An NPF transporter exports a central monoterpane indole alkaloid intermediate from the vacuole

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Plants sequester intermediates of metabolic pathways into different cellular compartments, but the mechanisms by which these molecules are transported remain poorly understood. Monoterpane indole alkaloids, a class of specialized metabolites that includes the anticancer agent vincristine, antimalarial quinine and neurotoxin strychnine, are synthesized in several different cellular locations. However, the transporters that control the movement of these biosynthetic intermediates within cellular compartments have not been discovered. Here we present the discovery of a tonoplast localized nitrate/peptide family (NPF) transporter from Catharanthus roseus, CrNPF2.9, that exports strictosidine, the central intermediate of this pathway, into the cytosol from the vacuole. This discovery highlights the role that intracellular localization plays in specialized metabolism, and sets the stage for understanding and controlling the central branch point of this pharmacologically important group of compounds.

Specialized metabolic pathways of plants are often compartmentalized. The monoterpane indole alkaloids (MIAs), a structurally diverse group of ~3,000 specialized metabolites that are produced across six plant families, include the anticancer agents vinblastine and vincristine, neurotoxin strychnine and antimalarial quinine1,2. While the chemical diversity of the MIAs is derived from enzymatic transformations on a central biosynthetic intermediate, strictosidine1, the spatial distribution of these biosynthetic enzymes in planta adds an additional layer of control and complexity to this pathway (Fig. 1).

In the medicinal plant Catharanthus roseus, it has been shown that MIA synthesis takes place in at least three distinct cell types, the internal phloem associated parenchyma, laticifers/idioiblasts and epidermal cells, with the epidermis hosting the final steps of strictosidine formation and many of the downstream reactions3. Notably, the epidermis-located steps also display compartmentalization at the intracellular level. The direct precursors of strictosidine, tryptamine and the monoterpane secologanin are synthesized in the cytoplasm and must be transported into the vacuole, where biosynthesis of the central intermediate strictosidine by the enzyme strictosidine β-glucosidase takes place3,4. The next enzyme in the MIA pathway, strictosidine β-glucosidase, is sequestered in the nucleus5. The separation of strictosidine synthase and strictosidine β-glucosidase has been hypothesized to serve as a firewall, preventing the inappropriate accumulation of strictosidine aglycone, which is a highly reactive dialdehyde that induces protein cross-linking6. Although the reasons for specific localization to the cytoplasm, vacuole and nucleus remain unresolved, this organelar separation implicates the need for transport of MIA intermediates into and out of the vacuole.

Here we identify transporter genes that are co-regulated with the early steps of the MIA pathway using a self-organizing map of C. roseus transcriptomic expression data. Three transporter candidates, two showing homology to multidrug and toxic compound extrusion (MATE) proteins (CrMATE1 and CrMATE2) and one showing homology to nitrate and peptide transport family (NPF) proteins (CrNPF2.9), were identified using this approach. Loss of function studies using virus-induced gene silencing (VIGS) and in vitro characterization in Xenopus laevis oocytes strongly suggest that CrNPF2.9 is responsible for the trans-tonoplastic movement of strictosidine out of the vacuole. The transporter is localized to the tonoplast membrane, which is consistent with this function. This marks the first identification of an intracellular transporter gene in MIA biosynthesis, highlights the emerging role of NPF transporters in plant specialized metabolism and provides insight into how localization confers an additional layer of regulation in specialized metabolism.

Results

Self-organizing maps used to identify candidates. To identify candidate transport genes, we utilized a publicly available RNA-seq database6 that has been used for identification of a number of genes in the C. roseus MIA pathway7. The expression profiles of genes from the transcriptomic dataset were clustered using a self-organizing map. This methodology has previously been employed to cluster large datasets8,9 as it provides an unbiased and unsupervised method for clustering. Nine contigs

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The import of secologanin and tryptamine into the vacuole, as well as the co-expression of these genes. Contigs within these five nodes were considered as candidates involved in MIA metabolism. Three putative transporters, one showing homology to an NPF transporter (CrNPF2.9), and two showing homology to MATEs (CrMATE1 and CrMATE2), were observed in these nodes and were selected for further study. Given the recent evidence that NPF transporters have a much broader substrate specificity than previously recognized10, we chose to focus on establishing whether CrNPF2.9 plays a role in MIA biosynthesis.

