Tobacco nicotine uptake permease (NUP1) affects alkaloid metabolism

Sherry B. Hildreth[a,b,1], Elizabeth A. Gehman[a,c,1], Haibing Yang[d], Rong-He Lu[a], Ritesh K C[a], Kim C. Harich[e], Shi Yu[a], Jinshan Lin[d], Jackson L. Sandoe[a,f], Sakiko Okumoto[a], Angus S. Murphy[d], and John G. Jelesko[a,2]

[a]Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg, VA 24061; [b]Biological Sciences Department, Virginia Tech, Blacksburg, VA 24061; [c]Battelle National Biodefense Institute, National Biodefense Analysis and Countermeasures Center, Fort Detrick, MD 21702; [d]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907; [e]Biochemistry Department, Virginia Tech, Blacksburg, VA 24061; and [f]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

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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 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 planta (19), and whole-plant nicotine levels are held at MeJA-responsive allometric 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 (NtJAT1) and multidrug and toxic compound extrusion (NtMATE1/2) transporters are vacuole-localized multisubstrate proton antiporters 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 (CjMDR1) 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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[1]S.B.H. and E.A.G. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: jelesko@vt.edu.

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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 pJGJ332 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 Physcomatrella 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 ^{3}H-adenine levels. In contrast, yeast cells harboring the *NUP1* cDNA showed significantly more ^{14}C-nicotine uptake activity (Fig. 1*A*) 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. 1*B* shows that the NUP1-specific ^{14}C-nicotine uptake activity was not efficiently competed with 10-fold excess of unlabeled kinetin (16.5% inhibition of ^{14}C-nicotine uptake activity). Therefore, NUP1 is not an ortholog of AtPUP1. In addition, 10-fold excess of atropine, anatabine, or anabasine resulted in statistically significant but not efficient competition for ^{14}C-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. 2*A*). Instead, the NUP1-GFP fluorescence colocalized with the plasma membrane marker PIP2A-mCherry fluorescence (Fig. 2*B*). 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).

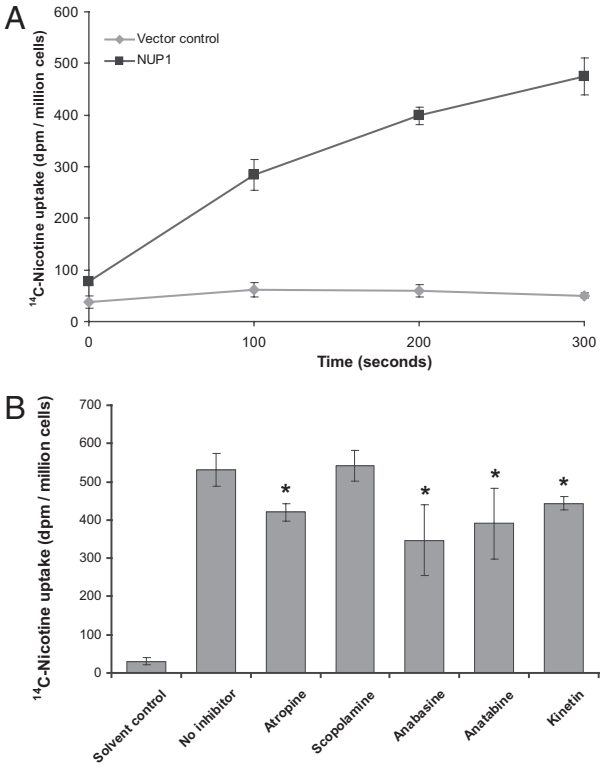

Fig. 1. Nicotine uptake assays in *S. pombe*. Error bars are SDs. Asterisks indicate ANOVA *P* values < 0.05 using Dunnett comparison with no inhibitor treatment. The experiments were replicated two times, each with four independent yeast transformants. (*A*) ^{14}C-nicotine uptake kinetics of *S. pombe*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

