Tobacco Nicotine Uptake Permease Regulates the Expression of a Key Transcription Factor Gene in the Nicotine Biosynthesis Pathway[1][C][W]

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The down-regulation of a tobacco (*Nicotiana tabacum*) plasma membrane-localized nicotine uptake permease, NUP1, was previously reported to reduce total alkaloid levels in tobacco plants. However, it was unclear how this nicotine transporter affected the biosynthesis of the alkaloid nicotine. When *NUP1* expression was suppressed in cultured tobacco cells treated with jasmonate, which induces nicotine biosynthesis, the *NICOTINE2*-locus transcription factor gene *ETHYLENE RESPONSE FACTOR189* (*ERF189*) and its target structural genes, which function in nicotine biosynthesis and transport, were strongly suppressed, resulting in decreased total alkaloid levels. Conversely, *NUP1* overexpression had the opposite effect. In these experiments, the expression levels of the *MYC2* transcription factor gene and its jasmonate-inducible target gene were not altered. Inhibiting tobacco alkaloid biosynthesis by suppressing the expression of genes encoding enzymes in the nicotine pathway did not affect the expression of *ERF189* and other nicotine pathway genes, indicating that *ERF189* is not regulated by cellular alkaloid levels. Suppressing the expression of jasmonate signaling components in cultured tobacco cells showed that *NUP1* acts downstream of the *CORONATINE INSENSITIVE1* receptor and MYC2, but upstream of *ERF189*. These results suggest that although jasmonate-activated expression of MYC2 induces the expression of both *NUP1* and *ERF189*, expression of *ERF189* may actually be mediated by *NUP1*. Furthermore, *NUP1* overexpression in tobacco plants inhibited the long-range transport of nicotine from the roots to the aerial parts. Thus, NUP1 not only mediates the uptake of tobacco alkaloids into root cells, but also positively controls the expression of *ERF189*, a key gene in the biosynthesis of these alkaloids.

Nicotine and structurally related pyridine alkaloids accumulate throughout the plant in *Nicotiana* spp., and contribute to chemical defense against insect herbivory (Steppuhn et al., 2004). Nicotine is synthesized from the amino acids Asp and Orn (Shoji and Hashimoto, 2011a; Dewey and Xie, 2013). Early in the NAD biosynthetic pathway, Asp is metabolized to nicotinate mononucleotide, which is incorporated into the pyridine ring of nicotine by unknown enzyme reactions. Quinolinate phosphoribosyltransferase (QPT) catalyzes the last step of this pathway to yield nicotinate mononucleotide (Katoh et al., 2006), and one of the duplicated *QPT* genes functions in nicotine formation, rather than in NAD biosynthesis (Shoji and Hashimoto, 2011b). The pyrrolidine ring of nicotine is synthesized from Orn by three enzymes: Orn decarboxylase, putrescine *N*-methyltransferase (PMT; Hibi et al., 1994), and *N*-methylputrescine oxidase. A nicotinate derivative from the Asp pathway and the *N*-methyl-Δ^1-pyrrolinium cation from the Orn pathway are then condensed into nicotine, but these reactions are poorly understood. Berberine bridge enzyme-like enzyme (BBL) is required for the late biosynthetic pathway of nicotine (Kajikawa et al., 2011).

Insect herbivory on tobacco (*Nicotiana tabacum*) leaves initiates the jasmonate signaling cascade, which ultimately activates nicotine biosynthesis genes in the roots (Shoji and Hashimoto, 2013). Tobacco uses canonical jasmonate signaling components, such as the jasmonate receptor CORONATINE INSENSITIVE1 (COI1) and the JASMONATE ZIM domain transcriptional repressors (JAZs), to activate structural genes involved in nicotine biosynthesis (Paschold et al., 2007; Shoji et al., 2008). The basic helix-loop-helix Leu zipper-type transcription factor MYC2 is released from JAZ-mediated repression upon jasmonate perception by COI1, and activates nicotine biosynthesis genes in *Nicotiana* spp. (Todd et al., 2010; De Boer et al., 2011; Shoji and Hashimoto, 2011c; Zhang et al., 2012). The evolutionarily conserved MYC2 transcription factor induces the expression of the master regulator genes of nicotine biosynthesis, which encode the *NICOTINE2* (*NIC2*)-locus ethylene response factor (ERF)-type transcription factors (Shoji and Hashimoto, 2011c). At least seven *ERF* genes encoding a subclade of the tobacco group IXa subfamily are clustered at the *NIC2* locus, also known as the *B* locus (Legg and Collins, 1971), and all of these genes are deleted in the low-nicotine tobacco *nic2* mutant (Shoji et al., 2010). The

[1] This work was supported in part by the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research no. 26440144 to T.S.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.114.251645

NIC2-locus *ERF* genes are expressed in the root and up-regulated by jasmonate with kinetics distinct among the locus members (Shoji et al., 2010). The *NIC2*-locus ERFs activate the transcription of nicotine biosynthetic genes by directly binding to GCC box-like elements in their promoter regions (Shoji and Hashimoto, 2011b, 2012; Shoji et al., 2010, 2013). Although these *ERF* homologs are likely somewhat functionally redundant, *ERF189* and its closest homolog *ERF199* are thought to be the major regulators of the nicotine biosynthetic pathway; overexpression of *ERF189* upregulates the expression of structural genes of this pathway to a greater extent than does that of the other *ERF* members examined (Shoji et al., 2010), and the expression patterns of *ERF189* and *ERF199* overlap with those of genes in the nicotine biosynthetic pathway (Shoji and Hashimoto, 2014; Shoji et al., 2010). In addition to containing ERF-binding sites, the promoters of nicotine biosynthesis genes contain functional binding sites for MYC2, which are required for their jasmonate-inducible expression (De Boer et al., 2011; Shoji and Hashimoto, 2011c). Thus, tobacco MYC2 has dual roles in nicotine biosynthesis; it positively regulates the expression of *NIC2*-locus ERFs and, together with these ERFs, directly binds to and activates the promoters of structural genes involved in nicotine biosynthesis (Shoji and Hashimoto, 2011c).

Several nicotine transporters have been reported in tobacco. Two highly homologous MULTIDRUG AND TOXIC COMPOUND EXTRUSION-family transporters (MATE1 and MATE2) are localized to the vacuolar membrane and transport tobacco alkaloids from the cytosol into the vacuole in alkaloid-synthesizing root cells (Shoji et al., 2009). *MATE1* and *MATE2* genes are coordinately regulated with nicotine biosynthesis genes with respect to spatial expression patterns in the root and *NIC2*-locus *ERF*-mediated expression (Shoji et al., 2009, 2010). Reducing the expression of *MATE1/MATE2* in tobacco plants renders root growth more sensitive to exogenously applied nicotine, indicating that these transporters ameliorate nicotine toxicity in root cells during the active production of nicotine (Shoji et al., 2009). Another tonoplast-localized MATE-type transporter, tobacco JASMONATE-INDUCIBLE ALKALOID TRANSPORTER1, also transports nicotine, and is preferentially expressed in leaves, suggesting that the transporter is involved in nicotine sequestration in the leaf vacuole (Morita et al., 2009).