In planta function by VIGS. To assess the physiological relevance of this transporter in planta, we used VIGS to transiently silence the expression of the CrNPF2.9 gene in C. roseus and screen for a metabolic phenotype11 (Fig. 3 and Supplementary Fig. 1). Silencing of the CrNPF2.9 transporter resulted in a dramatic increase in the level of strictosidine in C. roseus leaf tissue together with a significant decrease in vindoline and catharanthine, the end products of the MIA pathway (Fig. 3 and Supplementary Fig. 1). Silencing using a different fragment from a distinct region of the CrNPF2.9 gene resulted in the same phenotype (Supplementary Fig. 1). The accumulation of strictosidine is consistent with CrNPF2.9 acting as an exporter of strictosidine from the vacuole: silencing of CrNPF2.9 prevents the export of strictosidine, leading to its accumulation in the vacuole, while concomitantly leading to the decrease in the downstream alkaloids.

Additionally, VIGS of CrNPF2.9 resulted in cell death at the site of infection in the leaf tissue (Fig. 3 and Supplementary Fig. 2). This was validated by confocal microscopy of the leaf tissue after staining with both DAPI and propidium iodide (Supplementary Fig. 2). This cell death was not observed in empty vector controls, and has never been reported when any other known MIA biosynthetic gene has been silenced. We hypothesize that the substantial accumulation of strictosidine is cytotoxic to the plant, leading to cell death.

To further validate the distinct role of CrNPF2.9, we performed additional VIGS experiments with the other transporters that had been identified in the co-expression analysis (Supplementary Fig. 3). Simultaneous silencing of CrNPF2.9 and CrMATE1, as well as triple silencing of CrNPF2.9, CrMATE1 and CrMATE2, also resulted in the accumulation of strictosidine (Supplementary Fig. 3). In all cases, silencing experiments were consistent with a role of CrNPF2.9 in strictosidine metabolism.

The quantitative polymerase chain reaction (qPCR) was used to confirm the silencing of the putative transporter gene. mRNA levels could not be accurately measured on CrNPF2.9-silenced plants because of the cytotoxicity that is observed on CrNPF2.9 silencing. However, silencing of CrNPF2.9 was confirmed in the double and triple-silenced plants (which displayed less necrotic tissue) (Supplementary Fig. 3). The similar metabolic profiles that result when two distinct gene fragments were tested for CrNPF2.9 (Supplementary Fig. 1) suggest that off-target silencing is not responsible for the observed metabolic phenotype.

CrNPF2.9 is located at the tonoplast membrane. The subcellular localization of any protein directly impacts its physiological role, so a transporter involved in strictosidine biosynthesis must be localized to the tonoplast. To investigate the subcellular localization of CrNPF2.9, a carboxy (C)-terminal yellow fluorescent protein (YFP) fusion of CrNPF2.9 (CrNPF2.9–YFP) was co-expressed in C. roseus cell suspension cultures with a set of distinct subcellular compartment markers. These markers included C-terminal cyan fluorescent protein (CFP) fusions of three different proteins: the Arabidopsis two-pore K+ channel, AtTPK1 (TPK–CFP), which is known to localize to the tonoplast12–13, strictosidine synthase from C. roseus (STR–CFP), known to localize to the vacuole2 and the aquaporin Pip2A (PM–CFP), which is targeted to the plasma membrane26 (Fig. 4). In transiently transformed cells, CrNPF2.9–YFP co-localized to

Figure 1 | Localization of the MIA pathway in C. roseus. Biosynthesis of strictosidine occurs in the vacuole of epidermal cells in C. roseus, necessitating the import of secologanin and tryptamine into the vacuole, as well as the export of strictosidine from the vacuole. CrNPF2.9, identified in this study, is annotated in the figure. IPAP, internal phloem-associated parenchyma.
the same membrane as AtTPK1–CFP (Fig. 4a–d). Furthermore, CrNPF2.9–YFP was observed to surround the vacuole labelled with STR–CFP (Fig. 4e–h) and CrNPF2.9–YFP also appeared distinct from the plasma membrane labelled with PM–CFP (Fig. 4i–p). A similar pattern of localization was also observed in onion cells expressing CrNPF2.9–YFP (Fig. 4q–v). Therefore, it is highly likely that this transporter is localized to the tonoplast, a location compatible with a role in vacuolar transport.