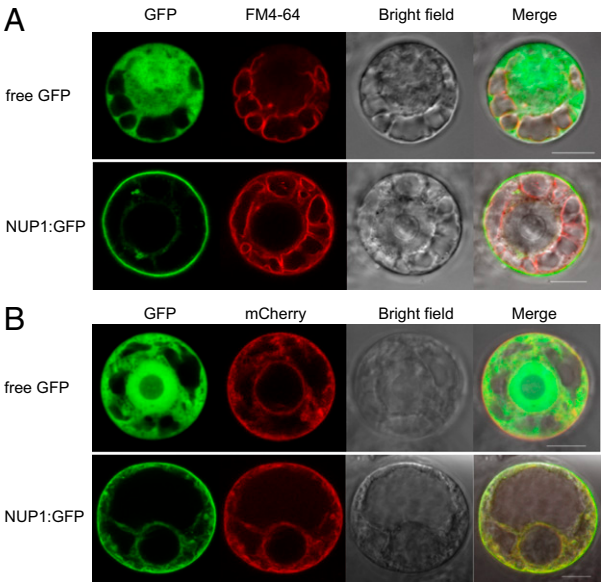

Fig. 2. Subcellular localization of NUP-GFP. (*A*) Tobacco BY2 protoplasts transiently expressing either free GFP or NUP1-GFP. The vacuolar membrane was labeled with FM4-64. The fluorescence of GFP (green), FM4-64 (red), and bright-field image (gray) was monitored using confocal microscopy and merged in the Merge column. (Scale bar: 10 μm.) (*B*) Colocalization of NUP1-GFP with plasma membrane aquaporin PIP2A-mCherry (red).

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

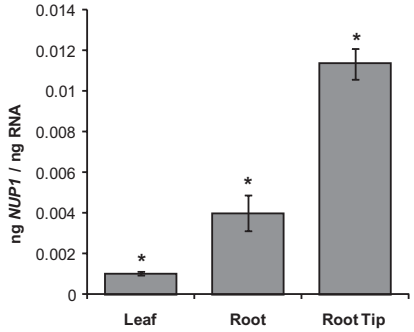

Fig. 3. Steady state *NUP1* mRNA levels in specific tissues. Tissues from 14 d hydroponically grown WT Xanthi plants were harvested, and *NUP1* transcript levels were quantified by QRT-PCR; $N = 4$, except for leaves, where $N = 3$. Error bars are SEM. The mean *NUP1* accumulation levels in each of the three tissues were significantly different from one another [general linear model (GLM) ANOVA; P value < 0.01].

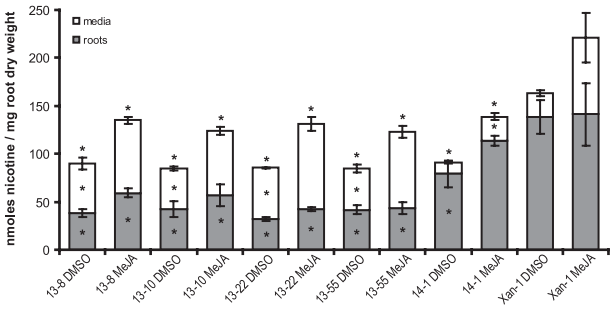

Fig. 4. Nicotine accumulation levels in hairy roots and root culture media. Open columns indicate normalized nicotine levels in the culture media, and gray columns indicate normalized nicotine levels in roots. Nicotine levels in the media were normalized to total hairy root mass at harvest as described in *SI Methods*; $N = 3$ independent replicates. Error bars indicate SEM. Asterisks indicate ANOVA P values < 0.05 using Dunnett comparison with Xan-1 for each respective treatment.

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. 6*A*).

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. 6*B* 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. 6*B*). 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