NICOTINE UPTAKE PERMEASE1 (NUP1) is a plasma membrane-localized tobacco transporter belonging to the purine uptake permease family (Hildreth et al., 2011). When expressed in yeast (*Saccharomyces cerevisiae*) cells or in cultured tobacco cells, NUP1 facilitates the import of tobacco alkaloids (nicotine and anatabine) from the culture medium into the cells (Hildreth et al., 2011; Kato et al., 2014). Although NUP1 preferentially transports pyridine alkaloids, it also transports pyridoxine and pyridoxamine (vitamin B_6; Kato et al., 2014). Unexpectedly, when NUP1 expression was suppressed in tobacco plants, total nicotine levels were reduced and seedling root growth was enhanced (Hildreth et al., 2011). Jasmonate-treated transgenic tobacco hairy root lines in which *NUP1* expression was suppressed (Hildreth et al., 2011) did not show statistically significant differences in the expression levels of five nicotine biosynthesis genes tested, including *PMT* and *QPT*. However, in the absence of exogenous jasmonate, some of these root lines exhibited reduced expression levels of nicotine biosynthesis genes encoding Orn decarboxylase, PMT, and quinolinate synthase. These results suggest the existence of a homeostatic control mechanism that maintains apoplastic nicotine levels in tobacco roots and indicate that NUP1 is unlikely to control nicotine biosynthesis directly.

In this study, we explored the enigmatic functions of NUP1 using transgenic tobacco lines, including cultured cells, hairy roots, and plants, in which *NUP1* was either efficiently suppressed or overexpressed. Transcriptome analysis of tobacco genes involved in jasmonate-inducible nicotine biosynthesis identified the *NIC2*-locus ERF transcription factors as the molecular targets of NUP1. Furthermore, we demonstrated that NUP1 affects the long-range transport of nicotine from the roots to the aerial parts of tobacco plants.

RESULTS

Expression Profiles of *NUP1*

NUP1 transcripts were reported to be more abundant in the roots, especially the root tips, than in the leaves of 2-week-old hydroponically grown tobacco 'Xanthi' plants (Hildreth et al., 2011). Furthermore, *NUP1* transcript levels in 4-week-old tobacco 'Burley 21' plants were higher in the roots than in the leaves and stems, although the transcript levels in the aerial parts of tobacco plants were considerably higher than those of genes encoding enzymes (e.g. *PMT*) and transporters (e.g. *MATE1/MATE2*) involved in nicotine biosynthesis and storage (Fig. 1A).

To examine the cell- and tissue-specific expression pattern of *NUP1*, we fused a 1.4-kb 5′-upstream regulatory region of *NUP1* to the *GUS* gene, and stably expressed this reporter construct (Pro_{NUP1}::*GUS*) in tobacco hairy roots. The GUS reporter was expressed throughout the root, but expression was greatest in the root tip region, including the root meristem and elongation zone (Fig. 1B). A 24-h exposure to exogenously applied methyljasmonate (MeJA; 100 μM) greatly enhanced GUS staining, consistent with the MeJA-induced expression of *NUP1* previously reported in tobacco hairy roots (Hildreth et al., 2011) and cultured cells (Kato et al., 2014). In cross sections of MeJA-treated roots prepared from the root tip region, GUS staining was strongest in the epidermal cells (Fig. 1C). GUS was expressed in root hairs, in the epidermal layer of the root cap (Supplemental Fig. S1), and in parenchyma cells in the stele.

Phenotypes of Tobacco Plants with Reduced or Increased *NUP1* Expression

Next, we examined whether down-regulation of *NUP1* decreased nicotine accumulation in tobacco plants, as

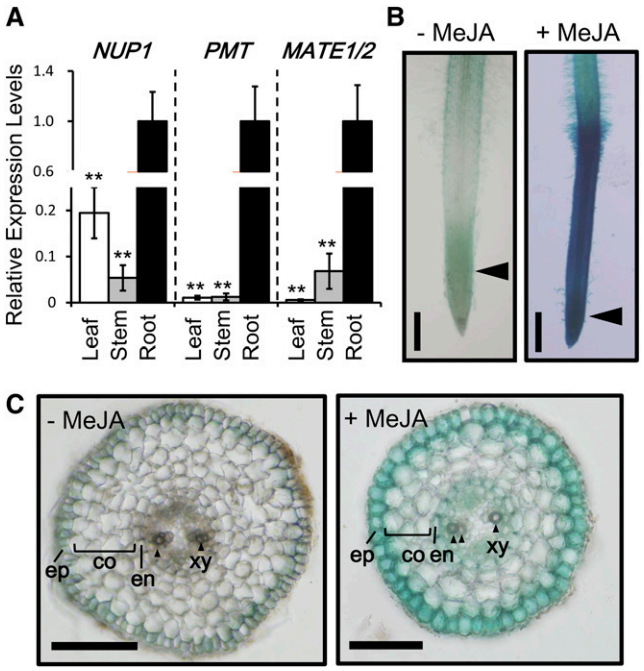

Figure 1. Expression patterns of *NUP1*. A, Quantitative RT-PCR analysis of *NUP1*, *PMT*, and *MATE1/MATE2* expression in the third leaf, stem (approximately 5 cm above the soil) and root of 8-week-old tobacco 'NC95' plants. The data are the mean values (±SD) for three independent plants. Significant differences between root values and other sample values were determined using Dunnett's test and are indicated by asterisks (double asterisks for $P < 0.01$). B and C, Histochemical GUS staining of tobacco hairy roots expressing Pro_{NUP1}::*GUS* grown in the absence (−) or presence (+) of 100 μM MeJA for 24 h. B, Hairy roots at the tip region. C, Cross sections of Pro_{NUP1}::*GUS* roots. Sections were made at the approximate positions indicated by arrowheads in B. co, Cortex; en, endodermis; ep, epidermis; xy, xylem. Arrowheads indicate xylem. Bars = 1 mm in B and 200 μm in C. [See online article for color version of this figure.]

reported for *NUP1*-RNA interference (RNAi) plants regenerated from tobacco hairy roots (Hildreth et al., 2011). Using *Agrobacterium tumefaciens*-mediated transformation of leaf discs, we generated two independent transgenic tobacco 'SR1' lines in which *NUP1* expression in the root was down-regulated to less than one-half of that in the wild type and two independent empty vector-transformed controls, collectively referred to as the control plants (Fig. 2A). The nicotine content was severely reduced in the leaves and roots of these RNAi lines (R1 and R2), to less than 20% of that in the control plants (Fig. 2, B and C), and was comparable in level to that reported in tobacco *COI1*-RNAi plants in which jasmonate perception was blocked (Shoji et al., 2008).

