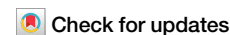


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Characterization of a vacuolar importer of secologanin in *Catharanthus roseus*



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Monoterpenoid indole alkaloid (MIA) biosynthesis in *Catharanthus roseus* is a paragon of the spatiotemporal complexity achievable by plant specialized metabolism. Spanning a range of tissues, four cell types, and five cellular organelles, MIA metabolism is intricately regulated and organized. This high degree of metabolic differentiation requires inter-cellular and organellar transport, which remains understudied. Here, we have characterized a vacuolar importer of secologanin belonging to the multidrug and toxic compound extrusion (MATE) family, named CrMATE1. Phylogenetic analyses of MATEs suggested a role in alkaloid transport for CrMATE1, and *in planta* silencing in two varieties of *C. roseus* resulted in a shift in the secoiridoid and MIA profiles. Subcellular localization of CrMATE1 confirmed tonoplast localization. Biochemical characterization was conducted using the *Xenopus laevis* oocyte expression system to determine substrate range, directionality, and rate. We can confirm that CrMATE1 is a vacuolar importer of secologanin, translocating 1 mM of substrate within 25 min. The transporter displayed strict directionality and specificity for secologanin and did not accept other secoiridoid substrates. The unique substrate-specific activity of CrMATE1 showcases the utility of transporters as gatekeepers of pathway flux, mediating the balance between a defense arsenal and cellular homeostasis.

The patterning of specialized chemical profiles throughout the plant body is complex and an indication of resource allocation and metabolic differentiation^{1,2}. It is also a means for dedicated storage³ and rapid deployment or activation of potentially toxic components^{4–6}, which requires inter-/intra-cellular transport. The movement of specialized metabolites can occur at a proximal or distal level, at a subcellular resolution, or encompassing the full range of plant organs. In many cases, transporters are required to facilitate movement across semi-permeable membranes, such as the vacuolar (tonoplast) or cellular/plasma membranes.

An intricate picture of compartmentalization has been developing in the study of monoterpenoid indole alkaloids (MIAs) in the medicinal and horticultural plant *Catharanthus roseus* (L.) G. Don^{1,2,7–9}. Many transporters have been identified for the multi-step biosynthetic pathway culminating in the anticancer bisindole alkaloids vinblastine and vincristine (Fig. 1).

A series of Nitrate Peptide Family (NPF) transporters (CrNPF2.4–2.6) have been characterized with H⁺-coupled import of the secologanin biosynthetic intermediates (deoxyloganic acid, loganic acid, loganin and secologanin), across the plasma membrane and into leaf epidermal cells¹⁰.

These transporters, or a subset, could putatively transport secoiridoids from the roots to the leaf, as the inter-organ accumulation of secologanin has been suggested¹¹. From the cytosol of leaf epidermal cells, secologanin and tryptamine are transported into the vacuole, where strictosidine synthase (STR) carries out a condensation reaction to form strictosidine^{12,13}. This vacuolar reaction is the first committed step of the MIA pathway, and the product is subsequently exported by the tonoplast-localized transporter, CrNPF2.9¹⁴.

Further downstream, an ATP Binding Cassette (ABC)-type transporter has been identified as a catharanthine plasma membrane exporter (CrTPT2) that is expressed predominantly in the epidermis of young leaves¹⁵. This class of TPT2 transporters, belonging to the pleiotropic drug resistance (PDR) subfamily, appears to be conserved across the Apocynaceae family, with an ortholog from the related species *Vinca minor* (VmTPT2/VmABCG1) characterized with a similar function¹⁶. These transporters seem to play a role in the accumulation of catharanthine and vincamine, respectively, with several reports suggesting that the idioblasts and laticifers are likely the final storage site^{1,9,17,18}.

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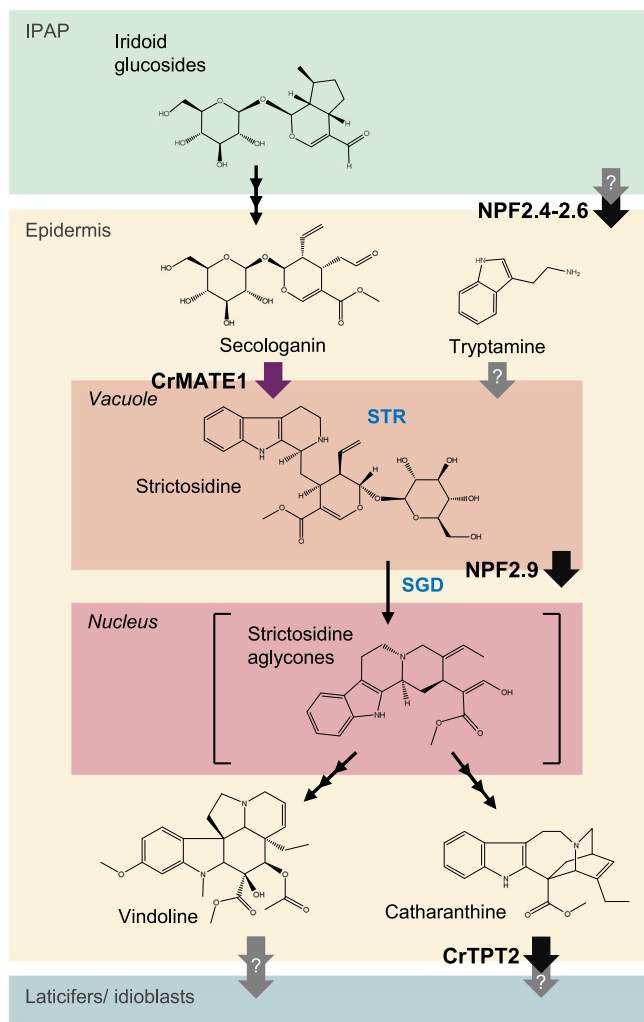


Fig. 1 | Compartmentalization of resolved and unknown transport steps in the *C. roseus* MIA pathway. Biosynthesis of iridoid glucosides derived from the MEP pathway occurs in the internal phloem-associated parenchyma (IPAP) and they are transported out via an unknown mechanism. Secoiridoids are moved into epidermal cells via CrNPF2.4-2.6¹⁰, where secologanin is imported into the vacuole by CrMATE1 (purple arrow). An unknown transporter concurrently imports tryptamine. In the vacuole, condensation of the two molecules by strictosidine synthase (STR) forms the central MIA intermediate, strictosidine, which is exported into the cytosol by CrNPF2.9¹⁴. Strictosidine-β-D-glucosidase (SGD) guides its substrate into the nucleus, where strictosidine aglycones accumulate. A series of enzymatic steps leading to catharanthine follows, where it is finally exported via CrTPT2¹⁵, accumulating in the laticifers/idioblasts, along with vindoline, mediated by unknown mechanisms. Uncharacterized transport steps are shown with gray arrows. Multiple reaction steps are shown with three conjoined arrows.

