Tobacco nicotine uptake permease (NUP1) affects alkaloid metabolism

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An effective plant alkaloid chemical defense requires a variety of transport processes, but few alkaloid transporters have been characterized at the molecular level. Previously, a gene fragment encoding a putative plasma membrane proton symporter was isolated, because it was coordinately regulated with several nicotine biosynthetic genes. Here, we show that this gene fragment corresponds to a Nicotiana tabacum gene encoding a nicotine uptake permease (NUP1). NUP1 belongs to a plant-specific class of purine uptake permease-like transporters that originated after the divergence of bryophytes but before or within the lycophytes. NUP1 expressed in yeast cells preferentially transported nicotine relative to other pyridine alkaloids, tropane alkaloids, kinetin, and adenine. NUP1-GFP primarily localized to the plasma membrane of tobacco Bright Yellow-2 protoplasts. WT NUP1 transcripts accumulated to high levels in the roots, particularly in root tips. NUP1-RNAi hairy roots had reduced NUP1 mRNA accumulation levels, reduced total nicotine levels, and increased nicotine accumulation in the hairy root culture media. Regenerated NUP1-RNAi plants showed reduced foliar and root nicotine levels as well as increased seedling root elongation rates. Thus, NUP1 affected nicotine metabolism, localization, and root growth.

Nicotine is a natural chemical defense compound that hyperstimulates insect acetylcholine receptors (1), resulting in rapid death or paralysis of nonadapted insects (2, 3). Under situations of moderate herbivory, Nicotiana sp. plants mount an effective nicotine defense that results in increased evolutionary fitness, but this response comes at a metabolic cost to the plants (4). For this reason, plants produce basal nicotine levels in the absence of herbivory, and these levels significantly increase in response to herbivory. Wounding is a primary response trigger in plant herbivory, and increased wounding levels induce nicotine biosynthesis (5, 6). Foliar wounding stimulates the production of jasmonic acid derivatives (7), such as methyljasmonic acid (MeJA), and these derivatives are transported to the roots through the phloem (6). Exogenous MeJA treatment alone is sufficient to increase nicotine biosynthetic levels (7) and increase the mRNA accumulation levels of transcripts encoding nicotine biosynthetic enzymes (8).

Nicotine is exclusively synthesized in the roots (9, 10). In particular, nicotine biosynthesis is localized to growing root tips (11). Restricted root growth in pot-bound plants eliminates an inducible nicotine chemical defense (5). Regulation of nicotine biosynthesis (12, 13) and nicotine biosynthetic gene expression (8, 14–18) are under the coordinate regulation of the A and B loci, also called NIC1 and NIC2, respectively (14). In addition to regulating nicotine biosynthetic genes, the A and B loci regulate a large and complex regulon of apparent stress response genes that are unrelated to alkaloid biosynthesis (15).

Nicotine produced in the roots is loaded into the xylem, where it accumulates to as high as 1 mM in the xylem sap (6). Nicotine is not appreciably degraded in plants (19), and whole-plant nicotine levels are held at MeJA-responsive allogenic set points (20, 21). Nicotine exhibits differential shoot accumulations over the course of plant development and seems to be mobilized by source-sink movement in the phloem (22, 23). In the leaf, nicotine accumulates in the vacuoles of mesophyll cells (24). Based on nicotine tissue accumulation profiles over plant ontology, one can infer nicotine uptake and efflux transport activities, but only two transporters capable of nicotine uptake have been characterized. Nicotiana tabacum jasmonate-inducible alkaloid transporter 1 (NJAT1) and multidrug and toxic compound extrusion (NtMATE1/2) transporters are vacuole-localized multisubstrate proton antiporers that mobilize nicotine into the vacuole (25, 26).

Plant tropane and monoterpenoid indole alkaloid biosynthesis are non-cell autonomous processes (27) requiring the efflux and uptake of alkaloid intermediates between different cell types. One apoplastic alkaloid uptake transporter has been characterized. Coptis japonica multidrug resistance 1 (CmMDR1) is an ATP-binding cassette (ABC) plasma membrane protein responsible for the uptake of berberine into rhizome cells, where it accumulates to high levels (28). In this report, we describe a category of plasma membrane alkaloid transporter, called nicotine uptake permease 1 (NUP1), that was highly selective for nicotine uptake, and lines with reduced NUP1 expression levels resulted in altered nicotine metabolism, localization, and increased root growth.