Because nicotine biosynthesis is largely blocked in the roots of *NUP1*-RNAi plants, it is challenging to examine the root-to-shoot translocation of nicotine in these plants. Therefore, we next generated two independent transgenic tobacco lines (referred to as O1 and O2) in which *NUP1* was overexpressed under the strong constitutive *Cauliflower mosaic virus* 35S promoter. The level of *NUP1* transcript in the roots of the *NUP1*-overexpressing plants was approximately 2-fold that of the control plants (Fig. 2D). Interestingly, the nicotine content in the roots of O1 and O2 was approximately 4-fold that of the control roots (Fig. 2F), whereas the foliar nicotine content was reduced to approximately 30% that of the control plants (Fig. 2E). These results indicate that *NUP1* overexpression inhibits the long-range transport of nicotine from the root to the aerial parts of tobacco plants. To test this possibility, we measured the alkaloid content in xylem sap collected from the base of tobacco stems of the various lines (Fig. 2G). The nicotine content in the xylem sap of *NUP1*-overexpressing plants was approximately 30% that of the control plants, suggesting that *NUP1* overexpression inhibits the xylem loading of nicotine in the root.

NUP1 Affects Nicotine Accumulation by Modulating the Expression of *ERF189* and Nicotine Biosynthesis Genes

To examine the molecular mechanisms by which NUP1 affects nicotine accumulation, we studied the expression of structural genes involved in nicotine biosynthesis (*PMT* and *QPT*) and transport (*MATE1/MATE2*) and their upstream regulatory genes in the jasmonate signaling pathway (*ERF189* and *MYC2*). In the roots of two *NUP1*-RNAi lines (R1 and R2), the transcript levels of *PMT*, *QPT*, and *MATE1/MATE2* were reduced to around one-half or less than one-half of those in the wild-type and vector control plants (Fig. 3A). The transcript levels of *ERF189* were similarly reduced to less than 40% of control plants in the RNAi roots, whereas those of *MYC2* were not significantly affected. Because *MYC2* is regulated not only transcriptionally but also posttranslationally, by its physical interaction with JAZ repressors (Chini et al., 2007), we analyzed the expression of the MYC2-regulated *PROTEINASE INHIBITOR II* (*PI-II*; Balandin et al., 1995; Shoji and Hashimoto, 2011c) to establish whether the transcriptional activator activity of MYC2 was affected in the silenced lines. *PI-II* expression was similar in the *NUP1*-RNAi and control lines, indicating that NUP1 regulates *ERF189* but not *MYC2*. By contrast, the expression of both *MYC2* and *ERF189*, and thus the expression of their downstream structural genes, was suppressed altogether in *COI1*-RNAi plants. COI1-dependent MeJA-induced up-regulation of *MYC2* has been reported in Arabidopsis (*Arabidopsis thaliana*; Lorenzo et al., 2004). We tested whether *ERF189* and its target genes would be up-regulated in *NUP1*-overexpressing plant lines (O1 and O2) subjected to MeJA treatment (Fig. 3B). The transcript levels of *PMT*, *QPT*, and *MATE1/MATE2* in *NUP1*-overexpressing roots were significantly higher than those in wild-type and control roots. The level of *ERF189* transcript was strikingly higher in the roots of *NUP1*-overexpressing plants than in those of the control plants, whereas the transcript levels of *MYC2* and *PI-II* did not differ significantly between the *NUP1*-overexpressing and control plants.

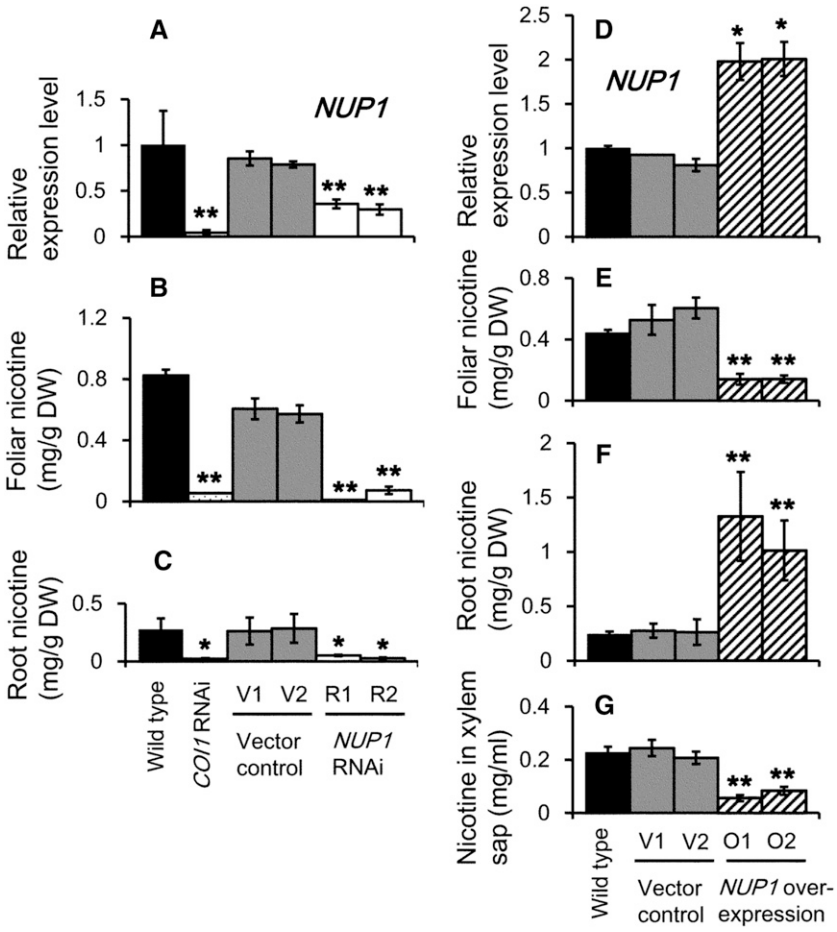

Figure 2. Down-regulation or overexpression of *NUP1* affects the accumulation and distribution of nicotine in tobacco plants. Down-regulation data are shown in A to C, whereas overexpression data are presented in D to G. A to C, Quantitative RT-PCR analysis of *NUP1* transcript levels in the roots (A) and foliar (B) and root (C) nicotine contents of 10-week-old wild-type, *COI1* RNAi, vector control (V1 and V2), and *NUP1* RNAi (R1 and R2) tobacco lines. D to G, Quantitative RT-PCR analysis of *NUP1* expression in the roots (D), and nicotine content of the leaves (E), roots (F), and xylem sap (G) of 10-week-old wild-type, vector control (V1 and V2), and *NUP1*-overexpressing (O1 and O2) tobacco lines. The data are the mean values (±SD) of three independent plants. Significant differences between the wild type and test samples were determined using Dunnett's test and are indicated by asterisks (single asterisk for $P < 0.05$ and double asterisks for $P < 0.01$). DW, Dry weight.