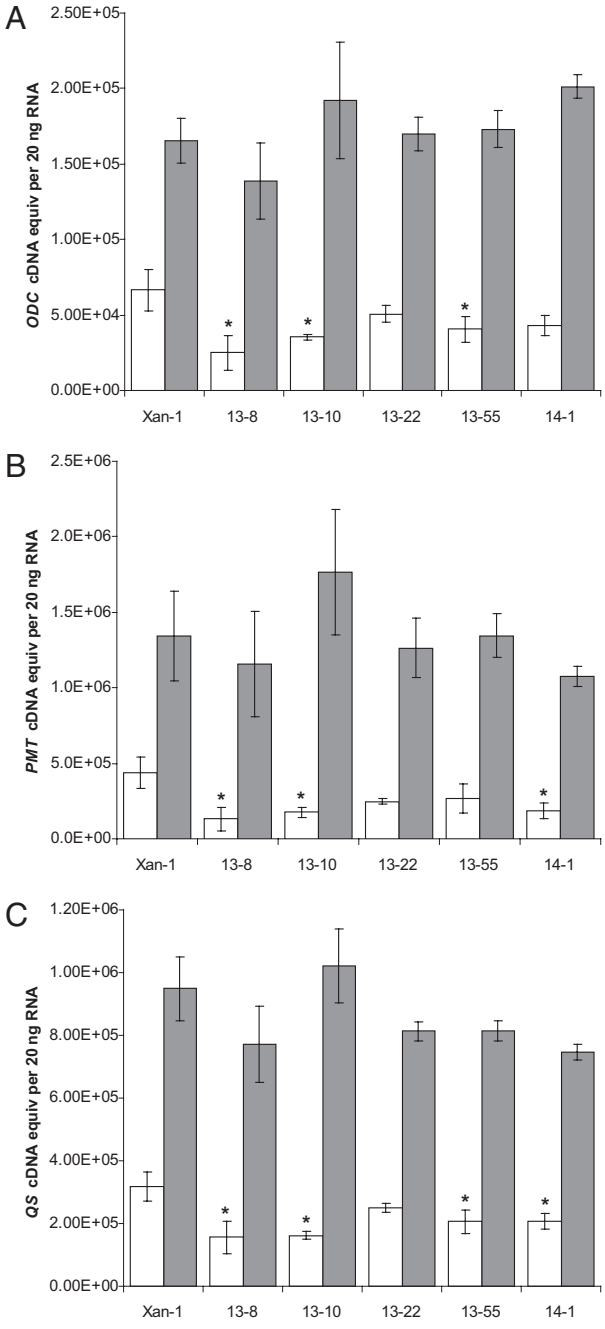

Fig. 5. Steady state nicotine biosynthetic gene mRNA levels in hairy root cultures. White columns are DMSO treatment, and gray columns are MeJA treatment. Error bars and asterisks are as described in Fig. 4; $N = 3$. (*A*) *ODC*. (*B*) *PMT*. (*C*) *QS*.

