps, rather than a single step, are blocked or markedly suppressed in the mutant, implying regulatory roles for the *NIC* loci (Saunders and Bush 1979; Shoji and Hashimoto 2013). Indeed, screening for genes suppressed in the mutant by three molecular approaches, cDNA subtraction (Hibi et al. 1994), differential display (Kidd et al. 2006; Shoji et al. 2009), and cDNA microarray (Kajikawa et al. 2011; Katoh et al. 2007; Shoji et al. 2010), led to the identification of nearly all of the known metabolic and transport genes involved in the nicotine pathway: *ODC*, *PMT*, *MPO*, *AO*, *QS*, *QPT*, *A622*, *BBL*, *MATE1*, *MATE2*, and *NUP1*.

Until recently, the molecular identities of the *NIC* genes had been long-standing questions. We found some clues to their identity through cDNA microarray analysis in a further search for genes suppressed in the low-nicotine mutant (Shoji et al. 2010). In addition to a series of structural genes of the biosynthetic pathway, the *ERF189* gene, which encodes a transcription factor of the APETALA2 (AP2)/ethylene response factor (ERF) superfamily (Nakano et al. 2006), was found to be severely suppressed in the *nic1nic2* mutant. In the tobacco genome, *ERF189* and its close homologs belong to group IXa of the AP2/ERF superfamily (Rushton et al. 2008), members of which can be further divided into clade 1 and the ERF189-containing clade 2 (Figure 2). Characterization of these ERFs revealed that at least seven members of clade 2, including ERF189, are deleted altogether in the *nic2* mutant (Figure 2); PCR did not detect the genes in the mutant genome and no expression of *ERF189* was detected by RT-PCR in the *nic2* mutant, while specific detection of the others is equivocal. Furthermore, since genetic analysis to examine linkage between the mutant allele and each gene deletion indicated a clustering of the deleted *ERF* genes

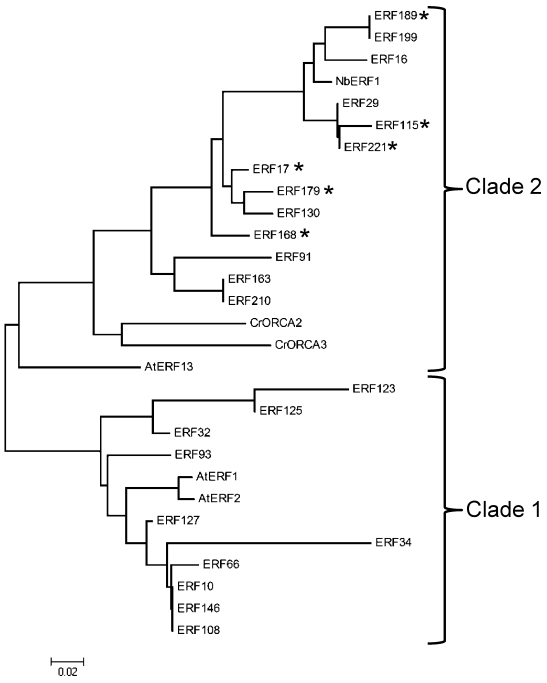

Figure 2. Phylogenetic relationship of group IXa ERF proteins. Based on the alignment of conserved DNA-binding AP2/ERF domain sequences, a phylogenetic tree was constructed by the neighbor-joining algorithm using MEGA4 (Tamura et al. 2007). The scale bar indicates the number of amino acid substitutions per site. Six of the seven ERFs whose genes are deleted in the *nic2* mutant are indicated by asterisks. Although the gene encoding ERF104 is also deleted in *nic2*, ERF104 is not included in this tree because a stop codon in the AP2/ERF domain suggests that *ERF104* is a pseudo-gene. AtERF1 (At4g17500), AtERF2 (At5g47220), and AtERF13 (At2g44840) are from *Arabidopsis thaliana*, CrORCA2 (AJ238740) and CrORCA3 (EU072424) are from *Catharanthus roseus*, NbERF1 (GQ859157) is from *N. benthamiana*, and others are from tobacco. Sequences of tobacco ERFs are available at TOBFAC (Rushton et al. 2008) under the same names.