A (D/E)X₃L(L/I) motif has been implicated in the localization of plant membrane proteins to the tonoplast membrane, and mutagenesis of this motif has directly proven its role in directing targeting to the tonoplast 13,17–19. CrNPF2.9 contains this motif at the amino (N) terminus (Supplementary Fig. 4). Additionally, CrNPF2.9 has a relatively acidic isoelectric point of 6.1. A lower isoelectric point has been used as a predictive indication of tonoplast localization 20,21. These data provide further support that CrNPF2.9 is likely to be tonoplastic.

CrNPF2.9 is co-expressed with MIA biosynthetic genes in leaf epidermis. Since strictosidine synthesis and deglycosylation occur in the leaf epidermis, cellular localization of CrNPF2.9 was examined by analysing its transcript distribution. A leaf epidermis-enriched fraction of mRNAs was generated, and the relative abundance of transporter messengers was evaluated by comparison with a whole leaf fraction. qPCR analysis showed that the epidermis fraction was strongly enriched with CrNPF2.9 transcripts. A similar enrichment was observed for strictosidine synthase and strictosidine glucosidase (Supplementary Fig. 5). The transcripts of hydroxymethylbutenyl 4-diphosphate synthase, known to accumulate in internal phloem-associated parenchyma, were not enriched in the epidermis fraction. The localization of CrNPF2.9 in the leaf epidermis, as observed for strictosidine synthase and strictosidine glucosidase, reinforces the proposed involvement in MIA biosynthesis.

Biochemical characterization of strictosidine export. As a putative vacuolar strictosidine exporter, CrNPF2.9, would, in planta, transport strictosidine from the acidic vacuolar lumen to the neutral cytosol. To validate this hypothesis, we expressed CrNPF2.9 heterologously in X. laevis oocytes and incubated the expressing oocytes in an uptake buffer containing strictosidine at pH 5. Hence, with respect to substrate and proton gradients, import of strictosidine from the acidic exterior to the neutral oocyte cytosol in principle mimics export across the plant cell tonoplast. Oocytes expressing CrNPF2.9 accumulated strictosidine to tenfold higher levels than control oocytes (Fig. 5a). Incubating CrNPF2.9-expressing oocytes in 100 µM strictosidine for varying time periods showed a linearly increasing accumulation that reached a stable plateau at ∼100 µM internal strictosidine concentration after approximately 90 min. In comparison, the concentration in control oocytes never increased above 6 µM, even after a prolonged 3 hours of incubation (Fig. 5b). This indicates that the main rate-limiting step for strictosidine accumulation in oocytes is import across the plasma membrane. Therefore, the main contribution to strictosidine accumulation in the oocytes stems from CrNPF2.9-mediated import across the plasma membrane. As internal strictosidine concentration in CrNPF2.9-expressing oocytes did not increase above the

Figure 2 | Self-organizing map of C. roseus transcriptomic data. Each circular node represents approximately 40 unigenes with the most similar expression profile. Furthermore, neighbouring nodes are related to each other by the similarity of their expression profile. The average expression profile of genes in the node is plotted within each node. The early MIA pathway genes strictosidine synthase, secologanin synthase, tryptophan decarboxylase, strictosidine β-glucosidase, geraniol 8-hydroxylase and loganic acid methyltransferase all localize to the nodes highlighted by a bold circle. Grey nodes represent the highest quality nodes.
Figure 3 | In planta silencing of CrNPF2.9. a. Representative total ion chromatogram (TIC) of leaf tissue extracts from Little Bright Eyes plants transformed with empty vector (VIGS-EV, black), and a vector designed to silence CrNPF2.9 (VIGS-CrNPF2.9, blue). The red box highlights the accumulation of strictosidine. b. Alkaloid profiles for tissue transformed with the VIGS-CrNPF2.9 vector relative to the empty vector control (VIGS CrNPF2.9 (n = 12), VIGS-EV (n = 10)). **P < 0.01. All data shown are mean ± s.e.m. c. Blackened leaf tissue that results upon silencing of CrNPF2.9 is indicated by a white arrow.