A recent report has suggested that a member of the multidrug and toxic compound extrusion (MATE) family, originally named CrMATE1 (referred to as SLTr), transports secologanin across the tonoplast, where it would be available to strictosidine synthase (STR)¹. Interestingly, CrMATE1 forms a cluster on chromosome 3 with STR and tryptophan decarboxylase (TDC), which catalyze consecutive reactions¹⁹. The role of this MATE was inferred solely based on virus-induced gene silencing (VIGS) of *CrMATE1* in young *C. roseus* leaves. The authors reported no change in secologanin levels but an increase in its reduced form, secologanol. It was reasoned that secologanol build-up was a by-product of secologanin remaining aberrantly in the cytosol. However, the authors could not conduct *in vitro* or *in vivo* transport assays due to the reported recalcitrance of the gene to heterologous expression.

In parallel, our group first became interested in this MATE protein after the publication of the *C. roseus* genome by Kellner et al.¹⁹. We further pursued characterization of the transporter when it was identified (CL1653Contig1, EST = 2) in a leaf epidermis (LE)-enriched transcriptome²⁰. Evidence from single-cell and idioblast-targeted transcriptomics has supported the theory that this transporter, along with several others, is enriched in epidermal, laticifer, and internal phloem-associated parenchyma (IPAP) cell types^{19,17}. However, our main challenge lay in providing an efficient heterologous platform for the direct characterization of CrMATE1.

Our analyses recapitulate the VIGS-based silencing of *CrMATE1*, which we conducted independently in two different *C. roseus* backgrounds, “Little Delicata” (LD) and “Pacifica White” (PW). Silencing of *CrMATE1* in both backgrounds led to significantly reduced secologanin and a drastic accumulation of secologanol, possibly due to spontaneous or endogenously catalyzed reduction of the aldehyde. We confirmed the tonoplast localization of CrMATE1 by visualizing the eGFP and YFP translational fusion proteins (at both N/C-termini) in *Nicotiana benthamiana*, along with multiple co-expressed controls. Finally, we biochemically characterized CrMATE1 using *Xenopus laevis* oocytes as a proxy for the plant vacuole. Using this expression platform, we confirmed that CrMATE1 is exclusively a vacuolar importer of secologanin, having tested other secoiridoid intermediates. Overall, these results establish the localization, directionality, substrate, and rate of this MATE secologanin transporter, improving our understanding of the mechanics of MIA spatiotemporal distribution.

Results

Phylogenetic and sequence analysis of MATEs

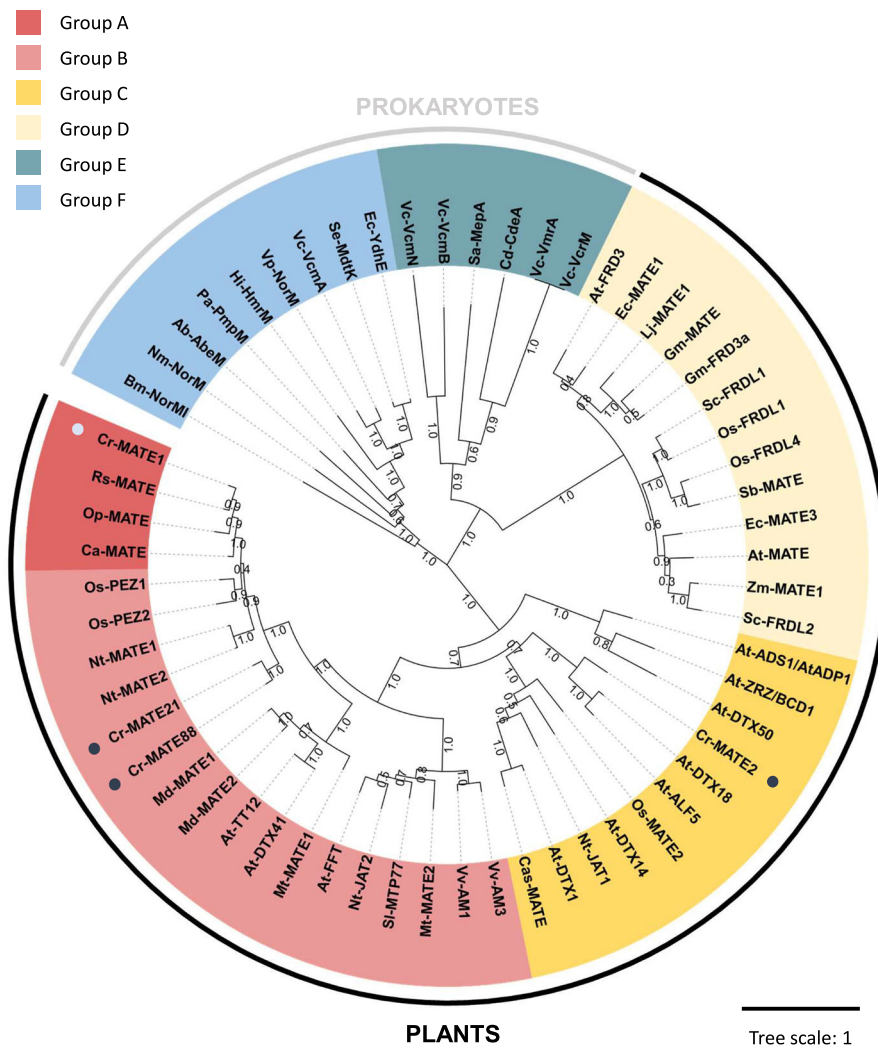
The characterization of *C. roseus* MATE paralogs was first approached by phylogenetic and structural analyses, compared to known and annotated homologs, ranging from alkaloid transporters in related species to prokaryotic MATEs (Fig. 2). In this phylogenetic tree, plant MATEs selected from 18 species formed four clades: A, B, C, and D. Group A includes CrMATE1 and other MIA-producing species of the Apocynaceae family, whereas group B includes many known transporters of alkaloids and flavonoids²¹. CrMATE1 appears closely related to NtMATE1 and 2, which have been characterized as transporters of nicotine in *Nicotiana tabacum*²². This clustering suggested a role for CrMATE1 in alkaloid transport. The selected prokaryotic MATEs from 11 species formed two separate clades, E and F.

MATE proteins have been described as forming a bilobate V-shape structure, with pseudo-symmetrical assembly of the N- and C-lobes²³. While MATE proteins belonging to group C have charged, hydrophilic residues in the V-shaped pocket (E136, E379, E384, and R508), group A/B members have hydrophobic or uncharged substitutions at some of these residues (Suppl. Figure 1). Here, we show that CrMATE1 and NtMATE1 share the same substitutions for two of the four hydrophilic residues conserved in group C. Hydrophilic residues E136, K275, and E379 are conserved in group A/B, while E384Q and R508V are replaced with uncharged amino acids for both CrMATE1 and NtMATE1.