at the *NIC2* locus, the molecular basis of the mutation is presumably the extensive chromosomal deletion of these so-called *NIC2*-locus *ERF*s. Genomic sequencing of the region will determine whether this assumption is correct. Apart from the seven deleted genes, the existence in the genome and possibly the expression of the other group IXa members are not affected by the *nic2* mutation, and so far no abnormality of any group IXa ERF tested has been found in the *nic1* mutant at either the genomic or the transcript level (Shoji et al. 2010, and our unpublished data). The ancestral origin of the *ERF* genes clearly explains why only a fraction of the clade 2 *ERF*s are missing in *nic2*. *NIC2*-locus *ERF* genes may be derived from *N. tomentosiformis*, one of the two diploid ancestors of allotetraploid *N. tabacum* (Clarkson et al. 2005), and a hypothetical equivalent of the *NIC2* locus from the other diploid ancestor, *N. sylvestris*, may contain the clade 2 *ERF* genes that are unchanged in *nic2*. The relatively mild phenotype of the *nic2* mutant may be attributed to the existence of such *N. sylvestris*-derived, functionally redundant *ERF* genes.

NIC2-locus ERFs and closely related factors have also been proposed to be nicotine regulators in other studies based on a high-throughput trans-activation assay of the *PMT* promoter using a transient expression system in tobacco protoplasts (De Sutter et al. 2005) and a functional screening using virus-induced gene silencing in *N. benthamiana* (Todd et al. 2010).

A regulon for nicotine biosynthesis

NIC2-locus ERFs directly up-regulate structural genes of the nicotine pathway, forming a nicotine biosynthesis regulon with their downstream target genes (Shoji et al. 2010; Shoji and Hashimoto 2011b; Shoji and Hashimoto 2012a). Over-expression of a certain member of *NIC2*-locus *ERF* genes increased alkaloid level dramatically in the low-nicotine *nic1nic*2 mutant and several-fold in wild-type tobacco, which accumulates nicotine to a high level even without the over-expression (De Boer et al. 2011; Shoji et al. 2010). Conversely, loss-of-function of *NIC2*-locus *ERF*s, achieved by collective down-regulation of the genes either by RNA interference or by expression of the *ERF*s under the control of a dominant-repressive motif, drastically decreased the alkaloid content (Shoji et al. 2010). Using the glucocorticoid receptor fusion system, we observed activation of the nicotine pathway genes in response to steroid-induced activation of *NIC2*-locus ERFs even in the presence of a protein synthesis inhibitor, suggesting that the structural genes of the pathway are activated directly by the ERF transcription factors (Shoji et al. 2010).

All *NIC*-controlled nicotine biosynthesis genes examined to date are immediate targets of *NIC2*-locus *ERF*s. At least eight GCC-like boxes recognized *in vitro* by *NIC2*-locus ERFs are present in the examined *NIC*-controlled promoter regions, and their consensus sequence, 5′-A/CGCA/CNNCCA/T-3′ (Shoji and Hashimoto 2012a), is similar but not identical to the sequence of the canonical GCC box, 5′-AGCCGCC-3′, a typical binding sequence for ERF factors, indicating that the *NIC2*-locus ERFs have a unique binding preference.

Based on the distribution of GCC-like boxes recognized by *NIC2*-locus ERFs in the *NIC*-controlled promoters, we could distinguish two *QPT* genes, *QPT1* and *QPT2*, that encode an enzyme involved in the formation of the pyridine ring that is utilized for both NAD and alkaloid synthesis (Shoji and Hashimoto 2011b). Only the *QPT2* promoter bears GCC-like boxes; in tobacco, three such boxes are in the proximal promoter region of *QPT2*, whereas none occur in that of *QPT1*. Accordingly, *QPT2* is controlled by *NIC2* locus-ERFs and is expressed in concert with other nicotine biosynthesis genes, while *QPT1* expression is nearly constant in all tissues examined (Ryan et al. 2012; Shoji and Hashimoto 2011b). We infer that the *QPT2* gene acquired the GCC-like boxes in its promoter to become involved in the nicotine biosynthesis regulon under the control of *NIC2*-locus *ERF*s, and that QPT2 supplies the pyridine ring that is increasingly demanded for nicotine synthesis, whereas the original role of sustaining metabolic flow for NAD synthesis is fulfilled by continuous *QPT1* expression.