Results

NUP1 Was Coordinately Regulated with Other Nicotine Biosynthetic Genes. A tobacco gene fragment with similarity to an Arabidopsis purine uptake permease 1 (AtPUP1) (29) was previously isolated during a transcriptional profiling screen for genes that are coordinately regulated by the A and B loci in N. tabacum (15). Plasmid pJGJ332 (GenBank accession no. DR752036) contains a cDNA fragment encoding a predicted polypeptide with similarity to AtPUP1 (BLASTX e-value = 1.35 e–22). AtPUP1 and AtPUP2 are responsible for the proton-mediated uptake of purines and cytokinins in Arabidopsis (29–31). Uptake of purines into yeast cells expressing the recombinant AtPUP1 transporter is inhibited 35% in the presence of nicotine, suggesting that nicotine might be partially recognized by AtPUP1 (29). Transcripts corresponding to the cDNA fragment in pJGJ332 increase in WT B21 root cultures (AABB genotype) treated with MeJA and are significantly reduced in mutant aabb roots (15). These mRNA expression patterns are


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characteristic of coordinated nicotine biosynthetic gene expression (8, 14–18, 32, 33). Based on these initial findings, we hypothesized that the gene represented by pJGI332 might encode a nicotine uptake permease (NUP). Two apparent full-length cDNAs (pEAT1 and pEAT5) were isolated from a tobacco root cDNA library. These cDNAs were 96% similar to each other and were provisionally named NUP1 and NUP2, respectively.

**NUP Homologs Are Present only in Vascular Plants.** The predicted NUP1 and AtPUP1 proteins showed 67% similarity and 56% identity. Both BLASTN and BLASTX homology searches of the nonredundant GenBank database using an e-value cutoff < 10^-4 identified homologs in some but not all plant species. BLASTP homology searches failed to identify NUP1 homologs in the whole-genome sequences of several single-celled eukaryotic algae: Ostreococcus lucimarinus (34), O. tauri (35), and Chlamydomonas reinhardtii (36). The bryophyte Physcomitrella patens genome did not encode any PUP/NUP-like homologs. The most primitive terrestrial plant genome encoding PUP/NUP-like proteins was the lycophyte Selaginella moellendorffii. In a Bayesian phylogenetic analysis (Fig. S1), the Selaginella PUP/NUP-like homologs clustered to a single well-supported clade that included angiosperm homologs. Thus, the absence of PUP/NUP homologs in unicellular algae and Physcomitrella and the monophyletic clustering of the Selaginella PUP/NUP homologs suggest that the plant-specific PUP/NUP transporters originated either just before or at about the same time as the origin of the lycophytes.

**NUP1 Is a Nicotine-Specific Uptake Transporter.** To test whether the NUP1 cDNA encoded a PUP ortholog, Schizosaccharomyces pombe cells expressing the NUP1 cDNA were assayed for radiolabeled adenine uptake activity. Fig. S2 illustrates that S. pombe cells with an empty vector, and those cells with a subcloned NUP1 cDNA accumulated comparable 3H-adenine levels. In contrast, yeast cells harboring the NUP1 cDNA showed significantly more 14C-nicotine uptake activity (Fig. 1A) relative to vector controls. Attempts to assay saturation uptake kinetics were unsuccessful, because exogenous 1 μM nicotine was toxic to the yeast cells (Fig. S3). Fig. 1B shows that the NUP1-specific 14C-nicotine uptake activity was not efficiently competed with 10-fold excess of unlabeled kinetin (16.5% inhibition of 14C-nicotine uptake activity). Therefore, NUP1 is not an ortholog of AtPUP1. In addition, 10-fold excess of atrazine, anatabine, or anabasine resulted in statistically significant but not efficient competition for 14C-nicotine uptake. Thus, heterologously expressed NUP1 was a nicotine-specific uptake transporter.

**NUP1 Localized Primarily to the Plasma Membrane.** The subcellular distribution of NUP1 was examined using a CaMV35S promoter NUP1-GFP gene fusion transiently expressed in tobacco Bright Yellow-2 (BY2) protoplasts cotreated with the vacuole-localizing dye FM4-64 (>12 h after dye treatment) or transiently coexpressed with the plasma membrane intrinsic protein 2A PIP2A-mCherry fusion protein. NUP1-GFP fluorescence was observed primarily at the plasma membrane and did not colocalize with the vacuole FM4-64 fluorescence signal (Fig. 2A). Instead, the NUP1-GFP fluorescence colocalized with the plasma membrane marker PIP2A-mCherry fluorescence (Fig. 2B). The free GFP fluorescence signal did not colocalize with either the FM4-64 or the PIP2A-mCherry fluorescence, confirming the expected nuclear and cytoplasmic localization of free GFP. Thus, NUP1-GFP predominately localized to the plasma membrane of tobacco BY2 protoplasts. In addition, steady state NUP1 transcript levels accumulated to the highest levels within 1 cm of the root tip (Fig. 3), and NUP1 mRNA levels were significantly greater in total roots than rosette leaves (ANOVA P value < 0.01).

**Fig. 1.** Nicotine uptake assays in S. pombe. Error bars are SDs. Asterisks indicate ANOVA P values < 0.05 using Dunnett comparison with no inhibitor treatment. The experiments were replicated two times, each with four independent yeast transformants. (A) 14C-nicotine uptake kinetics of S. pombe cells containing empty cloning vector (gray) and vector expressing NUP1 (black). (B) 14C-nicotine uptake and competition assays. Solvent control indicates 14C-nicotine uptake of cells with empty vector. All other columns contained vector expressing NUP1. All competitors were used at 10-fold excess relative to 14C-nicotine.

