Phytochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

# Tobacco NUP1 transports both tobacco alkaloids and vitamin B6

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ARTICLE INFO

*Article history:*
Available online xxxx

*Keywords:*
*Nicotiana tabacum*
Solanaceae
Tobacco
Transport assays
Transporter
Nicotine
Vitamin B6
PUP-family transporters

ABSTRACT

The purine permeases (PUPs) constitute a large plasma membrane-localized transporter family in plants that mediates the proton-coupled uptake of nucleotide bases and their derivatives, such as adenine, cytokinins, and caffeine. A *Nicotiana tabacum* (tobacco) PUP-family transporter, nicotine uptake permease 1 (NtNUP1), was previously shown to transport tobacco alkaloids and to affect both nicotine biosynthesis and root growth in tobacco plants. Since Arabidopsis PUP1, which belongs to the same subclade as NtNUP1, was recently reported to transport pyridoxine and its derivatives (vitamin B6), it was of interest to examine whether NtNUP1 could also transport these substrates. Direct uptake measurements in the yeast *Saccharomyces cerevisiae* demonstrated that NtNUP1 efficiently promoted the uptake of pyridoxamine, pyridoxine, anatabine, and nicotine. The naturally occurring (*S*)-isomer of nicotine was preferentially transported over the (*R*)-isomer. Transport studies using tobacco BY-2 cell lines overexpressing NtNUP1 or PUP1 showed that NtNUP1, similar to PUP1, transported various compounds containing a pyridine ring, but that the two transporters had distinct substrate preferences. Therefore, the previously reported effects of NtNUP1 on tobacco physiology might involve bioactive metabolites other than tobacco alkaloids.



## 1. Introduction

Nicotine (**1**) (Fig. 1) and its related pyridine alkaloids occur mainly in the *Nicotiana* genus and contribute to the chemical defense against insects (Shoji and Hashimoto, 2013). In *Nicotiana tabacum* (tobacco), these alkaloids are exclusively synthesized in the root, translocated to the aerial parts via the xylem, and stored in the vacuoles of leaf cells. Since berberine bridge enzyme-like oxidoreductases, which catalyze the last or a late step of nicotine (**1**) biosynthesis, are localized in the vacuoles, nicotine (**1**) is thought to be formed in the vacuoles (Kajikawa et al., 2011). Nicotine (**1**) thus appears to travel from its site of synthesis in the root vacuole to its distant storage site in the leaf vacuole, via the root cytosol, root apoplast, xylem sap, leaf apoplast, and leaf cytosol. Besides this long-distance transport within the plant, tobacco alkaloids and their biosynthetic intermediates may be transported across endomembranes of cellular organelles or plasma membranes. Multidrug and toxic compound extrusion (MATE)-type transporters sequester nicotine (**1**) in the vacuoles of tobacco roots, thereby decreasing cytoplasmic its toxicity during active nicotine (**1**) synthesis in the root (Shoji et al. 2009). Another MATE-type transporter is proposed to sequester nicotine (**1**) into the vacuoles of the leaf (Morita et al. 2009).

A plasma membrane-localized nicotine uptake permease (NtNUP1) was recently reported to import nicotine (**1**) from the apoplastic space, particularly in root tips (Hildreth et al. 2011). NtNUP1 belongs to a plant-specific class of purine permease-like transporters, which is classified into several subclades (Supplemental Fig. S1). RNAi-mediated suppression of *NtNUP1* in tobacco hairy root cultures caused nicotine (**1**) levels in the culture medium to increase and those in the root cells to decrease, consistent with the nicotine (**1**) uptake activity of NtNUP1 (Hildreth et al. 2011). Surprisingly, the total nicotine (**1**) content in the leaves and roots of NtNUP1-suppressed tobacco plants was significantly reduced, and the roots of NtNUP1-suppressed seedlings grew better than those of the control (Hildreth et al. 2011). When tobacco plants with highly reduced nicotine (**1**) levels were generated by suppressing expression of its biosynthesis genes, growth promotion of these transgenic plants was not observed (e.g., Chintaparkorn and Hamill, 2003; Xie et al. 2004). The requirement of NtNUP1 for optimal nicotine (**1**) biosynthesis and the inhibitory effect of NtNUP1 on root growth (Hildreth et al. 2011) cannot readily be explained by its transport activity for nicotine (**1**).

NtNUP1 belongs to the same subclade as *Arabidopsis thaliana* PUP1 in the PUP family of transporters (Supplemental Fig. S1).