Discussion

There has been tremendous recent progress in the elucidation of plant biosynthetic pathways. However, the mechanisms that control the localization of many plant pathways remain less clear. For example, many biosynthetic enzymes have been discovered and characterized for strictosidine and other MIA biosynthesis have been explored: ABC transporters have clearly shown to transport strictosidine with high affinity against its concentration gradient when expressed in X. laevis oocytes. The localization of CrNPF2.9 to the tonoplast in leaf epidermis is consistent with this role.

To date the NPF transporter family has been implicated in the movement of nitrate, peptides, glucosinolates, auxin, abscisic acid and gibberellic acid, and this work further expands the substrate repertoire of this family to include the alkaloid strictosidine. The characterized plant NPF transporters to date have primarily focused on substrate transport at the plasma membrane. A number of NPF transporters are present in the C. roseus transcriptome, but CrNPF2.9 appears to be the only one with a tonoplast membrane targeting sequence. Although there are Arabidopsis NPF transporters that are known to localize to the tonoplast membrane, such as AtPTR2, 4 and 6, the function of these transporters has not been elucidated. The characterization of CrNPF2.9 therefore provides the first evidence of the role of NPF transporters in the intracellular movement of specialized metabolites in planta.

It has been shown that an EXXE(R/K) motif in the peptide transporter from Shewanella oneidensis, PepT_M, is essential for the coupling of solute movement to the proton gradient. This motif is conserved in the Arabidopsis nitrate transporter NRT1.1 and in the Arabidopsis glucosinolate transporter NPF2.11; it has been shown through mutagenesis to contribute to coupling nitrate and glucosinolate movement to the proton gradient in NRT1.1 and NPF2.11, respectively. Notably, when mutated in the EXXE(R/K) motif, AtNPF2.11 loses its ability to accumulate glucosinolates against its concentration gradient. The CrNPF2.9 transporter does not contain this conserved motif (Supplementary Fig. 4), which is consistent with the inability of CrNPF2.9 to accumulate strictosidine against its concentration gradient. Such a limit on transport could have been retained during evolution to avoid the formation of high amounts of the reactive strictosidine aglycone and resulting cytotoxicity.

The discovery and characterization of CrNPF2.9 in C. roseus provides insight into the importance that intracellular metabolite flux.

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concentration in the external assay medium (Fig. 5b), the data indicate that the transporter cannot accumulate strictosidine against a concentration gradient. Based on the time course assays, an assay length of 20 minutes was chosen for estimating the CrNPF2.9 affinity constant towards strictosidine. Plotting transport rates as a function of increasing strictosidine concentrations yielded a saturation curve, which was fitted to the Michaelis–Menten equation (Fig. 5c) with an apparent affinity constant of ~110 μM. In addition, oocytes expressing CrNPF2.9 were incubated with the MIA intermediates secologanin and tryptamine, as well as the β-carboline alkaloid norharmane and the non-natural alkaloid tryptoline. These oocytes did not import these compounds, indicating specificity of CrNPF2.9 for strictosidine (Fig. 5d). In combination, our data show that CrNPF2.9 is a high-affinity transporter with specificity for strictosidine.
transport plays in the regulation of the MIA pathway. Notably, silencing of CrNPF2.9 and the subsequent accumulation of strictosidine causes extensive tissue death in *C. roseus* (Fig. 3 and Supplementary Fig. 2), suggesting that the intravacuolar accumulation of strictosidine is cytotoxic, and that CrNPF2.9 plays a key role in exporting this MIA intermediate from the vacuole. Both secologanin and...
Additionally, the heterologous expression of transport proteins for these steps have yet been identified. The transport processes that control the movement of alkaloid biosynthetic intermediates are generally not well characterized. This study shows that dedicated NPF transporters play a pivotal role in shuttling alkaloid intermediates between subcellular compartments. To date, an ABC transporter has been shown to be involved in the export of the MIA catharanthine to the leaf surface in C. roseus\textsuperscript{38}, and MATE-type transporters\textsuperscript{36-38} as well as a purine permease\textsuperscript{39} have been implicated in nicotine transport. Thus, the discovery of CrNPF2.9 further highlights that alkaloid localization is controlled by a wide variety of transporter types. The diversity of plant transporters, and an understanding of their substrate specificities and roles in metabolism, represents a potentially untapped resource for metabolic engineering and synthetic biology. As demonstrated in Arabidopsis, genetic manipulation of transporters can be used to affect the source to sink accumulation of antinutritional metabolites in the edible parts of crops\textsuperscript{28}. Additionally, the heterologous expression of transport proteins may be useful for biotechnological applications for metabolic engineering. There has been a concerted effort for the engineering of complex plant metabolic pathways in yeast, as exemplified by the production of strictosidine\textsuperscript{40} and benzylisoquinoline alkaloids in Saccharomyces cerevisiae\textsuperscript{41}. It is feasible that utilizing transport proteins for either the internal sequestration of intermediates in these metabolic pathways or for the export of toxic intermediates to the media on fermentation may improve titres of these important pharmaceuticals. The roles that transport proteins play in plant specialized metabolism have not been greatly studied. However, the decreasing costs of next-generation sequencing technologies for interrogation of plant genomes and transcriptomes will allow for the mining and subsequent characterization of transport proteins with a diverse array of substrate specificities.