Silencing of *CrMATE1* reduces secologanin content

Virus-induced gene silencing (VIGS) was conducted to suppress the expression of *CrMATE1* in *C. roseus* leaves, referred to hereafter as VIGS-MATE, compared to empty vector (EV)-infiltrated plants. Silencing was carried out in two *C. roseus* varieties, “LD” and “PW.” A 255 bp region was selected for silencing (Suppl. Figure 2A), and qPCR was used to confirm the silencing of *CrMATE1*. Expression of *CrMATE1* was reduced by 73 and 80% in LD and PW backgrounds, respectively (Fig. 3A, C). Additionally, potential off-target MATE paralogs (*CrMATE2*, *CrMATE21*, and *CrMATE88*) with high amino acid sequence identity (>50%) were examined in both backgrounds. A partial reduction (50%) in transcript levels was noted only for the distantly related *CrMATE2* in the PW background (Suppl. Figure 3B); overall, there were no consistently significant off-target effects.

Fig. 2 | MATE proteins form six clades (groups A–F). Groups A–D correspond to plant MATEs, and groups E and F correspond to prokaryotic MATE proteins. CrMATE1 was classified into Group A along with MIA-producing species from the Apocynaceae family. Two other *C. roseus* isoforms, CrMATE21 and CrMATE88, are in Group B. Closely associated are OsPEZ1, OsPEZ2 (rice phenolic exporters) and NtMATE1, NtMATE2 (nicotine biosynthesis and transport). A fourth *C. roseus* isoform CrMATE2, was classified in Group C, along with *Arabidopsis* transporters AtALF5 (an efflux transporter involved in detoxification) and AtDTX18 (hydroxycinnamic acid amide transporter). Phylogenetic trees were generated using MEGA11 software and the Maximum Likelihood method and annotated with iTOL v6.



Silencing of *CrMATE1* in both backgrounds led to a significant reduction in secologanin (Fig. 3B, D). Additionally, there was a drastic accumulation of secologanin, the reduced form of secologanin, which was present as a minor peak in the *EV* plants. Secologanin was identified by matching MS/MS spectra with a chemically produced standard (Suppl. Figure 2B–C). Levels of secologanin increased in silenced plants by 16 to 38-fold in LD and PW backgrounds, respectively. Downstream MIA profiles were also reduced differentially in the two varieties. In LD *VIGS-MATE* plants, catharanthine was significantly reduced by 22% (Fig. 3B). Conversely, silencing in PW yielded a notable reduction of vindoline and catharanthine by 41% and 45%, respectively (Fig. 3D). Tryptamine was not detected in initial scans as it typically accumulates 5–6 magnitudes lower than secoiridoids and MIAs. A targeted MRM scan for tryptamine revealed an affected profile in PW *VIGS-MATE* plants at an order of magnitude lower than the rest of the MIAs (ng/g FW versus mg/g FW) (Fig. 3D).

CrMATE1 is localized to the tonoplast

Using fluorescence confocal microscopy, CrMATE1 (N-terminal eGFP fusion) was shown to localize to the tonoplast. CrMATE1 displayed the hallmarks of tonoplast localization, including circular extensions or 'bulbs' into the lumen of the vacuole (Fig. 4A). A clear separation of fluorescence between adjacent cells was apparent, indicative of localization to the tonoplast, as compared to the plasma membrane (Fig. 4C). Furthermore, transvacuolar strands were noted streaming across the cellular confines (Fig. 4D). Chlorophyll autofluorescence was visualized concurrently with

CrMATE1-YFP, displaying the chloroplasts situated between adjacent vacuolar membranes (Fig. 4E–H). Tonoplast localization was further confirmed by co-expression of CrMATE1 (YFP fusion at either the N- or C-terminus) with a vacuolar marker fused to CFP²⁴ (Fig. 4I–P). CrMATE1 fluorescence is distinctly co-localized with the marker, as seen individually and in the merged images.

Finally, gene expression of *CrMATE1* in *C. roseus* was quantified in several tissues and developmental samples by qRT-PCR, including stem, root, flower, and leaf pairs (1–3) (Suppl. Figure 5). Expression was ubiquitous throughout the sampled tissues but with slightly higher expression in mature leaves and stems.

Biochemical characterization of secologanin transport

The results of *in planta* silencing and subcellular localization suggested that CrMATE1 was a vacuolar importer of secologanin. To confirm and characterize this proposed role, we tested substrate range, transport directionality, and rate using the *X. laevis* expression system with *MATE*-expressing and mock-injected (control) oocytes. By testing the directionality of substrate movement between oocytes (internal pH of 7) and the media at pH 5, this approach mimics *in planta* transport from the cytosol (pH 7) to the vacuole (pH 5). Thereby, export from the oocyte is a proxy for import into the vacuole and vice versa.

Following and informed by the results of the silencing experiments, substrate specificity assays were carried out individually with the secoiridoids, secologanin, loganic acid, and loganin. Substrate movement into the oocytes (vacuolar export) was not detected for any substrates (Fig. 5).

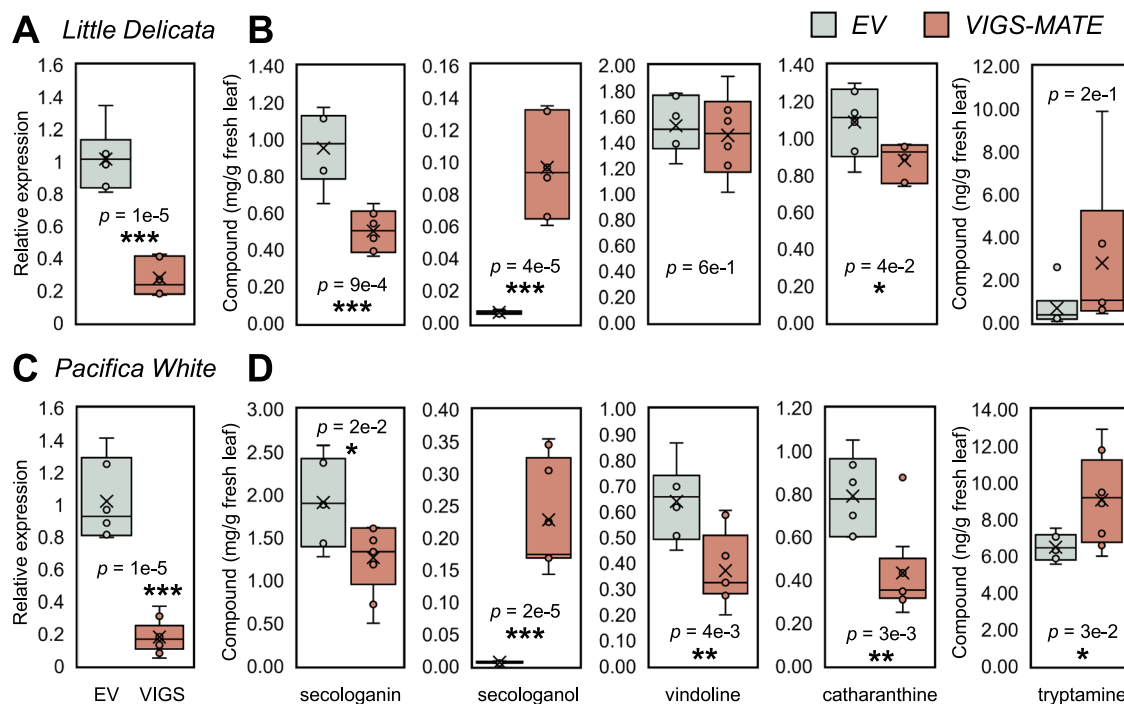


Fig. 3 | Silencing *CrMATE1* leads to significant changes in secoiridoid and downstream MIA pathways. A In the background “Little Delicata” (LD), *CrMATE1* transcripts were reduced by 73% (*VIGS-MATE*) compared to the empty vector (*EV*) controls. **B** Chemical profile of LD *VIGS-MATE* reveals a 50% reduction in secologanin, and a 16-fold increase in its reduced form, secologanol, compared to the *EV*. Vindoline was not affected and catharanthine was significantly reduced. **C** In “Pacifica White” (PW), *CrMATE1* expression was suppressed by 80%. **D** Chemical