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http://dx.doi.org/10.1016/j.phytochem.2014.05.011
0031-9422/

PUP1 was initially discovered as a plant transporter that complemented a yeast mutant deficient in adenine transport (Gillissen et al. 2000), and was shown to import adenine, cytosine, cytokinins, and related metabolites from the apoplast into the cytoplasm (Bürkle et al. 2003). More recently, PUP1 was shown to be a high affinity transporter of vitamin B6, which includes pyridoxine (**6**), pyridoxamine (**5**), and pyridoxal (**7**) (Szydlowski et al. 2013). The rather relaxed substrate specificity of PUP1 prompted us to investigate whether NtNUP1 transports vitamin B6 in addition to tobacco alkaloids. A better knowledge of NtNUP1 substrate preference is important for understanding the effects of NtNUP1 on plant metabolism and physiology.

## 2. Results

### 2.1. NtNUP1 transport assays in yeast

Previously, *Schizosaccharomyces pombe* cells expressing *NtNUP1* cDNA were used to measure the uptake of radioactive nicotine (**1**) into cells, and the inhibition of nicotine (**1**) uptake in the presence of excess amounts of non-radioactive competitors, such as other tobacco alkaloids and tropane alkaloids (Hildreth et al. 2011). Here, *NtNUP1* cDNA was expressed in *Saccharomyces cerevisiae* and the uptake of various metabolites was assayed by directly measuring their cellular contents using high-performance liquid chromatography (HPLC) or gas chromatography (GC). As an initial experiment, a C-terminal green fluorescent protein (GFP) fusion of NtNUP1 (i.e., NtNUP1-GFP) was expressed in *S. cerevisiae* to analyze the subcellular localization of NtNUP1 (Fig. 1a). NtNUP1-GFP was localized mainly to the plasma membrane and to endomembranes, possibly the tonoplast. Substantial localization of NtNUP1-GFP to the plasma membrane suggests the feasibility of conducting uptake assays using transgenic yeast cells. Transient expression of NtNUP1-GFP in onion epidermal cells indicated that NtNUP1 was localized exclusively to the plasma membrane (Supplemental Fig. S2), in agreement with the results of a previous report (Hildreth et al. 2011). Next, *S. cerevisiae* cells expressing either an empty vector (control) or *NtNUP1* cDNA was exposed to 250 μM of various plant metabolites (except for anatabine (**2**), which was administered at 50 μM), and the test metabolites was measured in yeast cells after a 4-h incubation period (Fig. 1b and c). (2′*S*)-Nicotine (**1a**) (the stereoisomer synthesized in tobacco plants) and anatabine (**2**) were taken up efficiently by *NtNUP1*-expressing yeast cells, to reach a cellular concentration of *ca.* 0.16–0.18 μmol $g^{-1}$ fr. wt, whereas the contents of these alkaloids in the control cells were less than 0.02 μmol $g^{-1}$ fr. wt. Uptake of hyoscyamine (**3**) (atropine) and scopolamine (**4**) by the *NtNUP1*-expressing cells was significantly enhanced ($P < 0.05$ and $P < 0.01$, respectively; Student's *t*-test) compared to the control cells, but the NtNUP1-mediated accumulation of these tropane alkaloids was low (less than 0.03 μmol $g^{-1}$ fr. wt). The flavonoid rutin (**10**) was not taken up by either *NtNUP1*-expressing or control yeast cells. These uptake properties of NtNUP1 in *S. cerevisiae* are similar to those reported in *S. pombe* (Hildreth et al. 2011).

Whether vitamin B6 could be transported by *NtNUP1*-expressing yeast cells, as reported for *PUP1*-expressing yeast cells (Szydlowski et al. 2013), was next investigated, Pyridoxine (**6**) and pyridoxamine (**5**) were both highly efficiently taken up by the *NtNUP1*-expressing cells, compared to control cells, reaching a concentration of 1.0 μmol $g^{-1}$ fr. wt during a 4-h incubation period (Fig. 1b and c). Uptake of pyridoxal (**7**), another form of vitamin B6, by NtNUP1 was significant ($P < 0.01$; Student's *t*-test) but low (*ca.* 0.03 μmol $g^{-1}$ fr. wt). These results show that NtNUP1 mediates the efficient uptake of some vitamin B6 derivatives, in addition to tobacco alkaloids, when expressed in yeast cells.