**Creation of NUP1-RNAi Whole Plants and Corresponding Hairy Root Lines.** To investigate the physiological role of NUP1, five transgenic tobacco lines containing NUP1-RNAi constructs were generated (NtEAT13-8, NtEAT13-10, NtEAT13-22, NtEAT13-55, and NtEAT14-1) in the Xanthi cultivar background. When independent T1 transgenic NUP1-RNAi shootlets were being regenerated, axenic leaves were removed and inoculated with Agrobacterium rhizogenes to generate matched T1 hairy root cultures. The control hairy root line Xan-1 was also derived from the Xanthi cultivar. Because the NtEAT13-8, -10, -22, and -55 lines gave very similar results, they will be subsequently referred to as the NtEAT13-x lines. Fig. S4 illustrates that the NtEAT14-1 hairy root line did not reduce steady state NUP1 mRNA levels as low as the levels of the four NtEAT13-x hairy root lines.

To access the impact of decreased NUP1 mRNA levels on nicotine production, the nicotine levels in both the hairy roots and their corresponding root culture media were measured during both noninducing (DMSO) and nicotine-inducing conditions (MeJA). As shown in Fig. 4, the four NtEAT13-x lines had significantly less nicotine in the roots during both DMSO and MeJA treatments (ANOVA Dunnnett comparisons to treatment-matched Xan-1 controls; P values < 0.05). Line NtEAT14-1 showed significantly less nicotine only in the DMSO-treated roots. The four NtEAT13-x hairy root lines treated with DMSO showed significantly increased nicotine levels in the media (Fig. 4). The nicotine levels in the media of MeJA-treated NtEAT13-x hairy roots were not significantly different from the levels of the Xan-1 control hairy root line. The fact that less total nicotine was produced by the
NUP1-RNAi lines became apparent when the combined nicotine levels in both the roots and media were considered together (Fig. 4). Therefore, the NUP1-RNAi hairy root lines accumulated less total nicotine on a per root mass basis.

Reduced Nicotine Accumulation Levels in NUP1-RNAi Lines Did Not Correlate Well with Reduced Nicotine Biosynthetic Gene Transcript Levels. Because the NUP1-RNAi hairy root lines showed overall reduced nicotine accumulation levels, the mRNA accumulation levels of five nicotine biosynthetic genes were assayed using quantitative RT-PCR (QRT-PCR). Nicotine is comprised of a pyridine ring conjugated to an N-methylpyrrolidine ring. The enzymes quinolinate phosphoribosyl transferase and quinolinate synthase (QS) contribute to the biosynthesis of the pyridine ring, whereas the enzymes ornithine decarboxylase (ODC), putrescine methyltransferase (PMT), and methylputrescine oxidase contribute to the formation of the N-methylpyrrolidine ring. Fig. 5 shows that ODC, PMT, and QS mRNA levels in the DMSO-treated hairy root cultures had significantly reduced steady state mRNA levels relative to the levels of WT Xanthi controls. The steady state mRNA levels of two other nicotine biosynthetic genes (quinolinate phosphoribosyl transferase and methylputrescine oxidase) and a housekeeping gene (β-ATPase) were not significantly different relative to Xanthi controls during the same condition (Fig. S5). In contrast, during nicotine-inducing MeJA treatment, no significant differences in any of the five nicotine biosynthetic gene transcript levels were observed relative to the Xan-1 controls similarly treated (Fig. 5 and Fig. S5). Thus, although reduced NUP1 transcript levels (Fig. S4) were well-correlated with total reduced nicotine accumulation levels in the hairy root cultures (Fig. 4), corresponding reductions of five nicotine biosynthetic gene transcript levels were not highly correlated with reduced nicotine accumulation levels during the two treatments.

Reduced NUP1 Expression Resulted in Decreased Foliar Nicotine Levels but Not a Reduced Capacity to Transport Nicotine to the Leaves. To determine the effect of reduced NUP1 expression on leaf nicotine accumulation levels, 20-d-old WT and T3 NUP1-RNAi plants grown on potting media were harvested and assayed for foliar nicotine accumulation levels. All five NUP1-RNAi lines showed reduced foliar nicotine levels compared with WT Xanthi plants, with three of five NUP1-RNAi lines showing statistical significance (Fig. 6A).