profile of PW *VIGS-MATE* shows a significant reduction in secologanin and a 38-fold increase in its reduced form, secologanol, compared to the *EV*. Vindoline and catharanthine were significantly reduced by 42% and 46%, respectively. Data represents the mean $\pm$ S.D. of $n =$ six biologically independent samples, except for PW *VIGS-MATE* (nine biologically independent samples). Data points are shown. One, two, and three asterisk(s) signify $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (Student's *t*-test).

However, in *MATE*-expressing samples, oocytic export (vacuolar import) was registered for secologanin but not loganin or loganic acid.

While *MATE*-expressing samples displayed complete secologanin translocation (90 min), control oocytes exhibited partial diffusion or leakage into the media. Separately, control oocytes were co-injected with secologanin and an internal standard, fluorescein, to assess the degree and specificity of leakage (Suppl. Figure 4). The passive, but minimal, leakage of each substrate was noted at 7% and 8%, respectively, at 1 minute; the leakage did not significantly change at 5 minutes. Therefore, the leakage of secologanin was partial and not significantly different compared to the internal standard; further, it was not equivalent to the complete translocation rendered by *MATE*-expressing oocytes.

A time-course assay (0–90 min, 6 time-points) further illustrated the rapid and complete translocation of secologanin into the media in *MATE*-expressing oocytes (25 min), compared to a slow and partial movement in control samples (Fig. 6A). Strikingly, we noticed the accumulation of secologanol in control oocytes over time, mirroring the chemotyping results from *VIGS-MATE* leaves (Fig. 6B). The rate of secologanin conversion to secologanol is faster than the passive diffusion or leakage of secologanin, where, at the end of a 90-minute incubation, there is 2-fold more secologanol in the control oocyte than secologanin. On the other hand, no secologanol is detected within the oocytes of the *MATE*-expressing samples. Furthermore, no secologanol is detected in the media in either set of samples. The rapid transport of secologanin by *CrMATE1* confirms its role as a highly efficient and substrate-specific vacuolar importer.

Discussion

Monoterpenoid indole alkaloid (MIA) biosynthesis in *C. roseus* is a complex pathway comprised of over thirty reactions that are highly compartmentalized, spanning multiple organelles and cell types (Fig. 1)^{3,7,8,25}. Despite our current, intricate level of understanding, gaps remain in the description of

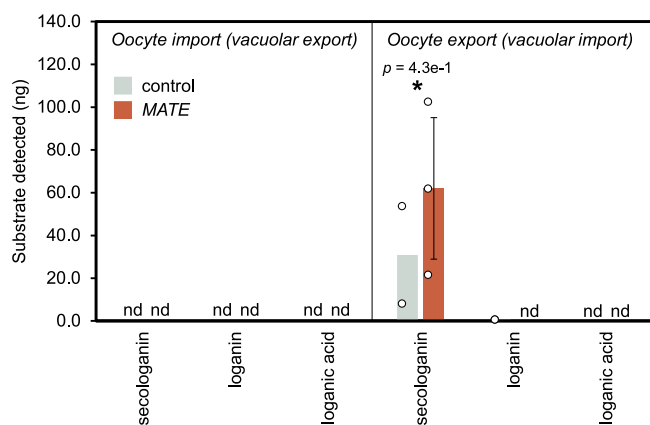
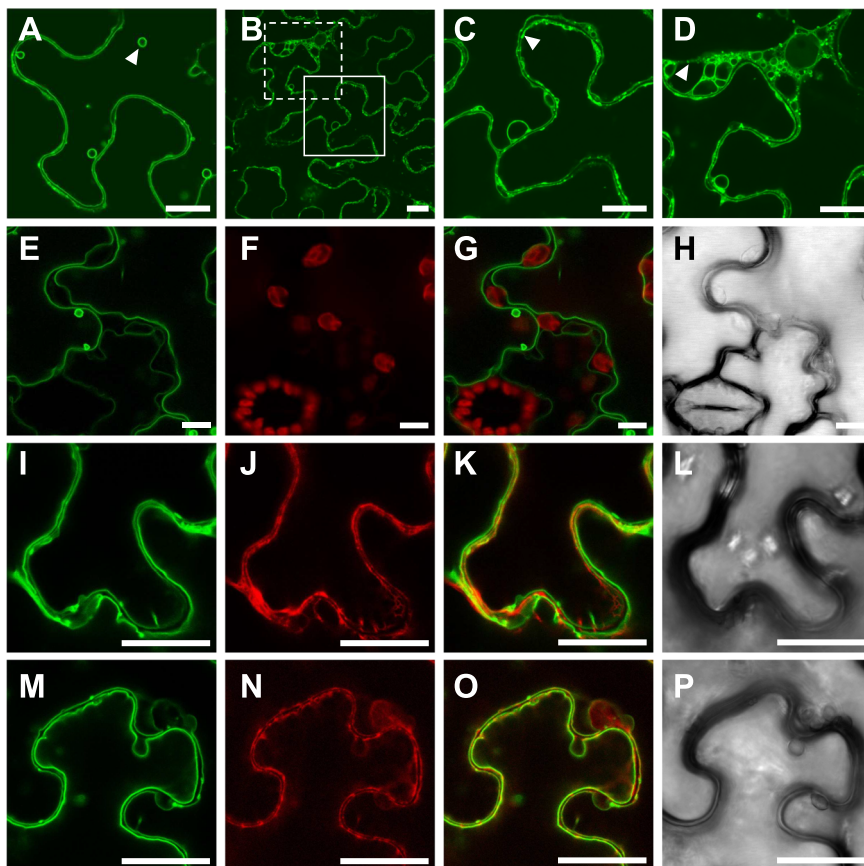
inter- and intra-cellular transport of intermediates. Previously, three transport steps have been characterized at the plasma membrane and tonoplast of epidermal cells, executed by NPF transporters (*CrNPF2.4-2.6* and *2.9*)^{10,14} and an ABC transporter, *CrTPT2*¹⁵. Here, we have characterized a *C. roseus* *MATE* transporter, *CrMATE1*, with a role in the vacuolar import of secologanin.