To substantiate these results obtained in transgenic tobacco plants, we generated tobacco cultured BY-2 cell lines in which *NUP1* expression was either down- or up-regulated. Although BY-2 cells do not biosynthesize alkaloids when cultured in growth medium containing auxin, they produce the alkaloids anatabine and, to a lesser extent, nicotine, when grown on auxin-free medium supplemented with MeJA (Shoji and Hashimoto, 2008). When grown under culture conditions that promote alkaloid biosynthesis, two independent RNAi lines (R3 and R4) exhibited *NUP1* transcript levels that were less than 30% those of control plants, whereas two independent overexpression lines (O3 and O4) had *NUP1* transcript levels that were more than 4-fold those of the control lines (Fig. 4A). The total amount of tobacco alkaloids in the culture (i.e. the sum of anatabine and nicotine in the cells and culture medium) was severely decreased in the *NUP1* down-regulated lines and was considerably increased in the *NUP1*-overexpressing lines, compared with those in the control lines (Fig. 4B). We calculated the relative ratios of tobacco alkaloids in the cells and in the medium, and found that the proportion of alkaloids in the medium was significantly increased in the RNAi lines, but was significantly decreased in the overexpression lines (Supplemental Fig. S2). Therefore, NUP1 not only promotes the uptake of tobacco alkaloids from the medium into the cells, but also positively regulates alkaloid biosynthesis. The expression level of an alkaloid biosynthesis gene (*PMT*), nicotine transporters (*MATE1/MATE2*), and their direct upstream regulator (*ERF189*) was highly decreased in the *NUP1* RNAi lines, but increased in the *NUP1*-overexpressing lines, although the *MATE1/MATE2* transcript level in the overexpression lines was only slightly increased and was not statistically different from that in the controls (Fig. 4C). The expression levels of *MYC2* and its downstream target gene *PI-II* were not affected by the down-regulation or overexpression of *NUP1*.

These results in transgenic plants and in cultured cells suggest that the *NIC2*-locus *ERF* transcription factor genes, but not *MYC2*, are regulated by NUP1. The changes in tobacco alkaloid levels and in the expression of genes involved in alkaloid biosynthesis in lines in which *NUP1* had either been down- or up-regulated are likely caused by changes in the transcript levels of *ERF189*, and possibly other *NIC2*-locus *ERF* genes.

Reducing Alkaloid Biosynthesis by Metabolic Blocks Does Not Affect *ERF189* Expression

NUP1 suppression reduces the uptake of tobacco alkaloids from the extracellular space, and is expected to

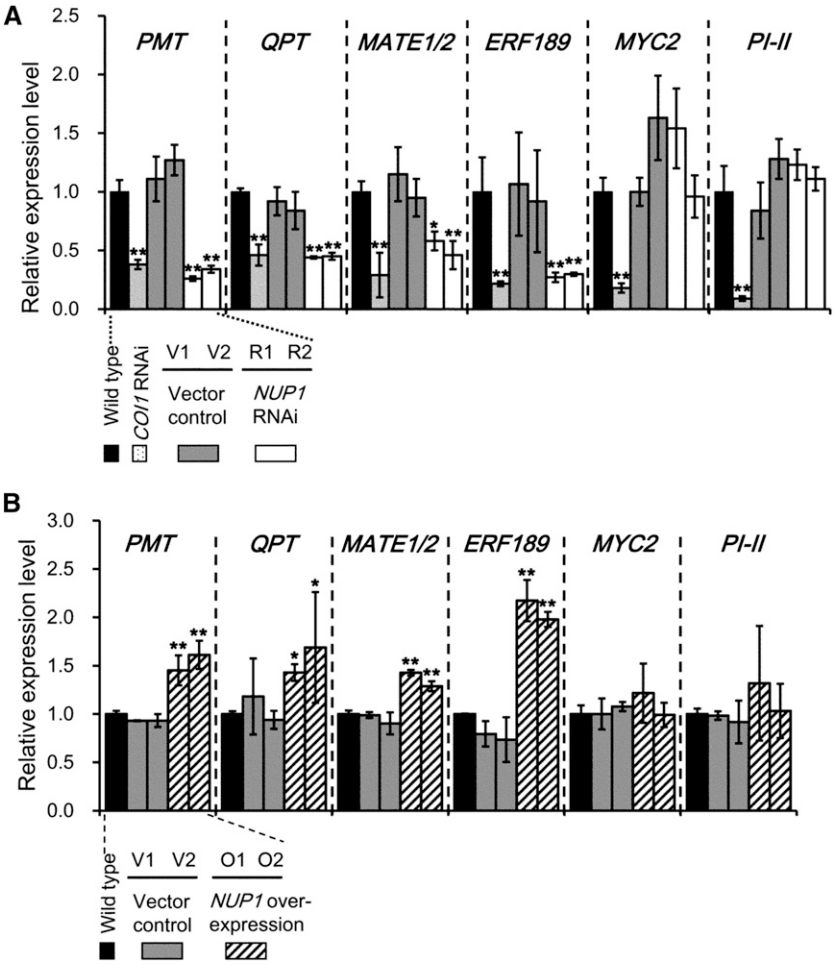

Figure 3. Down-regulation or overexpression of *NUP1* affects the expression of genes involved in the nicotine biosynthetic pathway in tobacco plants. A, Quantitative RT-PCR analysis of *PMT*, *QPT*, *MATE1/MATE2*, *ERF189*, *MYC2*, and *PI-II* in the roots of 10-week-old wild-type, *COI1* RNAi, vector control (V1 and V2), and *NUP1* RNAi (R1 and R2) tobacco lines. B, Quantitative RT-PCR analysis of *PMT*, *QPT*, *MATE1/MATE2*, *ERF189*, *MYC2*, and *PI-II* expression in the roots of 10-week-old wild-type, vector control (V1 and V2), and *NUP1* overexpressing (O1 and O2) tobacco lines. The data are the mean values (± SD) of three independent plants. Significant differences between the wild type and test samples were determined by Dunnett's test and are indicated by asterisks (single asterisk for $P < 0.05$ and double asterisks for $P < 0.01$).

lower their intracellular levels. To examine the possibility that a certain threshold level of cellular alkaloids is required to maintain the expression of the *NIC2*-locus *ERF* genes, we inhibited alkaloid biosynthesis by RNAi-mediated suppression of *QPT* (Xie et al., 2004). Suppressing *QPT* expression (Fig. 5A) in jasmonate-treated cultured tobacco BY-2 cells (Q1, Q2, and Q3) resulted in significant decreases in nicotine, anatabine, and anatalline (to below 30% of control values; Fig. 5B). Likewise, in *QPT*-suppressed tobacco hairy root lines (Q1 and Q2; Fig. 5D), accumulation of nicotine, nornicotine, and anatabine was highly reduced (Fig. 5E). In these alkaloid-deficient cell and root lines, the transcript levels of *ERF189* and of its target structural genes involved in nicotine biosynthesis and transport (*PMT*, *BBL*, and *MATE1/MATE2*) were not significantly different from those of the control plants (Fig. 5, C and F). Similar results were obtained in jasmonate-treated tobacco cells and tobacco hairy roots, in which expression of another nicotine biosynthesis gene (*BBL*; Kajikawa et al., 2011) was suppressed (Supplemental Fig. S3). We therefore conclude that the cellular levels of tobacco alkaloids do not affect the expression of their master transcription factor genes (e.g. *ERF189*) and the structural genes involved in nicotine biosynthesis, and that the transcriptional control of *ERF189* by NUP1 is not influenced by the cellular levels of tobacco alkaloids.