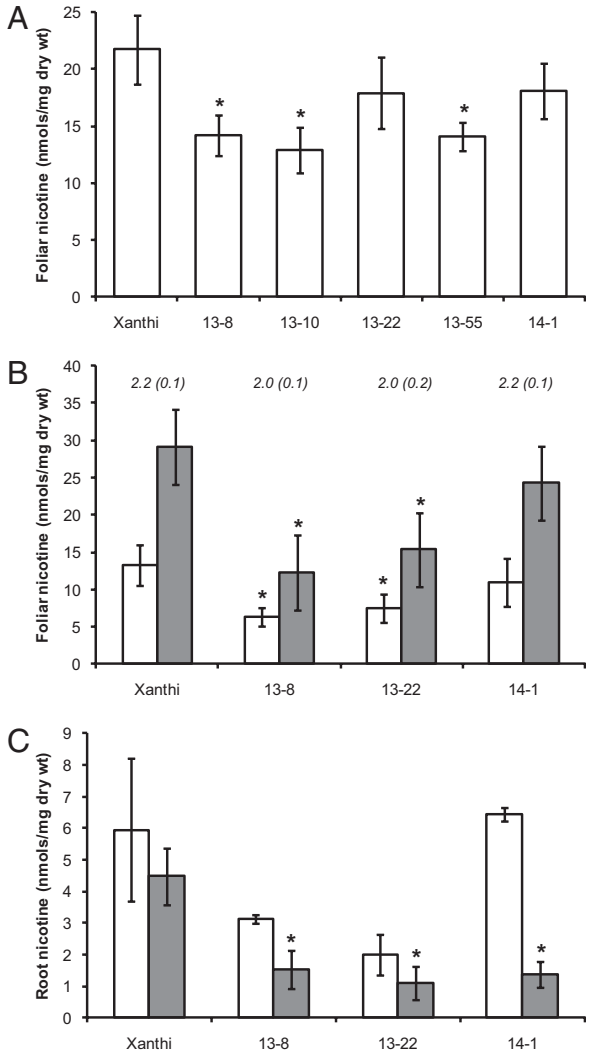

Fig. 6. Nicotine accumulation levels in leaves and roots. White columns are untreated controls, and gray columns are nicotine-treated. Error bars and asterisks are as described in Fig. 4; $N = 3$, unless otherwise noted. (*A*) Nicotine accumulation in leaves from 22-d-old plants grown in potting mix. (*B*) Nicotine accumulation in leaves from hydroponically grown plants treated with either control media or media supplemented with 1 mM nicotine; $n = 2$ for line 13-8 only. Italicized numbers indicate mean fold increase in nicotine levels because of nicotine feeding, with SE given in parentheses. (*C*) Nicotine accumulation in the roots of the same plants as *B*.

P values > 0.71). 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. 6*C*). 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

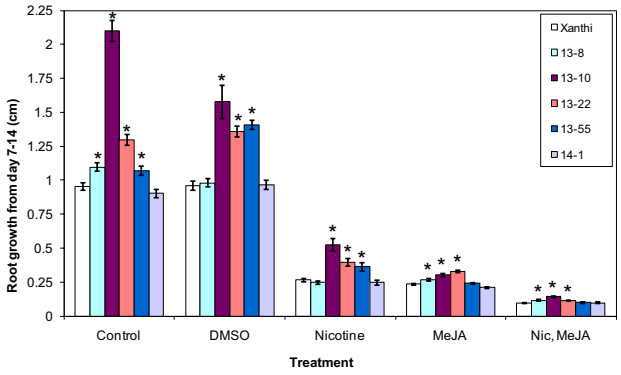

Fig. 7. Seedling root elongation assays. Error bars and asterisks are as described in Fig. 4. Treatment designations are the same as described in Fig. 4, with the following additions: control, unsupplemented media; nic, nicotine.

(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 NtEAT13-8 and -55 showed significantly longer roots during three and two treatments, respectively. Line NtEAT14-1 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 where 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. 1*B*); in contrast, NtJAT1 and NtMATE1/2 both efficiently transport tropane alkaloids (25, 26). *NUP1-RNAi* lines showed decreased nicotine accumulation levels (Fig. 4) and altered the root's capacity to retain nicotine (Figs. 4 and 6), whereas NtMATE1/2 reduced expression plants do not affect nicotine accumulation levels (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 hydrolysis 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 allometric set points (20, 21).

Methods

Detailed experimental methods are described in *SI Methods*. This section is a brief outline of the methods.

Isolation of *NUP1* and *NUP2* cDNAs. Plasmid pJGJ332 (15) and oligonucleotide primers oEAT1 (CAGTTTTGCTCCCCGTTAC) and oEAT2 (CTGCAAATATTTCAAGTGTT) were used to generate a dUTP-digoxigenin (Roche Diagnostics) probe that was used to screen a Burley 21 root phagemid cDNA library (43), resulting in plasmids pEAT1 (*NUP1*) and pEAT5 (*NUP2*; GenBank accession nos. GU174267 and GU174268, respectively).

Creation of *NUP1-RNAi* Transgenic Plants and Corresponding Hairy Root Cultures. A 448-bp PCR fragment derived from *NUP1*, with 97% identity to *NUP2* and subcloned into the RNAi vector pHANNIBAL (44), was eventually used to generate both stably transformed plants and hairy roots in the cultivar Xanthi using *A. tumefaciens* and *A. rhizogenes*, respectively.

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 Minitab version 14.13 (Minitab), most with a Dunnett comparison with treatment-matched controls.

Nicotine Transport Assays. The NUP1 coding region was subcloned into the *S. pombe* expression vector pREP41 (45), and the yeast cells were used in radiolabeled ^{14}C-nicotine (without and with unlabeled kinetin, tropane, and pyridine alkaloids) or ^{3}H-adenine uptake assays.

NUP1 Subcellular Localization. The NUP1 coding region was subcloned in frame to the *GFP* gene in pGFP-MRC (46) and used in transient BY2 protoplast transformations along with either FM4-64 dye or PIP2A-mCherry pm-rk (47). Florescence detection was performed by laser confocal microscopy.

Plant Growth Conditions. All plants were grown at 22 °C with 16 h light/d at 40 μEinstein m^{-2} min^{-1} in Promix potting mix 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.

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.

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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