Referred to as *SLTr* by Li and colleagues¹, they showed that silencing of *CrMATE1* in *planta* only increased the levels of secologanol; secologanin and reported MIAs were not significantly changed. Our group has performed silencing in two varieties of *C. roseus*, reporting consistent and drastic accumulation of secologanol and a moderate but significant reduction of secologanin. While there is a discrepancy in the decrease in secologanin levels (0.5 mg/g FW) compared to the accumulation of secologanol (0.1 mg/g FW), it is likely due to the repartitioning of this aberrant intermediate into other pathways or catabolism. Nevertheless, the magnitude of secologanol accumulation is significant and affected by *CrMATE1* silencing. The slight build-up of tryptamine was only recorded in one background (PW) and is likely an indirect impact of reduced secologanin import into the vacuole. The lower levels of secologanin within the vacuole likely led to a build-up of its metabolite partner in the STR-catalyzed condensation reaction.

We found that downstream MIAs were also affected by the silencing of *CrMATE1* but proved to be variety-dependent. LD was less susceptible to downstream profile changes, where only catharanthine was reduced, but the PW profile was reduced in both catharanthine and vindoline levels (Fig. 3B, D). This distinct metabolic shift was likely observed due to the strong suppression of *CrMATE1* (73–80%) in the selected varieties (LD and PW) and under our conditions. Taken together, the outcomes of the silencing experiments suggest a significant role for *CrMATE1* in controlling the flux of intermediates to the downstream MIA pathway.

Fig. 4 | CrMATE1 localizes to the tonoplast.

A–D eGFP was fused to the N-terminus of CrMATE1 and transiently expressed in *N. benthamiana* leaf epidermal cells: **A** CrMATE1 localized to the tonoplast, with circular extensions ‘bulbs’ into the lumen of the vacuole; **B** CrMATE1 localization to the tonoplast, revealing C tonoplast separation between adjacent cells, and **D** transvacuolar strands. Features are highlighted with white arrowheads. Magnified areas from panel **B** are denoted by white and dashed boxes, shown in panels **C** and **D**, respectively. **E–H** YFP was fused to the C-terminus of CrMATE1 and co-visualized with chlorophyll autofluorescence: **E** CrMATE1 expression, **F** chloroplasts, **G** merged image showing chloroplasts between adjacent vacuolar extensions **H** and brightfield image. Finally, **I–P** N- or C-terminal YFP fusions of CrMATE1 were co-expressed with a vacuolar marker CFP, showing co-localization; **I–L** YFP–CrMATE1; **M–P** CrMATE1–YFP co-localizing with the vacuolar marker. CrMATE1 fluorescence is shown as green, and marker/chlorophyll is shown as red. Subcellular localization was determined by surveying more than 50 cells in each of the three biologically independent samples. Scale bars indicate 20 μ m.

**Fig. 5 | Characterization of CrMATE1 substrate specificity and transport directionality.**

Substrate specificity for vacuolar export was measured in control (water-injected) oocytes and oocytes expressing CrMATE1. Oocytes (3 × 5) were incubated in Kulori buffer (pH 5), supplemented with 100 μ M of secologanin, loganin, or loganic acid. Vacuolar import was assessed by injecting substrates into oocytes to a final internal concentration of 1 mM for secologanin and 100 μ M for loganin and loganic acid. Each substrate was tested individually and assayed for 90 min. Substrate content in oocytes and media was determined by LC/Q-TOF MS. Data represents the mean $\pm$ S.D. of $n =$ three biologically independent samples. One, two, and three asterisk(s) signify $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (Student's t -test).

The lack of complete blockage in the biosynthesis of strictosidine and the MIAs is likely due to the remainder of CrMATE1 transcripts (20–27%) effecting residual translocation, which would diminish the chemical phenotype over the course of a VIGS experiment (>4 weeks). It could be

expected that a CrMATE1 knockout line would severely affect all related biosynthetic pathways and even lead to toxicity, with the building up of secologanin.

The off-target, partial silencing of CrMATE2 (Suppl. Fig. 3) is not likely to affect the movement of secologanin or related substrates due to the large evolutionary distance and low sequence similarity between the two paralogs (Fig. 2). Indeed, the phylogenetic analysis suggests that CrMATE2 is not involved in MIA or alkaloid transport. On the other hand, we cannot rule out the possibility of other vacuolar importers of secologanin; however, the significant build-up of secologanin in silenced plants suggests that CrMATE1 is of primary importance.

The substantial accumulation of secologanol in MATE-silenced plants appears to be a protective measure, reducing the cytotoxic aldehyde on secologanin, as the latter builds up in the cytosol due to a lack of transport. In parallel to our *in planta* observations, we noticed a similar build-up of secologanol in control oocytes. The injected secologanin in oocytes not expressing CrMATE1 was converted into the reduced form over the course of the experiment (Fig. 6B). We can rule out the spontaneous reduction of secologanin (at pH 5 or 7), as secologanol is only detected within the control oocytes. Different catalysts or abiotic factors might be responsible for the formation of secologanol in either setting. In contrast, MATE-expressing oocytes rapidly and completely translocate secologanin into the media (pH 5), where it was stable and unchanged.

Another issue was the apparent leakage into the media of secologanin in control oocytes (Figs. 5 and 6A). Using fluorescein as an internal standard, co-injected with secologanin, passive leakage was observed for both substrates in control oocytes (Suppl. Fig. 4). The leakage was minimal at 7–9% at two time points (1–5 min) compared to the near complete translocation of secologanin in MATE-expressing oocytes at 5 min (95%) in the rate assay (Fig. 6B). Therefore, it is unlikely that secologanin is passively diffusing; rather, it is likely leaking at the site of its injection or is carried out with the needle, which is heavily dependent on oocyte batch quality.