Epistasis Analysis of *NUP1* and Genes Involved in Jasmonate-Induced Nicotine Biosynthesis

To gain insight into the regulatory hierarchy of NUP1, ERF189, and jasmonate signaling components, we suppressed the expression of *COI1* or *MYC2* in tobacco BY-2 cells or in tobacco hairy roots and examined their expression levels after alkaloid-inducing MeJA treatment. When *COI1* was suppressed in cultured cells (Fig. 6A) and in hairy roots (Fig. 6B) in two independent transgenic lines (C1 and C2, and C3 and C4, respectively), the expression levels of *NUP1*, *PMT*, and *MATE1/MATE2* were all significantly down-regulated compared with the control levels. *NUP1* was also down-regulated in the roots of transgenic tobacco plants in which *COI1* was suppressed (Fig. 3A). In hairy root lines in which *MYC2* expression was suppressed (M1 and M2), the expression levels of *NUP1*, *PMT*, and *MATE1/MATE2* were also significantly down-regulated (Fig. 6C).

To suppress the function of partially redundant *NIC2*-locus *ERF* genes, we constitutively expressed a

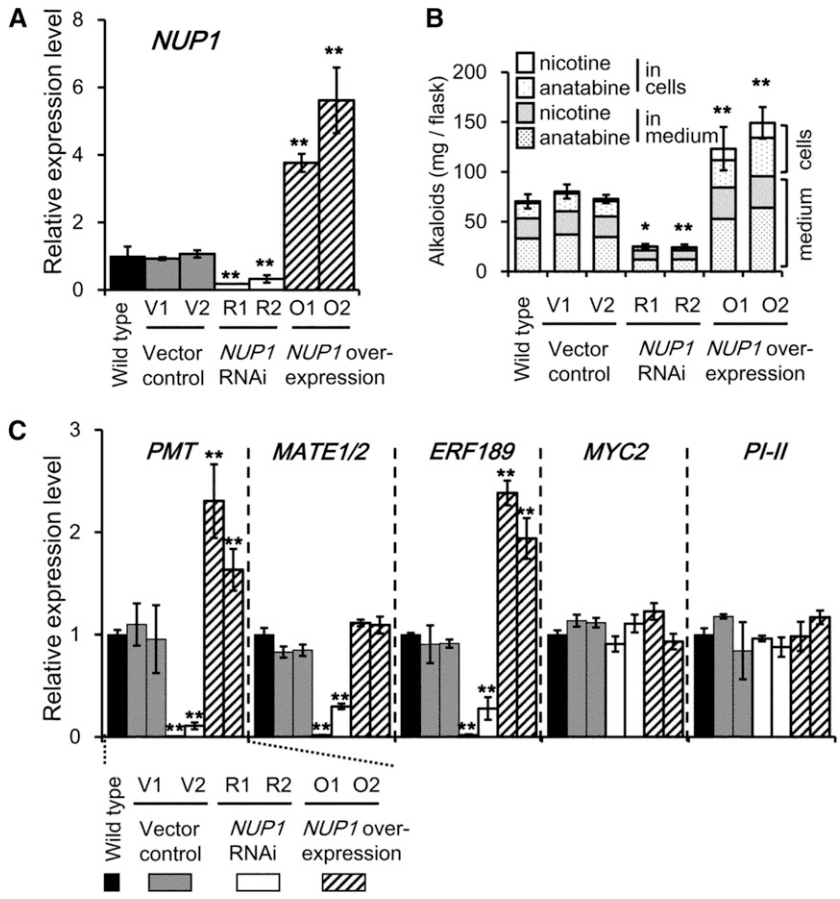

Figure 4. Down-regulation or overexpression of *NUP1* in cultured tobacco BY-2 cells affects the expression of genes involved in the nicotine biosynthetic pathway and the accumulation of alkaloids. A to C, Quantitative RT-PCR analysis of *NUP1* (A) and *PMT*, *MATE1/MATE2*, *ERF189*, *MYC2*, and *PI-II* (C) expression, and the amount of alkaloid in a culture flask (B) of wild-type, vector control (V1 and V2), *NUP1* RNAi (R1 and R2), and *NUP1* overexpressing (O1 and O2) BY-2 lines. Tobacco cells were treated with 100 μM MeJA for 24 h for RNA extraction and for 72 h for alkaloid analysis. The data are the mean values ($\pm$SD) of three biological replicates. Significant differences between the wild type and test samples were determined by Dunnett's test and are indicated by asterisks (single asterisk for $P < 0.05$ and double asterisks for $P < 0.01$).

dominant-negative form of ERF189 (ERF189-EAR; Shoji et al., 2010) in two independent transgenic lines (E1 and E2, and E3 and E4, respectively) of MeJA-treated tobacco BY-2 cells (Fig. 7A) or tobacco hairy roots (Fig. 7B). In both culture systems, the expression of ERF189-EAR effectively down-regulated *PMT*, as reported previously (Shoji et al., 2010), but did not affect the expression of *NUP1*. These results suggest that MeJA-induced expression of *NUP1* is mediated by the canonical jasmonate signaling components, including COI1 and MYC2, and that NUP1 acts upstream of the *NIC2*-locus *ERF* genes and their target structural genes involved in nicotine biosynthesis and transport.

DISCUSSION

NUP1 Is Expressed in Root Epidermal Cells

The *NUP1* promoter is primarily expressed in the epidermal cells of the root tip and in the outermost cell layer of the lateral root cap, with weaker expression in the parenchyma cells of the stele. Low but substantial expression of *NUP1* was also observed in the leaf and stem of tobacco plants, although *NUP1*-expressing cell types were not identified in the aerial organs in this study. These spatial expression patterns of *NUP1* in tobacco roots are distinct from those of known enzyme genes involved in nicotine biosynthesis, which are strongly expressed in the root cortex and not in the aerial parts (Shoji et al., 2000, 2002; Kajikawa et al., 2011; Shoji and Hashimoto, 2011b). Tobacco *MATE1/MATE2* genes, whose products sequester nicotine into the vacuole during active alkaloid biosynthesis, are also expressed in the root cortex, in a similar pattern as nicotine enzyme genes (Shoji et al., 2009). The spatially coordinated expression of these tobacco genes is likely controlled by key transcription factors for nicotine biosynthesis and accumulation, such as ERF189 (Shoji et al., 2010). The preferential expression of *NUP1* in the epidermis indicates that its transcriptional control does not depend on ERF189. Indeed, when *ERF189* and its related transcription factors were functionally suppressed, the expression of nicotine biosynthesis genes, but not of *NUP1*, was highly reduced (Shoji et al., 2010; Fig. 7). Thus, although NUP1 transports nicotine, its transcriptional regulation does not constitute an ERF189-mediated nicotine regulon.