Jasmonate signaling

Chemical defense based on nicotine has been well studied in ecological terms (Baldwin 1998; Steppuhn et al. 2004). In addition to a basal level of production, nicotine synthesis readily increases in response to insect attack or wounding through signaling mediated by jasmonates (JAs) (Baldwin 1989; Baldwin et al. 1996). Damage-induced JA elevation in the leaf initiates the signaling, and movement of JA through the phloem ensures the systemic spread of the signal (Baldwin et al. 1994). Yet JA's role as systemically transmitted signal is still controversial and other possibilities cannot be excluded. In *Nicotiana* roots and cultured cells, JAs coordinately up-regulate the nicotine metabolic and transport genes *ODC*, *PMT*, *MPO*, *AO*, *QS*, *QPT*, *A622*, *BBL*, *MATE1*, *MATE2*, *JAT1*, and *NUP1*, all of which except *JAT1* are also under the control of *NIC* genes (see above) (Shoji and Hashimoto 2011a; Shoji and Hashimoto 2013).

Molecular genetic studies using *Arabidopsis* have unveiled the basic framework of JA signaling from perception to gene activation (Browse 2009; Chung et al. 2009). A bioactive form of JA, JA-Ile, is perceived by a co-receptor complex of coronatine insensitive1 (COI1), an F-box component of an SCF-type E3-ubiquitin ligase complex (SCF^{COI1}), and JA ZIM-domain (JAZ) proteins, triggering ubiquitination of JAZs by the ligase activity and their subsequent removal by 26S proteasome-mediated degradation (Chini et al. 2007; Thines et al. 2007). JAZ sequences are not highly conserved except for the Jas and TIFY motifs, which are important for protein-protein interactions: homo- and heterodimer formation of JAZs depends on interactions involving the TIFY motif, whereas the Jas motif is at the interface for JAZ-COI1 and JAZ-MYC2 (see below) interactions (Chini et al. 2009; Melotto et al. 2008). JAZs interact with various transcription factors and thereby connect them through an adaptor protein, novel interactor of JAZ (NINJA), to a Groucho/Tup1-type co-repressor, which suppresses transcription of nearby targeted genes, possibly through chromosomal remodeling (Pauwels et al. 2010). The transcription factors are liberated from the repressor complex after JA-dependent removal of JAZs, and regulate downstream genes both positively and negatively (Dombrecht et al. 2007). A list of the transcription factors targeted by JAZs includes the basic helix-loop-helix (bHLH) family member MYC2 and the related MYC3 and MYC4; the MYB family members MYB21 and