Foliar nicotine accumulation is the product of at least two distinct processes: nicotine biosynthesis in roots and subsequent nicotine transport from roots to leaves. Hydroponically grown Nicotiana plants take up exogenous nicotine in the media and transport it to the leaves, resulting in increased foliar nicotine levels (37). If NUP1-RNAi plants were defective in transporting nicotine from roots to leaves, then they should display decreased differential accumulation of exogenous nicotine into the leaves of plants treated with exogenous nicotine at the roots. This hypothesis was tested by treating 14-d-old hydroponically grown Xanthi plants and three representative NUP1-RNAi lines with hydroponic media without or with 1 mM nicotine. Fig. 6B shows that NtEAT13-8 and NtEAT13-22 leaves had significantly reduced foliar nicotine levels during both control and nicotine treatment relative to treatment-matched Xanthi controls, whereas NtEAT14-1 leaves did not. Nevertheless, all plants treated with 1 mM nicotine in the hydroponic solution showed about a twofold increase in foliar nicotine levels relative to their untreated controls (Fig. 6B). The mean fold increase in foliar nicotine levels in the NtEAT13-8, NtEAT13-22, and NtEAT14-1 NUP1-RNAi lines were not significantly different from the increase of the Xanthi control (ANOVA Dunnett comparison; all
Therefore, the NUP1-RNAi mutant lines were not differentially affected in their ability to move exogenous nicotine from the media to the leaves. This finding suggests that the decreased foliar nicotine levels in lines NtEAT13-8 and NtEAT13-22 were likely caused by an overall decrease in steady state root nicotine levels. Indeed, two NUP1-RNAi lines showed lower root nicotine accumulation levels during both control and exogenous nicotine feeding conditions (Fig. 6C). The lack of statistical significance during control conditions may be because of a high degree of variation in the Xanthi controls, which had one sample with a high residual in the ANOVA analysis. In summary, results from the nicotine feeding experiments rejected the hypothesis that NUP1 directly affected root to shoot nicotine transport.

Reduced NUP1 mRNA Levels Correlated with Increased Seedling Root Growth. Induced de novo nicotine production confers a short-term reduction of *N. sylvestris* root growth (20). Exogenous nicotine treatment reduces both root and shoot biomass (37, 38) and root elongation (26). Given that NUP1 was a plasma membrane-localized (Fig. 2) nicotine uptake permease (Fig. 1), it was hypothesized that the NUP1-RNAi reduced expression lines might accumulate less nicotine and therefore, be less sensitive to the toxic effects of exogenous nicotine treatment to seedling root growth. WT and NUP1-RNAi plants were treated with nothing (control), DMSO, nicotine, MeJA, or nicotine and MeJA combined (Fig. 7). When factored across all genotypes, nicotine, MeJA, and combined nicotine and MeJA treatments resulted in significant reductions in seedling root length relative to the control treatment.
(ANOVA Dunnett comparison; P values < 0.001), whereas the control and DMSO treatments were not significantly different from each other (P value = 0.99). Thus, nicotine and/or MeJA significantly reduced root elongation of all genotypes. When factored over all treatments, genotype was a significant factor in the ANOVA model (P value < 0.001). Fig. 7 also shows that lines NtEAT13-10 and -22 seedlings had significantly longer roots relative to the corresponding Xanthi controls (ANOVA Dunnett comparison; P value < 0.05) during all treatments. Lines NIEAT13-8 and -55 showed significantly longer roots during three and two treatments, respectively. Line NIEAT14-1 root elongation was not significantly different from Xanthi controls during any treatment. Thus, reduced NUP1 mRNA levels (Fig. S4) correlated with less nicotine in the roots (Figs. 4 and 6) and roots that were less sensitive to the inhibitory effects of nicotine on root elongation (Fig. 7). Consistent with the observations that reduced NUP1 levels affected both root growth and nicotine content, NUP1 transcript levels were highest within 1 cm of the growing root tip (Fig. 3) where nicotine biosynthesis occurs.

**Discussion**

The nicotine-specific uptake activity of yeast cells expressing NUP1 (Fig. 1), the plasma membrane localization of NUP1-GFP in BY2 protoplasts (Fig. 2), and the preferential NUP1 mRNA accumulation in roots and root tips (Fig. 3) suggest that NUP1 is a plasma membrane transporter that moves apoplastic nicotine into the cytoplasm of tobacco root cells. The NUP1-RNAi lines showed correlations of reduced NUP1 mRNA accumulation levels (Fig. S4) with (i) reduced root nicotine accumulation levels (Fig. 4), (ii) redistribution of nicotine out of hairy roots and into the culture media (Fig. 4), (iii) decreased foliar nicotine levels (Fig. 6), and (iv) increased root growth (Fig. 7). Previous studies show a good correlation of concerted increases in the expression of multiple nicotine biosynthetic genes with increases in nicotine accumulation levels (8, 39, 40), indicating metabolic control mediated by the abundance of transcripts encoding nicotine biosynthetic enzymes. In contrast, the NUP1-reduced expression lines did not show a good correlation of five nicotine biosynthetic gene expression levels with reduced nicotine accumulation levels, suggesting that NUP1 may influence mechanisms of alkaloid metabolic control other than transcript abundance levels.

The reduced NUP1 mRNA expression lines did not completely suppress the inhibitory effects of nicotine and/or MeJA treatments on root elongation, suggesting that other mechanisms for nicotine uptake may be operating. The increased root elongation rates in the NUP1-RNAi lines might be because of either a decreased metabolic load conferred by less nicotine biosynthesis or less cytotoxicity resulting from lower cytoplasmic nicotine accumulation levels. The decreased root nicotine accumulation levels (Figs. 4 and 6) were not likely a simple dilution effect caused singularly by increased root growth, because (i) root growth is a requisite for nicotine biosynthesis (5, 11) and (ii) nicotine levels increase proportionally with increasing root mass (11). The low NUP1 mRNA levels in leaves (Fig. 3) do not necessarily preclude a secondary role for NUP1-mediated apoplastic nicotine uptake into leaf mesophyll cells.