The physiological functions of NUP1-mediated tobacco alkaloid uptake are not well understood. Epidermis-localized NUP1 may prevent apoplastic nicotine, which is synthesized in the cortex, from being secreted into the rhizosphere at the time of active nicotine synthesis or may even retrieve secreted nicotine back into the root tissues. *NUP1* overexpression in tobacco roots would be expected to decrease nicotine concentrations in the root

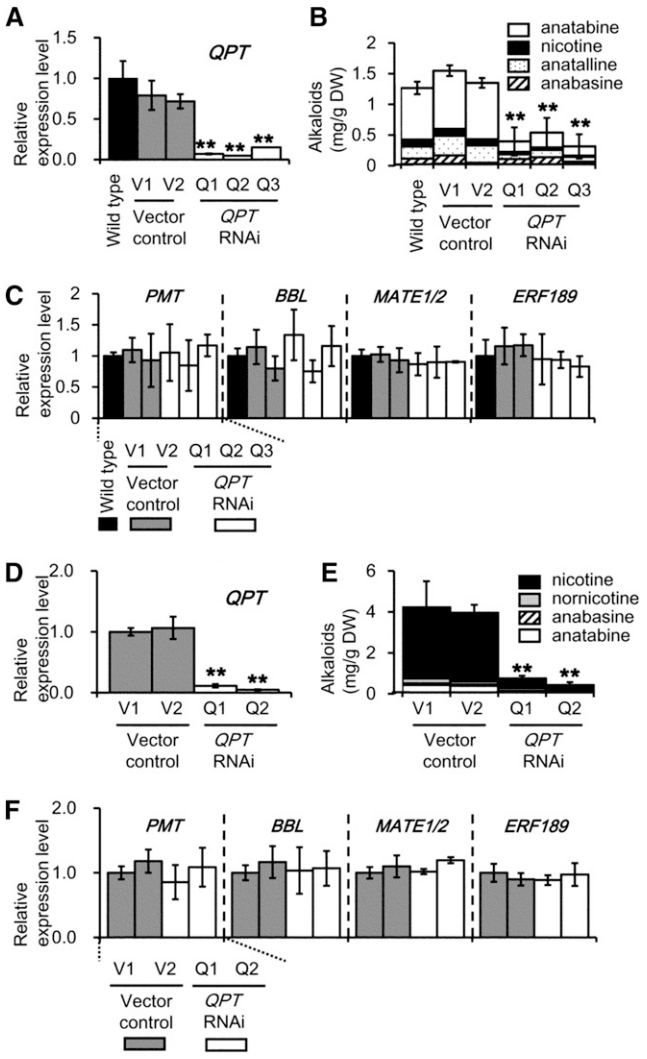

Figure 5. Down-regulation of *QPT* does not affect the expression of genes involved in alkaloid biosynthesis and transport. Data from cultured tobacco BY-2 cells are shown in A to C, whereas data from tobacco hairy roots are shown in D to F. Quantitative RT-PCR analysis of *QPT* (A and D), *PMT*, *BBL*, *MATE1/MATE2*, and *ERF189* (C and F) expression, and the accumulation of tobacco alkaloids (B and E) in wild-type, vector control (V1 and V2), and *QPT* RNAi (Q1, Q2, and Q3 in A–C, and Q1 and Q2 in D–F) transgenic lines. The cultured tobacco cells were treated with 100 μM MeJA for 24 h for RNA extraction and for 72 h for alkaloid analysis. The data are the mean values (±SD) of three biological replicates. Significant differences between the wild type and test samples were determined by Dunnett's test and are indicated by asterisks (double asterisks for $P < 0.01$).

apoplast, and may affect the root-to-shoot transport of nicotine via xylem. Indeed, in *NUP1*-overexpressing plants, the concentration of nicotine in the xylem sap was significantly decreased, resulting in decreased foliar nicotine contents and increased root nicotine contents (Fig. 2). These results indicate that NUP1 controls the apoplastic distribution of tobacco alkaloids in the root, and potentially influences the long-range shoot-bound transport of root alkaloids. Thus, it is noteworthy that *Nicotiana alata*, a wild *Nicotiana* sp. containing very low levels of foliar alkaloids, does not efficiently transport root-synthesized alkaloids to the aerial parts, and that this nontransporting phenotype (i.e. the retention of alkaloids in the root) is genetically dominant over the transporting phenotype (Pakdeechanuan et al., 2012). It would be interesting to determine whether *NUP1* or a functionally similar nicotine transporter is overexpressed in *N. alata* roots.

NUP1 Regulates Nicotine Biosynthesis via ERF189

The suppression of *NUP1* expression in tobacco hairy roots and the resulting regenerated plants unexpectedly reduced the total nicotine contents by unknown molecular mechanisms (Hildreth et al., 2011). Down-regulation of *NUP1* in cultured tobacco cells significantly decreased

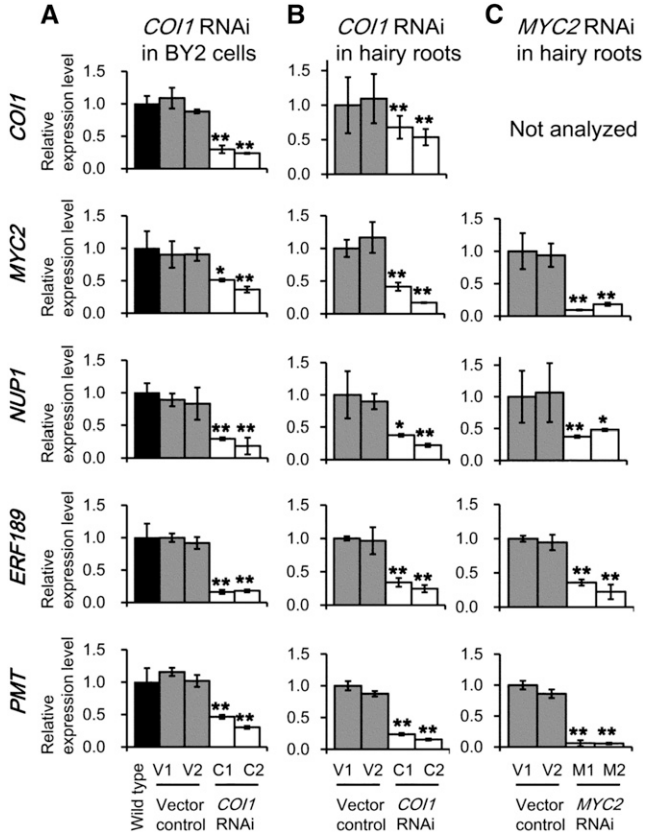

Figure 6. *NUP1* is regulated by the canonical COI1-MYC2 jasmonate signaling pathway. A to C, Quantitative RT-PCR analysis of *COI1*, *MYC2*, *NUP1*, *ERF189*, and *PMT* expression in *COI1* RNAi lines (C1 and C2, and C3 and C4, respectively) of cultured tobacco BY-2 cells (A) or tobacco hairy roots (B) and in *MYC2* RNAi lines (M1 and M2) of tobacco hairy roots (C). Tobacco cells or roots were treated with 100 μM MeJA for 24 h. The data are the mean values (±SD) of three biological replicates. Significant differences between the wild type (A) or vector controls (B and C) and the test samples were determined by Dunnett's test and are indicated by asterisks (single asterisk for $P < 0.05$ and double asterisks for $P < 0.01$).