To estimate initial uptake rates, tritium-labelled pyridoxine (**6**) and nicotine (**1**) at 50 μM were administered for 3 min. Yeast cells expressing either *PUP1* or *NtNUP1* were used to compare substrate preferences of these transporters. Since expression levels of functional PUP1 and NtNUP1 at the plasma membrane may be different, it is meaningful to compare uptake rates of two substrates in a particular yeast strain, but not to compare uptake rates of one substrate between two yeast strains. As shown in Table 1, both pyridoxine (**6**) and nicotine (**1**) were efficiently taken up by PUP1 and NtNUP1. PUP1's ability to transport nicotine (**1**) has been inferred from an uptake competition study in yeasts (Gillissen et al. 2000). These results herein show that PUP1 prefers pyridoxine (**6**) over nicotine (**1**), whereas NtNUP1 transports nicotine (**1**) more efficiently than pyridoxine (**6**), under the short-term uptake conditions.

Whether NtNUP1 displayed selectivity toward stereoisomers of nicotine (**1**) was also tested. (2′*S*)-Nicotine (**1a**) and (2′*R*)-nicotine (**1b**) (both at 125 μM) were mixed at a 1:1 ratio and supplied to the culture medium. After 4-h incubation, the nicotine (**1**) taken up by the yeast cells was analyzed using a chiral GC column, which separated the stereoisomers as distinct peaks (Fig. 2a). The amount of nicotine (**1**) taken up by the vector control cells was too low to be analyzed by this method. The nicotine (**1**) present in yeast cells expressing *NtNUP1* or *NtNUP1-GFP* though was greatly enriched in the (*S*) isomer, (**1a**) representing *ca.* 81% of the total nicotine (**1**) present (Fig. 2b). Thus, NtNUP1 preferentially transports the naturally occurring isomer of (2′*S*)-nicotine (**1a**) over the unnatural (2′*R*) (**1b**) form.

### 2.2. NtNUP1 transport assays in tobacco BY-2 cells

To validate the transport properties of NtNUP1, NtNUP1 was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in cultured tobacco BY-2 cells. Tobacco alkaloids (mainly anatabine (**2**)) are biosynthesized in BY-2 cell cultures only after jasmonate elicitation (Shoji and Hashimoto, 2008). In the present study, it was found that the expression level of *NtNUP1* in BY-2 cells was low when the cells were cultured in the absence of jasmonates, but increased several fold after jasmonate treatment (Fig. 3a), and that alkaloid biosynthesis, as evidenced by robust induction of an alkaloid biosynthetic gene, *PUTRESCINE N-METHYLTRANSFERASE* (*PMT*), was induced by addition of methyl jasmonate (**9**) (MeJA; Fig. 3b). NtNUP1-mediated transport activities can thus be estimated by comparing the activities in *NtNUP1*-overexpressing cells with those in wild-type or vector-transformed cells, cultured in the absence of jasmonates. Quantitative RT-PCR showed that two independent BY-2 cell lines transformed with the *CaMV 35S pro:: NtNUP1* vector had *NtNUP1* transcript levels that were *ca.* 27-fold (OX10 line) or 64-fold (OX18 line) higher than those in the wild-type or vector control cell lines (Fig. 4a). Two tobacco BY-2 cell lines was also generated that overexpressed *PUP1* (Fig. 4b), to compare the substrate specificity of NtNUP1 and PUP1. Tobacco cells were cultured in the presence of test compounds (50 μM), and the cellular concentrations of these compounds were measured after a 4-h period. The *NtNUP1*-overexpressing tobacco cells took up (2′*S*)-nicotine (**1a**) and anatabine (**2**) more efficiently than did the wild-type and vector control cells, whereas the *PUP1*-overexpressing cells did not accumulate (2′*S*)-nicotine (**1a**) (Fig. 5a). In this uptake assay, NtNUP1 transported greater amounts of anatabine (**2**) (3–4 μmol $g^{-1}$ dry wt) than of nicotine (**1**) (0.5–0.6 μmol $g^{-1}$ dry wt). Tropane alkaloids were transported by NtNUP1 at low efficiency (*ca.* 0.2 μmol $g^{-1}$ - dry wt for hyoscyamine (**3**), and less than 0.02 μmol $g^{-1}$ dry wt for scopolamine (**4**); data not shown). Rutin (**10**) was not transported by NtNUP1.

Please cite this article in press as: Kato, K., et al. Tobacco NUP1 transports both tobacco alkaloids and vitamin B6. Phytochemistry (2014), http://dx.doi.org/10.1016/j.phytochem.2014.05.011