**NUP1 Affects Different Physiologies than NtJAT1 and NtMATE1/2.** NUP1 showed several important differences from the tobacco MATE-type nicotine transporters. NUP1-GFP localized to the plasma membrane of BY2 protoplasts (Fig. 2), whereas NtJAT1 and NtMATE1/2 mostly localize to plant vacuole membranes (25, 26). NUP1 did not efficiently transport two tropane alkaloids (Fig. 1B); in contrast, NtJAT1 and NtMATE1/2 both efficiently transport tropane alkaloids (25, 26). NUP1-RNAi seedlings had roots that grew significantly faster regardless of treatment (Fig. 7) (one-way ANOVA), whereas NtMATE1/2 reduced expression plants do not affect root growth unless challenged with high exogenous nicotine levels (26). The relatively broad substrate recognition profiles, together with the vacuole localization of NtJAT1 and NtMATE1, are consistent with a role in sequestering toxic cations, including pyridine and tropane alkaloids, into the vacuole as part of a general detoxification physiology. In contrast, recombinant NUP1 showed a high degree of nicotine substrate specificity, and transgenic lines with decreased NUP1 mRNA accumulation levels affected several aspects of nicotine physiology.

There are similarities and differences in plant alkaloid transporters found in tobacco and *Coptis japonica*. In tobacco, the plasma membrane-localized NUP1 moved apoplastic nicotine into the cytoplasm. Similarly, the *C. japonica* CjMDR1 mobilizes the alkaloid berberine from the apoplast into the cytoplasm (28, 41). However, NUP1 and CjMDR1 differ in how the alkaloid uptake is energized. Based on similarity to AtPUP1 (29, 31), NUP1 most likely uses proton symport for the uptake of nicotine, whereas CjMDR1 uses the hydrolisis of ATP to energize the transport of berberine into the cytoplasm (28). Both tobacco and *C. japonica* use the antiport of protons from the vacuole in conjunction with MATE-type membrane transporters to move cytoplasmic alkaloids into the vacuole (25, 26, 42).

**NUP1 Affects Root and Shoot Nicotine Homeostasis.** *Nicotiana* sp. roots show three distinct nicotine transport processes. Most nicotine produced in roots is transported through the xylem (6) and dynamically accumulates in various above-ground tissues. However, some nicotine in roots is released into the rhizosphere (10), of which a portion could be taken back into the roots (37). Although the role of nicotine transport into the xylem is well-understood in terms of chemical defense, the ecological roles of nicotine release to and reabsorption from the rhizosphere are less well-understood. The release from and reabsorption of nicotine into root cells suggest that there are optimum root apoplastic nicotine accumulation levels and homeostatic control mechanisms to maintain them. Thus, NUP1 seems to be at a physiological nexus between root nicotine metabolism and root growth. In this light, the establishment and/or monitoring of apoplastic root nicotine levels by NUP1-mediated nicotine uptake might be important for maintaining well-documented—but poorly understood—whole-plant nicotine allelochemical set points (20, 21).

**Methods**

Detailed experimental methods are described in SI Methods. This section is a brief outline of the methods.
Creation of \textit{NUP1-RNAi} Transgenic Plants and Corresponding Hair Root Cultures. A 448-bp PCR fragment derived from \textit{NUP1}, with 97% identity to \textit{NUP2} and subcloned into the RNAi vector PHANFIBIL (44), was eventually used to generate both stably transformed plants and hairy roots in the cultivar Xanthi using \textit{A. tumefaciens} and \textit{A. rhizogenes}, respectively.

\textbf{Root Elongation Assays.} The root elongation assays were performed essentially as previously described (26). All statistical analyses were performed using the GLM ANOVA analysis in Minibit version 14.13 (Minitab) for the initial foliar nicotine assays, Gamborgs B5 media for the hairy root and root elongation assays, or Hoagland's solution for the exogenous nicotine feeding assays.

\textbf{Quantification of Nicotine Levels.} Nicotine levels were assayed by HPLC-UV (48) except for the hydroponically grown tissues, which were assayed by liquid chromatography-MS-MS.

\textbf{Quantification of Gene-Specific mRNA Levels in Hairy Root Cultures.} Steady state gene-specific transcript levels were estimated using QRT-PCR as described in ref. 15 and SI Methods.