**Methods**

**Generation of self-organizing maps.** The filtered transcriptomic data\textsuperscript{6} (contigs with FPKM (fragments per kilobase of exon per million fragments mapped) expression values of zero for more than half of the treatments or with zero expression variance across the samples were removed) was normalized to have a mean expression level of zero and unit variance across conditions. Self-organizing maps were implemented and visualized in R (R Core Team 2013, version 3.0.2) using the kohonen package\textsuperscript{42}. The map sizes were chosen to give approximately 40 contigs per node, with the map

![Figure 5](image-url)
shape selected such that the ratio between the two edge lengths was the same as the ratio between the two smallest odd divisors of the side lengths (3 : 3 mm) to ensure so that every node had the same number of neighbours. This avoided boundary effects occurring when the neighbourhood distance metric was calculated. To assess how well the generated self-organizing map fitted the data, two quality metrics were analysed. The first was the within-node distance, which is defined as the mean distance from the weight vector of a node to all its surrounding nodes. The smaller the within-node distance, the more accurately the node’s weight vector represents the samples mapped to the node. The other quality metric used was the internodal distance, defined as the sum of the distances from a node’s weight vector to the weight vectors of its neighbouring nodes. The smaller the value, the more similar the node’s weight vector is to the weight vectors of its surrounding nodes. In order for a node to be classified as high quality in this analysis, both of the described quality metrics for that node had to be in the lowest quartile compared with all nodes.

**Virus-induced gene silencing.**

The **VIGS constructs for CrNPF2.9**, as well as the **double silencing vector CrNPF2.9-CrMATE1** and the **triple silencing vector CrNPF2.9-CrMATE1-GMATE2**, were generated using a USER compatible VIGS vector pTRV2u. The USER compatible VIGS vector pTRV2u was digested with AsI I and NlBviCl, and an approximately 200–500 bp fragment of the desired gene, amplified from Ciona roseus cDNA using the primers in Supplementary Table 1, was USER cloned into the cut vector. For generation of the double (VIGS-CrnPF2.9-CrMATE1) and triple (VIGS-CrnPF2.9-CrMATE1-GMATE2) fusion vectors, USER fusion cloning was employed44,45. **Agrobacterium GV3101** strains containing pTRV1 (ABRC), pTRV2-emu vector, pTRV2-MgCl (f or silencing magnesium chelate) and pTRV2-gene of interest, were grown overnight in 5 ml LB supplemented with rifampicin, gentamycin and kanamycin at 28 °C. These cultures were pelleted at 3,000g, resuspended in Agrobacterium inoculation solution (10 mM MES, 10 mM MgCl2, 200 µM acetosyringone) to an A600 of 0.7, and incubated at 28 °C for 2 h. Transformants were confirmed by PCR using the gene-specific primers used to amplify the gene fragment. Strains containing pTRV2 constructs were mixed 1:1 with pTRV1 culture and this mixture was used to inoculate plants (Little Bright Eyes cultivar) by the pinch wounding method using a pair of nely bent forceps46. Plants (8–12, 2 months old) were inoculated for each construct, and the plants were grown at 25 °C in a 12 h photoperiod. The pTRV2u vector was used as a negative (empty) vector control, and the pTRV2-MgCl plants were used as a visual marker of the silencing response, with bleaching of the leaves occurring 21–25 days post inoculation. On silencing, the plant material was harvested, ground in a Retch ball mill under liquid nitrogen and stored at −80 °C before analysis by quantitative PCR (qPCR) and liquid chromatography mass spectrometry (LCMS). All of the VIGS experiments were replicated a minimum of three times per construct. All data were analysed by a pairwise Student’s t-test against the empty vector control tissue.