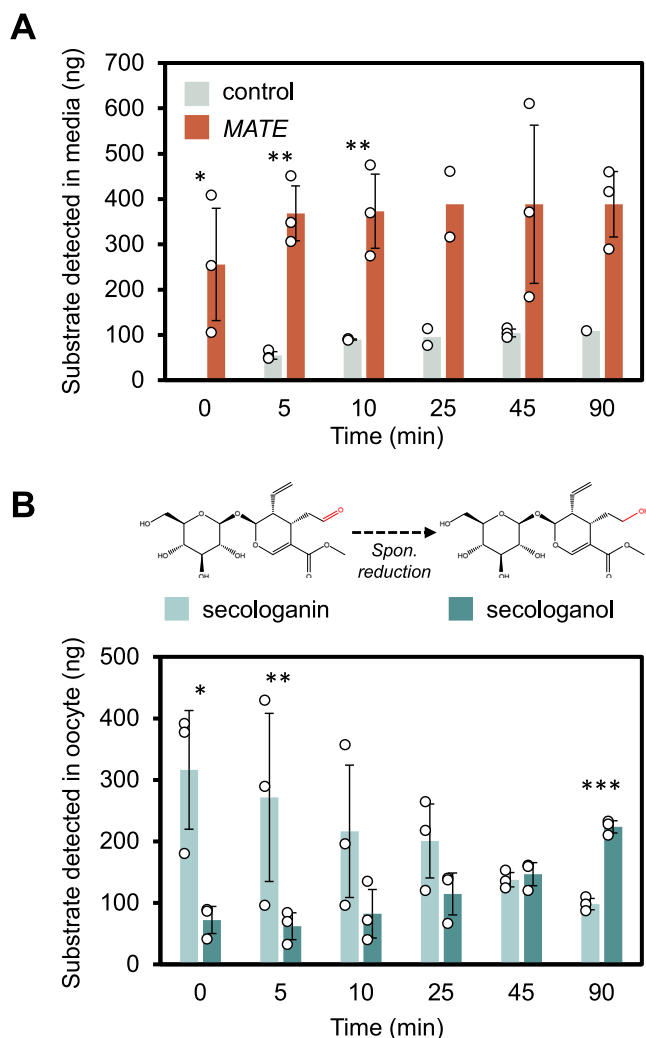


Fig. 6 | Rate of secologanin transport. **A** Secologanin transport over time in control (water-injected) oocytes and *MATE*-expressing oocytes. Oocytes (3×5) were injected with the substrate to a final internal concentration of 1 mM and incubated for indicated times from 0 to 90 min (pH 5 buffer). Substrate content in oocytes and media was determined by LC/Q-TOF MS. **B** The spontaneous reduction of secologanin was detected in control oocytes, shown by the schematic legend indicating the position where the reduction is occurring. Amounts of secologanol (light turquoise) are compared to secologanin (turquoise) at each time point. Data represents the mean $\pm$ S.D. of $n =$ three biologically independent samples. One, two, and three asterisk(s) signify $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (Student's t -test).

Repetition on multiple batches of oocytes (and frogs) confirmed that the partial leakage is consistent and not on the same magnitude as *MATE*-driven substrate translocation.

Finally, we confirmed the subcellular localization of CrMATE1 to the tonoplast with transient expression in *N. benthamiana* leaf epithelial cells (Fig. 4), consistent with its role as a vacuolar transporter. Meanwhile, the cognate transcript of *CrMATE1* is ubiquitously present throughout sampled tissues but with slightly higher expression in leaf tissues, where we expect a correlated higher degree of protein activity (Suppl. Fig. 5). Indeed, leaf vacuoles are major sinks of alkaloid accumulation, and their translocation across the tonoplast for storage has also been characterized in *N. tabacum* MATE transporters NtMATE1/2 and NtJAT1/2^{22,26,27}. In the case of MIA biosynthesis, CrMATE1 plays a role in controlling metabolic flux by delivering secologanin to its committing condensation reaction in the vacuole. Payne et al.¹⁴ show that the disruption of subsequent strictosidine export from the vacuole leads to cytotoxicity. Therefore, transvacuolar movement,

in either direction, for both substrates and the product, could be an effective gatekeeping mechanism for this vital, yet physiologically harmful, compound.

Although the leaves are the primary source of MIAs in *C. roseus*, biosynthesis also occurs in other organs, including stems, flowers, and roots. Interestingly, secologanin and the preceding secoiridoids are mobile intermediates, moving from WT roots to low-secoiridoid mutant shoots in a grafted scion¹¹. Therefore, the secologanin pool for strictosidine production in the leaves might not be sourced in situ, but could accumulate from inter-organ transport, potentially mobilized by another transporter(s) in conjunction with the epidermal importers, CrNPF2.4-2.6. Furthermore, the shuttling of secologanin into root-localized pathways, producing such compounds as vincadifformine, hörhammericine, and tabersonine-derivatives might require further unique transport proteins^{28,29}. Overall, there remain salient gaps in our comprehension of inter-organelle and inter-cellular transport in MIA biosynthesis, including the vacuolar import of the other constituent for strictosidine formation, i.e., tryptamine.

A phylogenetic analysis of MATE family proteins showed CrMATE1 clustering with orthologs from other MIA-producing species *Rauvolfia serpentina*, *Ophiorrhiza pumila*, and *Camptotheca acuminata* in group A (Fig. 2)³⁰. This grouping suggests potential secologanin import activity for these uncharacterized Apocynaceae family orthologs. Tonoplast importers (CrMATE1, NtMATE1, NtMATE2, NtJAT2) cluster closely in groups A and B (Fig. 2), sharing several hydrophobic substitutions in their V-shaped pockets (Suppl. Fig. 1). While a general V-shape structure is conserved among various MATEs, the nature of the pocket, including charge, polarity, and hydrophobicity, is highly variable. The crystal structure of CasMATE from the plant *Camelina sativa* has shed light on the role of critical residues within this pocket that determine substrate selectivity²³. The overall increased hydrophobicity of MATE proteins in group A/B, within the substrate pocket, is consistent with their putative role in transporting specialized metabolites with bulky aromatic rings. An exploration of the evolution of this capacity and its further narrowing to accept substrates, such as secologanin, could benefit from large-scale phylogenomic studies³¹.

Transporters are crucial in the matrix of specialized metabolism, acting as mediators of metabolic flux. Their activity adds yet another layer of regulation, often modulating levels of defense potency in the small molecules they shuttle; in this regard, transporters are also crucial in balancing the homeostatic parameters of plant cells, safeguarding defenses without compromising cellular health³². The complete enzymatic resolution of the MIA pathway has facilitated its reconstitution into microbial and non-native plant systems, where recent efforts have made tremendous progress in the heterologous production of valuable MIAs^{33–36}. However, biosynthesis outside of the native context often exposes many shortfalls, when conferring plant-derived pathways to microbial hosts, including a liability for by-product formation and a loss of carbon^{37,38}. Retrieving pathway intermediates or substrates “lost” to the media can improve product titers. In this context, the utility of transporters has been demonstrated by the inclusion of benzylisoquinoline alkaloid (BIA) uptake permeases (BUPs) in engineered yeast strains producing morphine and related intermediates³⁹. The inclusion of BUPs in the co-cultured modules of the pathway increased titers up to 300-fold, allowing separation of the metabolic burden into three strains. Whether *C. roseus* transporters can improve heterologous production of MIAs has yet to be examined; however, it could be a notable consideration in providing an alternative supply chain for the valuable and life-saving compounds offered by this unassuming plant.

Experimental procedures

Ethics statement

We have complied with all relevant ethical regulations for animal use. All work involving animals was carried out under the McGill University Animal Use Protocol 2022-7758, authorized by the Animal Care Committee of the Office of Research Ethics and Compliance, McGill University.

Statistics and reproducibility

All experiments are conducted on a minimum of $n = 3$ biologically independent samples and analyzed by a two-tailed, unpaired Student's t -test. Gene expression and chemical profiles from VIGS were analyzed from the average of $n = 6$ or $n = 9$ biological replicates. Subcellular localization was determined by surveying more than 50 cells in each of the 3 biologically independent samples. Oocyte assays were conducted with 3 biological replicates, each containing 5 oocytes, repeated on a minimum of two batches of oocytes from different *Xenopus laevis* individuals.