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\textbf{References.}
Supporting Information

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SI Methods

Isolation and Characterization of Nicotine Uptake Permease 1 and 2 cDNAs. Plasmid pGJ332 (1) and oligonucleotide primers oEAT1 (CAGTGGTCCTCCCGGTTAC) and oEAT2 (CTGGCATATTTCAAGTTGT) were used to generate a dUTP-digoxigenin (Roche Diagnostics) probe that was hybridized to a Burley 21 root phagemid cDNA library (2). Hybridizing phagemids were isolated, and corresponding plasmids were excised and sequenced using primers: M13 Forward, M13 Reverse, oEAT3 (GGCTTCGACTGGTCTGTG), oEAT4 (GATTGCGACTGTTGCTGTT), oEAT5 (TAGAGAAAATCATCAAGACCGT), oEAT6 (TATAGAAATAATCAGAAGCCAGT), oEAT7 (GTAACGGCCGGC-AAAACGT), oEAT8 (CTTACTCTTGTCGCCTCCTGTGC), oEAT10 (TTACAAACGGTGTTGATG), oEAT11 (GCTCTG- GCTTTTATTC), and oEAT12 (GCTTGGATAGAAGG- GTATAAC). The nucleotide sequences from the cDNA inserts in plasmids pEAT1 (NUP1) and pEAT5 (NUP2) were deposited into GenBank (accession nos. GU174267 and GU174268, respectively).

Creation of NUP1-RNAi Transgenic Plants and Corresponding Hairy Root Cultures. Oligonucleotides oEAT24 (TCTAGACTCGACT-CTGGT) and oEAT25 (ATCGATGGTCATTTCAAGTTGCTCT) were used in standard Taq PCR reactions with pEAT1 as the template to amplify a 488-bp PCR fragment internal to the NUP1 coding region, which showed 97% identity to NUP2 and would efficiently hybridize with both sequences. The resulting PCR fragment was subcloned using Xhol-KpnI and ClaI-XbaI restriction sites into pHANNIBAL (3), resulting in plasmid pEAT24. A 2.5-Kb NotI fragment containing the NUP1-RNAi construct was filled in using Klenow Fragment and subcloned into pCAMBIA2300 cut with Smal, resulting in a CaMV35S promoter-NUP1-RNAi transgene subcloned in both orientations, which resulted in pEAT13 and pEAT14, respectively. Plasmids pEAT13 and pEAT14 were conjugated from Escherichia coli into Agrobacterium tumefaciens LBA4404 using the plRKR2013 helper plasmid (4). LBA4404 containing either pEAT13 or pEAT14 was used for RNA extraction for quantitative RT-PCR. Each hairy root culture was independently replicated four times, each on a different day. Statistical analyses were performed using the general linear model (GLM) ANOVA analysis in Minitab version 14.13 (Minitab) with a Dunnett comparison with treatment-matched Xanthi controls.

Root Elongation Assays. The root elongation assays were performed as previously described (6) with the following modifications. The solidifying agent in the solid media was 0.8% Difco Granulated Agar (Becton Dickinson). Approximately 30 plants of each line were initially grown on 0.5x Gamborgs B5 media with 1.5% sucrose and then transplanted after 7 d to the same media supplemented with nothing (i.e., control), 0.2% DMSO, 2 mM nicotine, 100 μM MeJA, or nicotine and MeJA combined in 150-mm Petri plates that stood on edge in a Percival CU-36L4 (Percival Scientific) with a 14-h light/10-h dark cycle at 25°C. Seven days after transfer to the treatment plates, the Petri plates were photographed, and the length of the primary roots was measured using the Image J software (http://rsb.info.nih.gov/ij/). The experiment was independently replicated four times, each on a different day. Statistical analyses were performed using the general linear model (GLM) ANOVA analysis in Minitab version 14.13 (Minitab) with a Dunnett comparison with treatment-matched Xanthi controls.

Nicotine and Adenine Yeast Uptake Assays. An NcoI site was introduced into the NUP1 ATG codon by PCR using oligonucleotide primers oGJ199 (GTCTAGACCTAGGAAACATCCGATTTAAGCA) and oGJ200 (GCAAGAGGTATAAGGGTGAGTG); the modified fragment was subcloned into pEAT1 as a XbaI-NdeI fragment to create pGJ392. The full-length NUP1 region then subcloned into pTM1 to create pGJ393. An NcoI-SalI NUP1 fragment was cut from pGJ393 and ligated into NdeI-SalI sites in the Schizosaccharomyces pombe expression vector pREP41 after the NcoI and NdeI ends in the polylinker were blunted. NUP1 was placed under the control of the nmt41-inducible promoter (7). Either vector pREP41 or pREP41-NUP1 was introduced into S. pombe strain YF016 (h-, leu1-32, ura4-C190T, ade7::ura4). Four separate positive transformants were selected in Edinburgh minimal medium (EMM) without Leu and confirmed with PCR. S. pombe cells were grown to OD600 = 2.0 in EMM containing 15 μM thiamine. The thiamine was removed by washing two times with EMM, and cells were transferred to fresh EMM and incubated for 19 h (final OD600 = 1–2) to induce the expression of the NUP1 protein. Cell pellets were washed one time and resuspended in EMM (pH 5.6). Cell density was adjusted to OD600 = 2. Cells were kept at 4°C in all the following steps, except for the 30°C incubation; 1 μL 13C-nicotine (0.1 μCi, final concentration ~20 nM; specific activity = 54 Ci/mmol) (Moravek Biochemicals) was added into 100 μL cells and incubated at 30°C for 0, 100, 200, and 300 s. 3Hadenine (40 Ci/mmol and 1 μCi/mL) (American Radiolabeled Chemicals, Inc.) uptake assays were performed using the same procedure as the above nicotine uptake assays.