**qPCR of VIGS tissue.** Approximately 100 mg of plant tissue was used for extraction of RNA from each replicate in a VIGS experiment using a Qiagen RNeasy plant mini kit in accordance with the manufacturer’s instructions. For each set of pTRV2u plants, a minimum of eight plants was selected, including the plants that yielded the most pronounced silencing phenotype, for RNA extraction and qPCR. RNA quality was assessed on a 1% agarose gel and the concentration was measured on a NanoDrop ND-1000. cDNA for qPCR from each replicate was synthesized using the manufacturer’s instructions. For each primer set, a standard curve was generated to ensure amplification efficiency had a linear relationship with cDNA concentration, with only primer pairs giving a linear regression (R2) value of 0.99 used. The primer efficiency values generated in this study were between 99–101%.

**Liquid chromatography mass spectrometry of VIGS tissue.** Ground leaf tissue was weighed and collected into 200 µl of methanol containing 40 µl caffeine or 10 µl escin as an internal standard and incubated at 60 °C for 2 h. After a 30 min centrifugation step at 5,000g, an aliquot of the supernatant (25 µl) was mixed with an equal volume of water and analysed on a Thermo Finnigan instrument equipped with a Deca XP ion trap detector or a Shimadzu IT-TOF. In both systems the column used was a Phenomenex Kinetic 5 µm C18 100 Å (100 × 2.10 mm × 5 µm) and the binary solvent system consisted of 0.1% TFA and ACN. The mobile phase gradient down to 10% ACN and 6 min isocratic at 10% ACN, 1 min gradient down to 30% ACN, 4 min gradient up to 100% ACN, 6 min isocratic at 100% ACN, 1 min gradient down to 10% ACN and 6 min isocratic at 10% ACN. The compounds were monitored by UV 241 nm, and the fractions collected at the retention time 13.5 min. The resulting fractions were lyophilized and analysed by NMR.

**Subcellular localization.** The full length CrNPF2.9 gene was subcloned into the pcDNA3.1 cassette vector as a YFP fusion, and the Arabidopsis AtTPK1 was subcloned into the into the pcSA-cassette vector as a CFP fusion. The vacuolar STR–CFP and the plasma membrane FM–CFP (CD3-1002) markers were described previously5,6. All vectors used a 335 promoter for expression. Transient transformation of C. roseus cells by particle bombardment and fluorescence imaging were performed following the procedures previously described6,9. C. roseus cells were bombarded with DNA-coated gold particles (1 µm) and 1,100 psi rupture disc at a stopping-screen-to-target distance of 6 cm, using the Bio-Rad PDS1000/He system and 100 ng of each plasmid per transformation. For the transient transformation of onion cells, 100 µg ACN, 1 µm gradient down to 100% ACN, 6 min isocratic at 100% ACN, 1 min gradient down to 10% ACN and 6 min isocratic at 10% ACN. The compounds were monitored by UV 241 nm, and the fractions collected at the retention time 13.5 min. The resulting fractions were lyophilized and analysed by NMR.