Phylogenetic analysis

The full-length amino acid sequences of 60 selected MATEs (Suppl. Table 1) were aligned using Geneious Prime or MUSCLE, and a Maximum Likelihood tree was constructed using MEGA 11 software⁴⁰. Trees were generated by applying “neighbor-joining” and “BioNJ” algorithms followed by a matrix of pairwise distances estimated using the Jones-Taylor-Thorton (JTT) model, and then selecting the topology with superior log likelihood value, with the highest log likelihood tree shown (-31865.33). A discrete Gamma distribution was used to model evolutionary rate differences among sites with 5 discrete gamma categories (+G, parameter = 2.5865). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.40% sites). The tree is drawn to scale with branch lengths measured in the number of substitutions per site and partial deletion applied with a 95% site coverage cutoff. Subsequent visualization and annotation were performed using iTOL v6⁴¹.

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) was performed using *C. roseus* var. “LD” and “PW” seedlings, which were grown in a controlled chamber at 25 °C (16/8 h photoperiod). *Agrobacterium tumefaciens* (strain GV3101) cells either harboring *pTRV2-MATE* (VIGS-MATE), *pTRV2-empty vector* (EV), or *pTRV2-CrPDS* (phytoene desaturase) were grown overnight (28 °C) and harvested by centrifugation. Cells were resuspended in infiltration buffer (10 mM MES pH 5.6, 10 mM MgCl₂, 0.2 mM acetosyringone) to an OD₆₀₀ of 1.5 and were cultured at 28 °C for 2.5 h. Cell suspensions of *pTRV1* and *pTRV2* were mixed, and a syringe needle was dipped in the mixture and used to penetrate the 4-week-old *C. roseus* seedlings underneath the shoot apical meristem. After penetration, an additional 0.12 mL of suspension was used to flood the wounded area. Thus, we produced plants co-transformed with *pTRV1* and EV (6 in LD, 6 in PW) or VIGS-MATE (6 LD, 9 PW) constructs. The infected seedlings were grown at a lower temperature of 20 °C until the *pTRV2-CrPDS* control seedlings started to show strong leaf bleaching (approximately 4 weeks post-injection). Silenced leaf pairs were harvested, split along the main vertical vein, and stored by flash-freezing. One half was used for RNA isolation using Trizol[®] reagent (Thermo Fisher, USA) and qRT-PCR studies⁴². The other half was used to extract leaf alkaloids; filtered extracts (5 µL) were submitted to LC-MS/MS for MIA quantifications. Changes in chemical profiles were evaluated by a two-tailed, unpaired Student's t -test.

Gene expression analysis of VIGS tissues

Quantitative Real Time-PCR (qRT-PCR) was performed on an Agilent AriaMx RealTime PCR instrument using the SensiFAST SYBR No-ROX qPCR 2X master mix (FroggaBio, Canada) according to the manufacturer's protocol. The settings for the qRT-PCR (10 µL, 5 ng total RNA) included 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The standard 2^{−ΔΔCT} method was used to quantify gene expression levels normalized to the expression of the *C. roseus*, 60S ribosomal RNA housekeeping gene, *Cr60SrRNA*⁴². Gene expression changes were analyzed by a two-tailed, unpaired Student's t -test from independent biological samples and the average of a minimum of six technical replicates.

Subcellular localization

For Fig. 4A–D, the full-length *CrMATE1* gene was cloned into the entry vector, *pDONR-Zeo*, followed by recombination into the destination vector *pSITE-2CA*⁴³, for N-terminal fusion with eGFP. The following panels in Fig. 4E–P were produced by cloning *CrMATE1* into *pEarleyGate-104* (N-terminal) and *pEarleyGate-101* (C-terminal) for fusion with YFP. Vectors were constructed using Gateway[®] Cloning technology (Invitrogen, USA). All constructs were confirmed by sequencing, and the corresponding plasmids were transformed into chemically competent *A. tumefaciens* cells (GV3101). Overnight cultures were grown to an OD₆₀₀ of 0.4–0.8 and resuspended in infiltration buffer to an OD₆₀₀ of 1.0 (10 mM MES/KOH solution pH 5.6, 10 mM MgCl₂, and 0.15 mM acetosyringone). Agroinfiltration was performed using a syringe without a needle on the abaxial side of the leaf according to the protocol by Sparkes et al.⁴⁴.

Nicotiana benthamiana plants were grown for 6 weeks in a controlled chamber at 22/20 °C (16/8 h photoperiod) and light intensity of 150 µmol/m²/s. Plants were sampled three days after infiltration, and leaf epidermal samples were visualized with a Leica SP5 CLSM equipped with a Radius 405 nm laser (Leica Microsystems, Germany). GFP and YFP fluorescences were assessed with excitation at 488/514 nm and emission at 510/530 nm, respectively, for *CrMATE1* visualization. RFP and CFP fluorescences were assessed with excitation at 558/458 nm and emission at 583/470 nm, respectively, for visualization of the tonoplast marker²⁴. Chloroplast autofluorescence was visualized with excitation 405 and emission at 619 nm. Samples were viewed with a 63× water immersion objective (microscope slide with coverslip thickness 1.5; 0.17 mm).

In vitro RNA transcription

The full-length coding sequence of *CrMATE1* was cloned into the *pTD2* oocyte expression vector⁴⁵ and confirmed by sequencing, containing the gene of interest flanked by 5'- and 3'- *X. laevis* β-globin UTRs and a 3' poly-A tail, respectively. Primers (Suppl. Table 2) were used to amplify *CrMATE1* CDS, plus flanking sites, with Q5[®] High-Fidelity DNA Polymerase (New England Biolabs, USA) from the *pTD2-MATE* construct, followed by gel-purification, using the Monarch[®] DNA Gel Extraction Kit (New England Biolabs, USA). In vitro transcription of this template was performed using the mMACHINE T7 kit (Invitrogen, USA), precipitated with lithium chloride, and dissolved in RNase-free water. The final cRNA (complementary RNA) concentration was adjusted to 500 ng/µL for injection purposes.

Xenopus laevis oocyte extraction and injection

Xenopus laevis individuals used in this study were maintained following the McGill University Animal Use Protocol 2022-7758. Adult female frogs (Xenopus1, USA) housed in the Xenopus Housing 603 System (Technoplast, Italy) were anesthetized in 0.15% MS-222, pH 7.3 (Sigma-Aldrich, USA). For oocyte extraction, an incision was made on the side of the abdomen and oocytes were prepared according to standard protocol⁴⁶. Lobes were placed in a Ca²⁺-free OR2 solution (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES buffer, pH 7.3) and separated into individual oocytes using fine tweezers. A 90-min incubation at RT on a tube rotator was followed by supplementation with 10 mg/mL collagenase type II (Sigma-Aldrich, USA) in Ca²⁺-free OR2 and, finally, stopped by washing in Ca²⁺-free OR2. Defolliculated oocytes were transferred to ND96 solution (NaCl 96 mM, KCl 2 mM, CaCl₂ 1.8 mM, MgCl₂ 1 mM and HEPES 5 mM, pH 7.3), supplemented with 2.5 mM sodium pyruvate (Sigma-Aldrich, USA)⁴⁶. For injections, glass capillaries were pulled into injection needles (World Precision Instruments, USA), and the tips were clipped using tweezers. Thus prepared, needles were first backfilled with oil, followed by cRNA aspiration. Oocytes were injected the same day as the surgery with 50.6 nL (25 ng per injected gene) of cRNA using a Nanoject injector (Drummond Scientific, USA). Control oocytes were injected on the same day with 50.6 nL of water. After injection, oocytes were incubated for 3 days at 18 °C to reach peak protein expression before assaying.