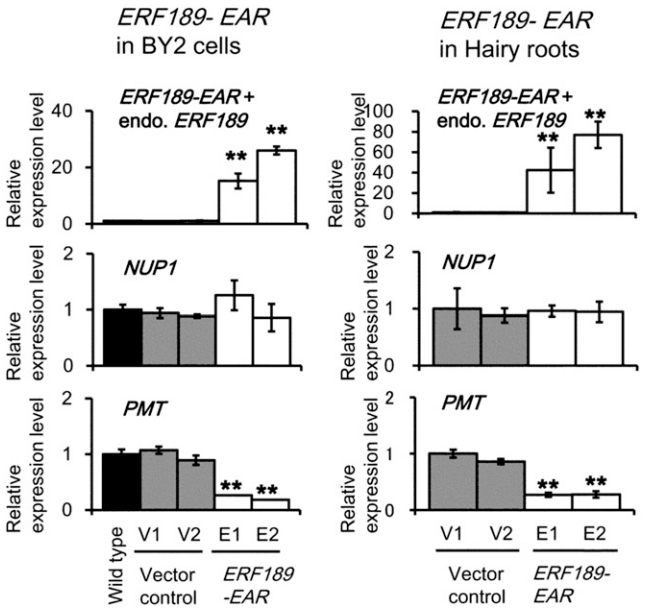

Figure 7. *NUP1* is not regulated by *ERF189*. Quantitative RT-PCR analysis of *ERF189*, *NUP1*, and *PMT* expression in cultured tobacco BY-2 cells or tobacco hairy roots of wild-type, vector control (V3 and V4, and V5 and V6, respectively), and ERF189-EAR overexpressing (E1 and E2, and E3 and E4, respectively) lines. An expression assay for *ERF189* detected transcripts of both the *ERF189-EAR* transgene and endogenous *ERF189*. Tobacco cells or roots were treated with 100 μM MeJA for 24 h. The data are the mean values (±SD) of three biological replicates. Significant differences between the wild type (A) or vector controls (B) and the test samples were determined by Dunnett's test and are indicated by asterisks (double asterisks for $P < 0.01$).

the total amounts of tobacco alkaloids in the culture, whereas *NUP1* overexpression increased these amounts (Fig. 4B), indicating that NUP1 either promotes alkaloid biosynthesis or inhibits alkaloid degradation.

Expression analysis of *PMT* and *QPT* revealed that these enzyme-encoding genes involved in nicotine biosynthesis are positively regulated by *NUP1* in plant roots and cultured cells (Figs. 3 and 4). Moreover, the vacuolar-localized nicotine transporter genes *MATE1/MATE2* are similarly regulated by *NUP1*. Because these structural genes involved in nicotine biosynthesis and transport are the direct targets of the *NIC2*-locus *ERF* genes (Shoji et al., 2010), we analyzed the expression of a representative *NIC2*-locus gene, *ERF189*, which was found to be up- and down-regulated by the up- and down-regulation of *NUP1* expression, respectively. Although *ERF189* expression is regulated by MYC2, modulating *NUP1* expression levels did not affect the expression of *MYC2* (Figs. 3 and 4). The expression levels of *PI-II*, a gene that functions downstream of MYC2, did not change upon modulation of *NUP1* expression, indicating that MYC2 was not activated at the transcriptional or post-transcriptional levels under these conditions. Therefore, the regulation of *ERF189* by NUP1 is not mediated by MYC2. The functional suppression of ERF189 and the related ERF subclade members in the ERF189-EAR overexpressing lines confirmed that NUP1 acts upstream of ERF189, but not vice versa (Fig. 7).

Hildreth et al. (2011) did not find reduced transcript levels of nicotine enzyme genes encoding Orn decarboxylase, quinoline synthase, QPT, PMT, and *N*-methylputrescine oxidase in the jasmonate-treated hairy root lines induced from NUP1-RNAi tobacco plants. Upon exogenous jasmonate treatment, *NUP1* transcript levels in their NUP1-RNAi roots increased severalfold from the untreated values, although the increased levels were still lower than the levels in wild-type tobacco roots (Hildreth et al., 2011). The elevated levels of *NUP1* transcript might have obscured the impact of *NUP1* suppression in their experimental conditions. It should be noted that, in the absence of jasmonate, the expression levels of *ORN DECARBOXYLASE*, *QUINOLINE SYNTASE*, and *PMT* were significantly suppressed in several NUP1-RNAi hairy root lines (Hildreth et al., 2011).

NUP1 is known to be up-regulated in hairy root and other cultures by the exogenous application of jasmonates (Hildreth et al., 2011; Kato et al., 2014). In this study, we found that the jasmonate-mediated induction of *NUP1* relies on COI1 and MYC2 (Fig. 6). These results are summarized in Figure 8. After jasmonate perception by COI1, activated MYC2 induces the expression of the *NIC2*-locus *ERF* genes (e.g. *ERF189*), as well as of *NUP1* and general jasmonate-responsive genes (e.g. *PI-II*). However, the jasmonate-elicited induction of *ERF189* is modulated by the expression levels of *NUP1*. Successfully induced ERF189 and MYC2 then bind to and activate structural genes involved in nicotine biosynthesis.

It is unknown how a nicotine transporter (i.e. NUP1) affects the expression of a transcription factor gene *ERF189*. Inhibiting the biosynthesis and accumulation of tobacco alkaloids by suppressing the expression of

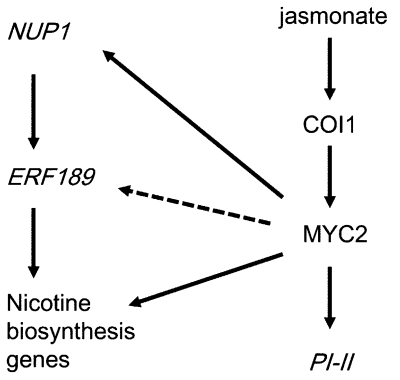

Figure 8. Model of *NUP1*-mediated regulation of tobacco alkaloid biosynthesis. Bioactive jasmonate is perceived by COI1 and activates MYC2, which induces the expression of general wound-inducible genes (e.g. *PI-II*) and nicotine biosynthesis genes. MYC2 and the *NIC2*-locus transcription factors (e.g. ERF189) specifically regulate nicotine biosynthesis. This study demonstrates that jasmonate-induced expression of *ERF189* requires the MYC2-mediated expression of *NUP1*. Previously reported MYC2-dependent *ERF189* expression (dashed arrow; Shoji and Hashimoto, 2011c) may be mediated by NUP1.

nicotine biosynthesis enzyme genes (i.e. *QPT* and *BBL*) did not affect *ERF189* expression (Fig. 5), indicating that the cellular levels of tobacco alkaloids are unlikely to mediate the transcriptional activation function of NUP1. Because the uptake substrates of NUP1 are not restricted to tobacco alkaloids (Kato et al., 2014), unidentified bioactive metabolites that are secreted from MeJA-treated tobacco cells or are present in the culture medium might be transported by NUP1. Alternatively, NUP1 might possess a unique biochemical function distinct from, and in addition to, its transporter activity. In this scenario, a *NUP1* mutant in which the transporter activity is abolished may activate ERF189. It would be interesting to explore whether Arabidopsis NUP1-related transporters (i.e. PURINE PERMEASE1 [PUP1], PUP2, and PUP3) have biological functions that are not readily explained from their known transporter activities.