NUP1 Subcellular Localization. The NUP1 translation termination codon was replaced with a serine codon followed by an in-frame ATG codon within an NcoI site by using pEAT1 as the template DNA and oligonucleotide primers oEAT1 (CAGTTTGTGCT-CCCGTTACC) and oEAT9 (TCTTGGTCACTTACCA-TGGAAAACAGATTTCGATGTTG) in PCR reactions that generated a modified fragment. The resulting PCR fragment was
subcloned into pEAT1 as a BsmBI-XhoI fragment. A GFP gene was excised as an Ncol-XhoI fragment from pCAMBIA1302 and subcloned into the modified pEAT1 plasmid described above to yield plasmid pEAT11 (NUP1::GFP gene fusion). The NUP1 coding region was excised from pEAT11 as an EcoRI-Ncol fragment and subcloned into plasmid pGFP-MRC (8) that was similarly cut to create plasmid pGJ402. This cloning strategy resulted in the deletion of the tobacco mosaic virus (TMV) transcription enhancer from pGJ402. Bright Yellow-2 (BY2) protoplasts were generated (9) and transiently transformed with either plasmid pGFP-MRC or pGJ402 (10). Transformation of protoplasts with pGFP-MRC or pGJ402 and PIP2A-mCherry pm-rk (11) was performed using a polyethylene glycol-mediated method as previously described in ref. 10. To label the tonoplast with FM4-64 dye (T13320; Invitrogen), BY2 cells were incubated in BY2 growth medium containing 20 μM FM4-64 for 30 min, washed, and then, incubated in the growth medium for an additional 2 h before protoplasting and polyethylene glycol-mediated DNA transformation. The localization of the expressed proteins was visualized with a confocal microscope (LSM 510 META with Axio Observer Z1; Zeiss) equipped with lasers suitable for GFP (Argon ion laser, 488 nm), FM4-64, and mCherry (Helium-Neon laser, 543 nm). Fluorescence images were acquired using appropriate bandpass (BP) and longpass (LP) filters (BP 505–530 for GFP, LP 560 for FM4-64, and BP 585–615 for mCherry) at least 12 h after FM4-64 dye treatment, at which time the FM4-64 fluorescence was mostly confined to the tonoplast.

**Plant Growth Conditions.** WT and T3 homozygous NUP1-RNAi seeds were surface-sterilized with 50% bleach, washed with sterile distilled water, and germinated on Gamborg's B5 media incubated at 22 °C with 16 h light/d at 40 μEinsteins m⁻² min⁻¹ for 7 d. Single 2-d-old seedlings were transplanted to 3.5-in pots containing Premix potting mix (Premier Horticulture) and grown at the same conditions used for germination. All above-ground portions of 10 20-d-old rosette stage plants were harvested and immediately frozen in liquid nitrogen. The hairy roots and foliar tissues from potting mix grown plants were analyzed by HPLC (12). Hydroponically grown 10-d-old seedlings were placed in Styrofoam floats and propagated in aerated Hoagland's solution for 14 d. The hydroponically grown plants were sorted into pairs of equivalent-sized plants, with one plant's roots immersed in 50 mL Hoagland's solution and the other plant's roots immersed in Hoagland's solution supplemented with 1 mM nicotine for 2 d. After this treatment period, the plants were removed from the media, roots were blotted on absorbent tissue, tissues were separated into roots and shoots, and they were placed into 50-mL plastic tubes that were subsequently flash-frozen in liquid nitrogen.

**Quantification of Nicotine Levels.** Frozen tissues were ground to a fine powder using a liquid nitrogen-cooled mortar and pestle. Homogenized plant tissue was lyophilized and stored −20 °C in a desiccated chamber. The nicotine in the foliar and hairy root tissues was extracted using a simple aqueous extraction procedure and quantified by HPLC (12) using a Waters 2695 Separations (Waters Corp) unit, a Zorbax Eclipse XDB-C18 4.6 × 250 mm 5-μm column (Agilent Technologies), and a Waters 996 photodiode array detector. Authentic nicotine standards (SIGMA) were used to generate a standard curve, and nicotine levels were quantified using the Waters Empower Pro software (build 1154).

The nicotine levels in hairy root culture media were normalized to total dry root mass at the time of harvest. This normalization was accomplished by measuring the nicotine concentration in the hairy root culture media and the volume of culture media and calculating the total nanomoles of nicotine in the media divided by the total hairy root dry weight. The total root dry weight was calculated by measuring the fresh hairy root weight multiplied by an empirically derived constant of 0.077 mg dry roots mg⁻¹ fresh roots. This calculation allowed for the direct comparison of nanomoles nicotine milligram⁻¹ root dry weight in both the hairy roots and the nicotine levels released into the media over the course of the 24-h treatments. In short, the hairy root cultures were a closed system, in which all nicotine present in both the roots and media was counted and observed differences in the combined root and media nicotine levels (normalized to root mass) indicated differences in net nicotine accumulation.