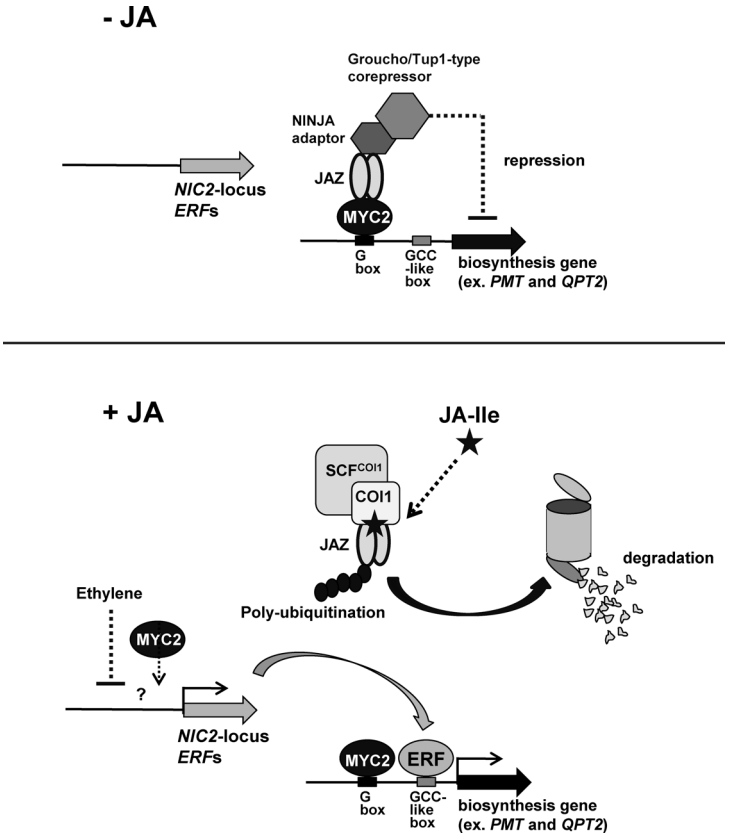

Figure 3. Model of JA-mediated regulation of nicotine biosynthesis. In the absence of JA signal input, JAZs interact with MYC2 and recruit a Groucho/Tup1-type co-repressor, which suppresses genes targeted by MYC2, through an adaptor protein NINJA. After JA-Ile is recognized by a co-receptor complex of COI1 and JAZ, JAZs are ubiquitinated by an SCF complex containing COI1 and then degraded by the 26S proteasome. The removal of JAZs allows downstream MYC2 to activate the genes for nicotine biosynthesis, either directly by binding to the corresponding promoters at the G box or indirectly by activating *NIC2*-locus ERFs. *NIC2*-locus ERF transcription factors up-regulate all metabolic and transport genes of nicotine pathway by binding at GCC-like boxes in their promoters. Ethylene signaling may suppress the transcription of *NIC2*-locus *ERF*s to negatively regulate the JA response of nicotine biosynthesis.

MYB24, which are required for stamen development; and complex-forming bHLH and R2R3-MYB family factors that mediate anthocyanin accumulation and trichome initiation (Chini et al. 2007; Fernandez-Calvo et al. 2011; Qi et al. 2011; Song et al. 2011; Thines et al. 2007).

The basic components in JA signaling are conserved evolutionarily and *Nicotiana* COI1, JAZs and MYC2 are all required for JA-induced nicotine formation (see below for MYC2) (Figure 3). Suppression of *Nicotiana COI1* effectively impaired JA- and wound-induced nicotine formation along with other JA responses (Paschold et al. 2007; Shoji et al. 2008). Like their *Arabidopsis* counterparts, *Nicotiana JAZ* transcripts and proteins are induced and degraded, respectively, in response to JA (Oh et al. 2012; Shoji et al. 2008). Expression of truncated forms of JAZs lacking the C-terminal Jas motif, which may act in a dominant-negative manner by forming non-functional JAZ dimers, also clearly inhibited nicotine induction by JA (Shoji et al. 2008). Interestingly, RNA interference-mediated knockdown of a single JAZ member in *N. attenuata*, *JAZh*, significantly reduces nicotine levels, but enhances other JA-dependent responses (Oh et al. 2012). These latter observations, which seem contradictory at first glance, implicate mutual cross-regulation of *JAZ*s, where JAZh may negatively regulate other *JAZ*s that act as repressors of the nicotine response.

Transcriptional regulators for the jasmonate response

JA positively regulates nicotine pathway genes at the transcriptional level (Shoji et al. 2000a). As revealed for *PMT* and *QPT2* genes, JA-mediated induction of these genes depends on two distinct *cis*-elements in their proximal promoter regions, the G box and GCC-like box (Oki and Hashimoto 2004; Xu and Timko 2004), which are recognized by MYC2 and *NIC2*-locus ERFs, respectively (De Boer et al. 2011; Shoji et al. 2010; Shoji and Hashimoto 2011b; Shoji and Hashimoto 2011c; Todd et al. 2010; Zhang et al. 2012) (Figure 3). These transcription factors are also induced at the transcript level by JA and cooperatively activate their target promoters when expressed transiently in tobacco cells. Suppression of the transcription factor genes decreases

both alkaloid level and the expression of genes involved in nicotine biosynthesis. While MYC2 is directly targeted by JAZ repressors and plays wide-ranging roles in JA signaling unrestricted to nicotine regulation, *NIC2*-locus ERFs are more specialized to regulating the nicotine pathway and do not interact with tested JAZs in the yeast two-hybrid assay (De Boer et al. 2011; Shoji and Hashimoto 2011c).