**Preparation of epidermis enriched fraction and transcript distribution analysis.** An epidermis-enriched fraction was obtained from a modified version of a procedure previously described37. Briefly, a cotton swab was dipped into a carborundum powder (particle size < 300 grit, Fischer) and used to abrade both the lower and the upper epidermis layers of young C. roseus leaves. Abraded leaves were dipped in 4 ml of Trizol (Life Technologies) for 5 in a 15 ml centrifuge tube. A total of 3 × 10 leaves were abraded and RNAs were extracted according to the manufacturer’s protocol. The RNA pellet resulting from the isopropanol precipitation was washed with 70% ethanol and re-suspended in 10 µl of RNase-free water. Excess sugars were removed by precipitation with 10% ethanol for 5 min on ice and centrifugation for 5 min at 15,000g and 4 °C. The supernatant was recovered and precipitated with 0.5 volume of sodium acetate 3 M, pH 5.2 and 2.5, vortexed , washed with 70% ethanol at 28 °C for 20 min and re-suspended with 20 µl of RNase-free water. Total RNAs from whole young leaves were also extracted with Trizol (Life Technologies) according to the manufacturer’s protocol. RNA from both fractions was quantified using a NanoDrop ND-1000 and 1 µg was retro-transcribed with the ReverTraFirst Strand cDNA Synthesis Kit according to the provider’s instructions (Thermos Fisher Scientifics). Gene expression levels were measured by qPCR run on a CFX96 Touch Real-Time PCR System (Bio-Rad). Each reaction was performed in a total reaction volume of 25 µl containing an equal amount of cdNAs (1 in 3 dilution), 0.05 µM forward and reverse specific primers (Supplementary Table 4), and 1 × DyNaMo ColorFlash Probe qPCR Kit (Thermo Fisher Scientifics). The amplification programmes was 95 °C (polymerase heat activation), followed by 40 cycles of 95 °C for 5 s and 60 °C for 40 s. Quantification of transcript copy number was performed with calibration curves and normalization with the C. roseus 40S Ribosomal protein 59 reference gene and expressed relatively to the amount of transcript measured in the whole leaf fraction. All amplifications were performed in triplicate and repeated at least on two independent biological repeats.

**Production and purification of strictosidine.** Strictosidine synthase was purified as described46. Strictosidine was generated enzymatically by incubation of 4 mM tryptamine (Sigma), 2 mM purified secoecgonin (Sigma) and 2 µM strictosidine synthase in 50 mM phosphate buffer pH 7.4 buffer, in a temperature of 37 °C at 37 °C overnight. This reaction was monitored for strictosidine production and consumption of secoecgonin by LCMS. The solution was purified by solid phase extraction, eluted in 100% methanol, and dried under vacuum. The dried product was resuspended in H2O, filtered with 2 µm filters and purified by preparative high-performance liquid chromatography (HPLC) in 2 ml aqueous buffer in a total volume of 2 ml, eluted with a Dionex Ultimate 3,000 pump, with a variable wavelength UV detector. The column used for chromatographic separation was a Phenomenex Luna 5 µ (C18) 250 × 30 mm, with the binary solvent system consisting of 0.1% TFA and ACN. The elution programme was the following: 1 min isocratic at 10% ACN, 8 min gradient up to 30% ACN, 4 min gradient up to 100% ACN, 6 min isocratic at 100% ACN, 1 min gradient down to 10% ACN and 6 min isocratic at 10% ACN. The compounds were monitored by UV 241 nm, and the fractions collected at the retention time 13.5 min. The resulting fractions were lyophilized and analysed by NMR.
Expression and functional characterization of CnNPF2.9 in X. laevis oocytes. The full-length coding sequence of the CnNPF2.9 gene was cloned into the USER-cleaving compatible X. laevis expression vector, pNB1u™ and verified by sequencing. Primers (SI) were used to amplify CnNPF2.9 CDS, including the surrounding T7 promoter and the Xenopus-β-tubulin gene’s 5′- and 3′-UTRs via PCR from the Xenopus pNB1u expression vector. cDNA was in vitro transcribed from the purified linear PCR products using the MuMLV mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific). The resulting cRNA was purified using LiCl precipitation and adjusted with RNase-free water to a concentration of 0.5 µg µL⁻¹.