The nicotine levels from hydroponically grown plants were assayed by liquid chromatography-MS-MS. These samples were fractionated using an Agilent 1200 Series HPLC using a Thermo Scientific Hypersil Gold 100 × 4.6 mm 3-μm particle size column, with a mobile phase of 0.1% formic acid/acetonitrile and 0.1% formic acid (99/1) and a flow rate of 1 mL/min. Nicotine had a retention time of 2 min in a 6-min run. Nicotine was detected using an Applied Biosystems 3200 Q-Trap mass spec spectrometer operated in positive ion multiple reaction monitoring mode with the following parameters: 75 ms dwell time each, declustering potential of 40 V, entrance potential of 5 V, collision energy of 20 V, collision cell exit potential of 2 V, curtain gas pressure of 35 psi, collision cell gas setting of 6, ion spray voltage of 4,500 V, ion source temperature of 600 °C, ion source gas 1 pressure of 70 psi, and ion source gas 2 pressure of 60 psi. Mass spectrometric data were processed using the Analyst version 1.4.2 software. Nicotine levels were quantified by creating a calibration curve injecting authentic nicotine standards and measuring the detector response (in counts per second) for the m/z 163.2 to m/z 132.2 transition. The response for the m/z 163.2 to m/z 106.1 transition was used as a qualifying signal to confirm that the chromatographic peak detected and measured was caused by nicotine.

**Quantification of Gene-Specific mRNA Levels in Hairy Root Cultures.** Quinolinate phosphoribosyltransferase, quinolinate synthase, and purine permease-like PUP/NUP1-like transcripts in hairy root cultures were quantified by quantitative RT-PCR as previously described (1). NUP1 mRNA levels were quantified in hydroponically grown plants using oligonucleotide primer pair oJGJ224 (CCTTCATTGCTACTTTCATTATGTTTGT) and oJGJ225 (TTCAGTTGTTGTTAGGATATTAAGAG) and modified thermocycling conditions in which the annealing, synthesis, and detection are all performed at 60 °C using a PEAT1 dilution series as a standard curve. Omithin decarboxylase transcript levels were quantified using plasmid pDRG58 (13) and oligonucleotide pair oJGI203 (GTTTACATCCGGCACCAGGTT) and oJGI204 (AA-AAAAACGACCCGTTTACAG). Methylputrescine oxidase 1 transcript levels were quantified using plasmid pWG115 (14) and oligonucleotide pair oJGI205 (TCACACATGGTCTCCTGGTG-GG) and oJGI206 (TCAACAGCGGCCAGGAGTATTA). Putrescine methyltransferase mRNA levels were assayed by quantitative RT-PCR as previously described (13).

Fig. S1. Bayesian phylogeny of PUP/NUP homologs. Homologs were identified in BLASTP homology searches of the National Center for Biotechnology Information RefSeq and Joint Genome Institute Selaginella site using an e-value cutoff < e^{-4}. Sequences were aligned using MAFFT and subjected to a 5 million-generation MrBayes run (using the JTT + I + G + F model). Cladogram was manually rooted on Selaginella-containing clade. The posterior probability for each furcation is shown on each node. Each sample is coded by the first three letters of the genus, underscore, the first three letters of the species, underscore, and an arbitrary sequential number.
Fig. S2. Adenine uptake assays in S. pombe. Error bars are SDs (n = 4). (A) \(^3\)H-adenine (25 nM) uptake kinetics of S. pombe cells containing empty cloning vector (◇) and vector expressing NUP1 (■). (B) \(^3\)H-adenine uptake assays at 25 and 50 nM for 5 min.

Fig. S3. Yeast nicotine toxicity assays. Yeasts were grown in EMM liquid media for 2 d and diluted with EMM to an OD\(_{600}\) of 0.5. The yeasts were then treated with different concentrations of nicotine (200 nM, 600 nM, and 1 \(\mu\)M) for 10 min at 29 °C, diluted in EMM, spotted (5 \(\mu\)L) onto EMM plates, and incubated at 29 °C for 4 d.
Fig. S4. Steady state NUP1 mRNA levels in hairy root lines. White columns are DMSO treatment, and gray columns are MeJA treatment; *N = 3 independent replicates. Bars indicate SEM. Asterisks indicate ANOVA $P$ values < 0.05 using Dunnett comparison with Xan-1 for each respective treatment.

Fig. S5. Steady state mRNA levels in hairy roots. White columns are DMSO treatment, and gray columns are MeJA treatment. Error bars and asterisks are as described in Fig. 4; *N = 3. (A) Quinolinate phosphoribosyl transferase (QPT). (B) Methylputrescine oxidase (MPO). (C) β-ATPase.