Little is yet known about how *NIC2*-locus ERFs are regulated. Some *NIC2*-locus *ERF* transcripts are immediately induced by JA, while others are gradually induced at later time points (Shoji et al. 2010). Ethylene signaling may integrate with JA signaling in the transcriptional regulation of *NIC2*-locus *ERF*s (Figure 3), since JA-mediated induction of the *ERF* transcripts along with those of downstream structural genes is suppressed by ethylene precursor treatment, even though these suppressive effects are incomplete and only apparent after prolonged treatment (Shoji et al. 2000b; 2010). When *MYC2* was silenced by RNA interference in tobacco hairy roots, all *NIC2*-locus *ERF*s and thus their downstream structural genes were down-regulated. In contrast, *MYC2* expression level was not altered either in *nic* mutants or in transgenic tobacco hairy roots in which a dominant-repressive form of a *NIC2*-locus ERF was over-expressed. These results suggest that *MYC2* regulates *NIC2*-locus *ERF* genes, but not vice versa (Shoji and Hashimoto 2011c) (Figure 3). Further studies are necessary to address how individual *NIC2*-locus *ERF* genes are regulated by MYC2, either directly or indirectly, and whether they are regulated at levels other than transcription.

Regulatory function of proteins related to tobacco NIC2-locus ERFs

Tobacco *NIC2*-locus ERFs are related to *Catharanthus roseus* ORCA2 and ORCA3 (Figure 2), which are known to regulate the JA response of terpenoid indole alkaloid biosynthesis in this species (van der Fits and Memelink 2000). The GCC-like boxes to which tobacco *NIC2*-locus ERFs bind can also be recognized by ORCA3 (Shoji and Hashimoto 2012a). *ORCA3* is responsive to JA (van der Fits and Memelink 2000), and *C. roseus* MYC2 directly activates *ORCA3* expression by recognizing a G box in its promoter (Zhang et al. 2011). These results indicate functional similarities between tobacco *NIC2*-locus *ERF*s and *C. roseus ORCA*s, in terms of their DNA-binding properties and of how they are regulated by JA and MYC2, and imply that functionally similar ERFs can be recruited to regulate JA-inducible metabolism in at least two distinct plant lineages, tobacco (Solanaceae) and periwinkle (Apocynaceae) (De Geyter et al. 2012; Shoji and Hashimoto 2012b).

Arabidopsis has three ERFs of group IXa, AtERF1, AtERF2, and AtERF13 (Figure 2), all of which are inducible by JA. AtERF1 and AtERF2 included in clade 1 are well-known founding members of AP2/ERF superfamily and can recognize a canonical GCC box (5′-AGCCGCC-3′) found in various defensive genes (Fujimoto et al. 2000). AtERF13 that belongs to clade 2 including tobacco *NIC2*-locus ERFs and periwinkle ORCAs, was reported to be involved in abiotic tolerance (Lee et al. 2010). These facts suggest wider defensive roles of group IXa ERFs that are not restricted to JA-inducible alkaloid regulation.

Perspectives

We hope that the knowledge on nicotine regulation could be applicable to genetic engineering of other phytochemicals. Reflecting the defensive functions of natural products, elicitors that induce JA production and JA itself have been widely used to improve the productivity of useful phytochemicals in plant cells and tissue cultures (Blechert et al. 1995; Gundlach et al. 1992; Yukimune et al. 1996). It would be interesting to reveal whether the same kinds of ERFs as those described above are recruited for JA-dependent regulation of valuable phytochemicals. If they are, engineering aimed at transcription factors (Grotewold 2008) may give us promising ways to dynamically improve metabolic flow in selected pathways.

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Addendum

After the submission of this manuscript, Shoji et al. (2013) reported similar but divergent DNA-binding specificities of *NIC2*-locus ERFs and related transcription factors, such as ORCA3, AtERF1, and AtERF13. In this study, amino acid residues in the DNA-binding domain critical for such divergence were also determined.

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