MATERIALS AND METHODS

Plant Materials and Transformation

Nicotiana tabacum 'Burley 21' or 'Petit Havana' line SR1 plants were grown in the greenhouse. The tobacco BY-2 cell suspension was cultured as described (Nagata et al., 1992). To induce alkaloid biosynthesis, 4-d-old BY-2 cells were first rinsed extensively to remove 2,4-dichlorophenoxyacetic acid and then transferred to fresh auxin-free medium at an inoculum density of a 10-mL packed cell volume per 90 mL of culture medium. After incubation for 12 h, MeJA was added to a final concentration of 100 μM. All samples were frozen in liquid nitrogen immediately after harvest and were kept at −80°C until use.

Transgenic tobacco plants (SR1) were produced using *Agrobacterium tumefaciens* strain EHA105 and a leaf disc transformation protocol (Horsch et al., 1985). Transgenic plants of the T1 generation were analyzed and nontransgenic T1 progeny were excluded from the analysis. Tobacco BY-2 cells were transformed as described (An, 1985) using *A. tumefaciens* strain EHA105. Tobacco hairy roots were induced from tobacco 'SR1' leaf discs by *Agrobacterium rhizogenes* strain ATCC15834 and cultured in liquid Gamborg B5 medium at 25°C, as described (Shoji et al., 2010).

Plasmid Construction

The 5′-upstream regulatory region (1.4 kb) of *NUP1* was obtained by PCR using genomic DNA extracted from tobacco 'Burley 21.' PCR primers (Supplemental Table S1) were designed based on the genome fragment sequence C32375 in the tobacco Methylation Filtered Genome TGI:V.1 (http://solgenomics.net). After the identity of the cloned *NUP1* promoter region was confirmed by sequencing, it was flanked by the *Hin*dIII and *Bam*HI sites, and then cloned into pBI121 to generate Pro_{NUP1}::*GUS*.

To generate the *NUP1*-RNAi vector, two complementary DNA (cDNA) fragments of *NUP1* (+750 to +1,129, relative to the translation initiation ATG) were placed in front of a *pyruvate dehydrogenase kinase* intron and just after it in the reverse orientation in the pHANNEBAL vector (Wesley et al., 2001), and the resulting RNAi expression cassette was inserted into pBI121 at the *Bam*HI and *Sac*I sites. The *QPT1*-RNAi vector was constructed in a similar way, using the QPT1 cDNA fragment (+1 to +510). The sequences of the PCR primers used for cloning are listed in Supplemental Table S1.

Transformation vectors used to overexpress *NUP1* (Kato et al., 2014), to down-regulate *COI1* (Shoji et al., 2008), *MYC2* (Shoji and Hashimoto, 2011c), or *BBL* (Kajikawa et al., 2011), or to dominantly suppress the expression of *ERF189*-related genes (Shoji et al., 2010) have been reported.

Histochemical GUS Assay

GUS activity was detected histochemically and cross sections of roots were prepared as described previously (Shoji et al., 2000). GUS-stained cultured roots were rinsed with 20 mM phosphate buffer, pH 7.0, embedded in 5% (w/v) agar containing 1 mM dithiothreitol and 20 mM phosphate buffer (pH 7.0), and sliced into 75- to 100-μm-thick cross sections using a microslicer (DTK-1500; Dohan EM). For the sections in Supplemental Figure S1, roots were embedded in Technovit 7100 (Heraeus Kulzer) and sliced to give 10-μm-thick cross and longitudinal sections using a MICROM HM335E Rotary Microtome (Thermo Fisher Scientific). The GUS-stained sections were then counterstained with neutral red. Images of the stained tissues and sections were captured with an SZX12 (Olympus) or an Eclipse E-1000 (Nikon) microscope, equipped with a DP-70 digital camera (Olympus).

Quantitative Reverse Transcription PCR

Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) from samples that had been ground in liquid nitrogen. cDNA was synthesized from 1 μg of total RNA using Super Script II Reverse Transcriptase (Invitrogen) and an oligo(dT) primer. The cDNA templates were amplified using a LightCycler 480 (Roche) with SYBR Premix Ex Taq (Takara) under the following conditions: 95°C for 10 min, followed by 55 cycles of 94°C for 10 s, 55°C for 10 s, and 72°C for 15 s. *EF1a* was used as a reference gene. The PCR primers used are listed in Supplemental Table S2. The *QPT* primers amplified both *QPT1* and *QPT2* (Shoji and Hashimoto, 2011b), while all four BBL members, BBLa, BBLb, BBLc, and BBLd, were collectively detected by the BBL primers (Kajikawa et al., 2011).

Alkaloid Analysis

The alkaloids in freeze-dried plant samples were purified as described (Shoji et al., 2009). When BY-2 cell cultures and hairy root cultures were harvested, the culture medium was separated from the cultured samples after filtering through a paper filter (0.26-mm thickness × 55 ø; Advantech) and centrifuged at 17,400*g* for 10 min. The supernatant was then applied directly to an Extrelut-1 column (Merck), eluted with chloroform, and dried as described (Shoji et al., 2009). Purified tobacco alkaloids were analyzed by gas-liquid chromatography (Shoji et al., 2009). The xylem sap was collected from the base of 8-week-old tobacco plants as described (Pakdeechanuan et al., 2012) and was analyzed for alkaloids in the same manner as described above for alkaloids in the culture medium.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GU174267 (NUP1), AB433899 (COI1), HM466975 (MYC2), AB827951 (ERF189), AB004323 (PMT), AJ748263 (QPT1), AJ748263 (QPT2), AB604219 (BBLa), AM851017 (BBLb), AB604220 (BBLc), AB604221 (BBLd), AB286961 (MATE1), AB286962 (MATE2), Z29537 (PI-II), and D63396 (EF1α).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Histochemical GUS staining of Pro_{NUP1}::*GUS* tobacco hairy roots.

Supplemental Figure S2. *NUP1* expression affects the distribution of tobacco alkaloids in cells and the culture medium.

Supplemental Figure S3. Down-regulation of *BBL* does not affect the expression of genes involved in alkaloid biosynthesis and transport.

Supplemental Table S1. PCR primers used for vector construction.

Supplemental Table S2. Quantitative reverse transcription-PCR primers used in this study.

Received October 6, 2014; accepted October 22, 2014; published October 24, 2014.

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