Defoliated X. laevis oocytes at maturation stage V–VI were purchased from Ecocyte bioscience, Germany. Oocytes were injected the day after surgery with 25 ng cRNA and were incubated for 3–4 days at 18°C before assaying. For transport assays, oocytes were pre-incubated for 5 min in Kulori Buffer (90 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM MES, pH 5.5) to facilitate intracellular steady-state pH before transferral to Kulori buffer pH 5.1 containing one of the tested compounds (strictosidine, secoligan, trypamine, norharmane and tryptoline) and were generally stopped after 90 min unless otherwise stated by washing in 5 × 20 ml Kulori buffer without substrate. Each assay consisted of 15 oocytes and in pools of five oocytes were homogenized in 50 µl 50% methanol. Following precipitation (20,000g for 10 min), supernatants were frozen overnight and centrifuged (20,000g for 10 min). For analysis by UHPLC/QTMS (triple quadrupole mass spectrometry), supernatants were then additionally filtered through 0.2 µl filter discs (Millipore, cat. no. MAHVN45500) and diluted three times in H₂O before LC/MS analyses (see below). The concentration of compounds in oocytes was calculated by relating peak areas to standard dilution curves and assuming an oocyte volume of 1 µl.

LCMS analyses of compounds used in oocyte uptake experiments. In Fig. 5a–c, stricdiscine was measured using UHPLC/QTMS on an AdvanceTM-UHPLC/EVOQTMeTLite-QQ-ToF-MS instrument (Bruker) equipped with a C18 reversed phase column (Kinetex 1.7 µm XB-C18, 10 cm × 2.1 mm, 1.7 µm particle size, Phenomenex) by using a 0.05% formic acid in water (v/v) (solvent A)–0.05% formic acid in ACN (v/v) (solvent B) gradient at a flow rate of 0.4 ml min⁻¹ at 40°C. The gradient applied was as follows: 2% B (0.9 min), 2–85% B (0.5 min), 100% B (0.5 min), 100–2% B (1 min) and 2% B (1.4 min). Stricdiscine was ionized by electrospray ionization with a spray voltage of +3.500 V and a heated probe temperature of 360°C. The following multiple reaction monitoring transitions were monitored: (+)351 > 514 (91 V) (quantifier), (+)351 > 144 (34 V) and (+)351 > 252 (25 V). Quantification was based on external standard curves for stricdiscine measured in control oocyte extracts. In Fig. 5d, stricdiscine, secoliganin, trypamine, norharmane and tryptoline were measured by LCMS using Agilent 1100 Series LC (Agilent Technologies) coupled to a Bruker HCT/Ultro ion trap mass spectrometer (Bruker Daltonics). A Zorbax SB-C18 column (Agilent; 1.8 µm, 2 × 50 mm) was used at a flow rate of 0.2 ml min⁻¹. The oven temperature was maintained at 35°C. The mobile phases were as follows: A was water with 0.1% (v/v) HCOOH and 50 µM NaCl and B, ACN with 0.1% (v/v) HCOOH. The gradient programme was as follows: 0–0.5 min, isocratic method at 0% B; 0.5–7.5 min, linear gradient from 0% to 90% B; 7.5–8.5 min, linear gradient from 90% B to 100% B; 8.5–11 min, isocratic method at 100% B; 11.6–17 min, isocratic method at 2% B. The flow rate was increased to 0.3 ml min⁻¹ in the interval 11.2–13.5 min. The MS was run in positive electrospray mode. For processing of the LCMS data, DataAnalysis 4.1. (Bruker Daltonics) was used.

Data availability. Sequences for the three transporters mentioned in the study have been deposited in NCBI (CrMATE1 (KT372304), CrMATE2 (KT372305) and CnNPF2.9 (KT372303)). Transcriptional data used in this study are available at http://medicinalplantgenomics.msu.edu.

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Author contributions
R.M.E.P. made the initial discovery of CrNPF2.9 and designed and carried out all silencing experiments; D.X. and H.H.N.E designed and carried out kinetic experiments; E.F., M.I.S.T.C., T.D.B. and V.C. designed and carried out localization experiments; F.G.-F., S.E.O., H.H.N.E, V.C. and B.A.H. contributed to the conception and design of the experiments; R.M.E.P., D.X., E.F., M.I.S.T.C., A.O., T.D.B, V.N., M.B., C.E.O, D.M.J., E.C.T. and A.P. contributed to the acquisition of data; R.M.E.P., D.X., E.F., M.I.S.T.C., F.G.-F., V.C., H.H.N.E and S.E.O. contributed to data analysis and interpretation; R.M.E.P. and S.E.O. drafted the manuscript and all authors critically revised and approved the final version of the manuscript for publication.

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Competing interests
The authors declare no competing financial interests.