Biochemical characterization of CrMATE1

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) to ensure intracellular steady-state pH before transferral to Kulori containing one of the tested compounds (secologanin, loganin, and loganic acid). Assays testing oocyte import were conducted by incubating control (water-injected oocyte) and MATE-expressing oocytes in Kulori buffer containing 100 μ M of individual substrates. Oocyte export assays were conducted by injecting the test substrate into the oocyte to reach a final internal concentration of 1 mM for secologanin and 100 μ M for loganin and loganic acid, assuming an internal oocyte volume of 1 μ L. A 2-min recovery period following substrate injection in ND96 (pH 7) was performed prior to transfer into Kulori buffer (pH 5). Assays were stopped after 90 min, unless stated otherwise, by washing the oocytes in 3 $\times$ 20 mL Kulori buffer without substrate. To assess leakage, fluorescein was co-injected with secologanin as an internal standard in control oocytes at a final internal oocyte concentration of 1 mM and assayed at the same conditions. The same injection needle was used for injecting control and MATE-expressing oocytes in the oocyte export assays for each substrate respectively.

Media was collected and concentrated using a Savant SpeedVac DNA130 rotary-vacuum evaporator (Thermo Scientific, USA). Each assay was conducted with 3 biological replicates, each containing 5 oocytes. Pools of 5 oocytes were homogenized in 50 μ L of 50% methanol. Homogenates were precipitated by centrifugation at 20,000g for 10 min, frozen overnight, and followed by centrifugation at 20,000g for 10 min, where supernatants of oocyte and media extracts were used for LC/QTOF-MS analyses. The presence and quantification of target compounds (substrates) within the oocytes and in the media were calculated using calibration curves of authentic standards. Changes in substrate movement observed in control versus MATE-oocytes were evaluated by a two-tailed, unpaired Student *t*-test. Data from the rate assay (Fig. 6) was normalized with the dilution factor to calculate mass and corrected by a coefficient to remove technical error.

Liquid chromatography-mass spectrometry

In planta samples from VIGS were measured with an Agilent Ultivo Triple Quadrupole LCMS (Agilent, USA) equipped with an Avantor® ACE® UltraCore™ SuperC18™ column (2.5 μ m, 50 $\times$ 3 mm). For alkaloid detection from the variety “PW,” the following solvent system was used: solvent A, MeOH:ACN:1 M ammonium acetate:water at 29:71:2:398; solvent B, MeOH:ACN:1 M ammonium acetate:water at 130:320:0.25:49.7. The following linear gradient (8 min, 0.6 mL/min) was used: 0 min: 80% A, 20% B; 0.5 min: 80% A, 20% B; 5.5 min: 1% A, 99% B; 5.8 min: 1% A, 99% B; 6.5 min: 80% A, 20% B; 8 min: 80% A, 20% B. The solvent system for alkaloids extracted from the variety “LD” was as follows: solvent A, MeOH:ACN:5 mM ammonium acetate at 6:14:80; solvent B, MeOH:ACN:5 mM ammonium acetate at 24:64:10. The following linear elution gradient was used: 0–0.5 min: 99% A, 1% B at 0.3 mL/min; 0.5–0.6 min: 99% A, 1% B at 0.4 mL/min; 0.6–8.0 min: 1% A, 99% B at 0.4 mL/min; 8.0–8.3 min: 99% A, 1% B at 0.4 mL/min; 8.3–10.0 min: 99% A, 1% B at 0.3 mL/min. The photodiode array detector recorded from 200 to 500 nm. The MS/MS was operated with a gas temperature of 300 °C, gas flow of 10 L/min, capillary voltage of 4 kV, fragmentor of 135 V, and collision energy of 30 V in both polarities.

The Qualitative Analysis 10.0 software by Agilent was used for all LC-MS/MS analyses. The analytes were either dissolved in methanol or methanol:water in an equal volume ratio. Compounds were identified and quantified using peak areas (UV 280 nm: catharanthine; UV 300 nm: vindoline) using calibration curves for authentic standards. Secologanol was produced by reducing 150 μ g secologanin with trace amounts of sodium borohydride dissolved in 150 μ L methanol at room temperature for 1 h, which resulted in quantitative conversion. Secologanin and secologanol in VIGS experiments were quantified by standard curves of respective chemical standards recorded using MRM *m/z* 411 to 249 and *m/z* 413 to 251 ion transitions. Target MIA compounds were calculated per fresh sample weight.

Samples from *X. laevis* oocyte assays were measured using an Agilent 1290 Infinity II LC system coupled to the 6545 Q-TOF MS (Agilent Technologies, USA). The LC separation was conducted on a Poroshell 120 EC-C18 analytical column (Agilent, 2.1 $\times$ 5 mm, 1.8 μ m). Samples (1 μ L) were injected into the column, running on the mobile phases A (0.1% formic acid in water, 5 mM ammonium acetate) and B (MeOH:ACN 50:50 vol, 0.1% formic acid, 5 mM ammonium acetate). The LC gradient used was 0–0.5 min: 10% B; 0.5–2 min: ramp to 25% B; 2–4 min: ramp to 40% B; 4–6 min: ramp to 100% B; 6–8 min: 100% B; 8.5 min: 10% B; 8.5–10 min: 10% B at a flow rate of 0.4 mL/min. For targeted analysis, samples were run in negative ion mode: capillary voltage 3500 V, fragmentor voltage 175 V; skimmer 65 V; sheath gas temperature 350 °C; sheath gas flow 12 L/min; Nebulizer 45 psig; scan rate 2 spectra/s; and a mass range of 50–1100 *m/z*. The MassHunter Quantitative Analysis software by Agilent was used for all LC/Q-TOF MS analyses.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Source data are provided with this paper in Supplementary Data 1. Accession numbers used in this study include *CrMATE1*: CRO_T006097/CRO_03G032350.1/AQM73450.1.

Code availability

No custom code is used or reported in our study. Open source and commercial software used for data analyses includes MEGA11, MUSCLE 3.8.31, iTOL v6, and Agilent MassHunter Software Suite.

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Author contributions

F.L. performed the biochemical characterization and LC/Q-TOF MS data analyses. M.S. performed VIGS. Y.Q. supervised VIGS work and advised on the analysis of results. C.-J.H.-H. performed *X. laevis* surgeries. C.-J.H.-H. and V.M. provided training for the *X. laevis* expression system. R.B. supervised and provided advice for the *X. laevis* expression system. L.L. performed LC/Q-TOF, supervised by S.B. M.D. performed initial subcellular localization, repeated by X.B.-T., A.C., and F.L. M.D. performed phylogenetic and qPCR analyses. The work began under the guidance of V.D.L. at Brock University and continued with his support. F.L. and M.D. wrote the paper with input from all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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