The emerging field of transport engineering of plant specialized metabolites
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From a biotechnological perspective transport processes represent attractive targets for modulation of metabolite levels and are the foundation for the emerging field of transport engineering. Potential applications of transport engineering include control of metabolite accumulation in a tissue-specific manner in crop plants as well as increased yields of commercially valuable compounds produced in synthetic biology approaches. Within specialized metabolism, recent advances include identification of not only vacuolar but now also plasma membrane-localized transporters and neo-functionalization of members of primary metabolite transporter families to include specific roles in transport of specialized metabolites. As glucosinolates are specialized metabolites of the model plant Arabidopsis, glucosinolate transport processes emerge as a model system for studying transport of specialized metabolites.

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Introduction
Plants synthesize a vast number of specialized metabolites, which enable these sessile organisms to interact and adapt to the ever-changing environment. Some of the compounds are high-value nutraceuticals and pharmaceuticals, which has primed a desire to establish production platforms through engineering of the requisite biosynthetic pathways in various host organisms. In agriculture, toxic defense compounds in edible parts of crops (e.g. seeds or tubers) reduce their nutritional value [1,2]. Previous approaches to reduce the amounts of toxic defense compounds have focused on abolition of biosynthetic pathways [3–7]. Often, however, these approaches negatively affect plant fitness owing to impact on processes such as susceptibility to biotic or abiotic stresses [1,8,9]. Specialized metabolites are often stored in specific tissues, cell types or subcellular compartments, which are spatially distinct from the sites of biosynthesis [10]. This necessitates intracellular and intercellular transport of both pathway intermediates and end products. From a biotechnological perspective, targeting of transport processes potentially offers the means to control the accumulation of specialized metabolites in a tissue-specific manner without compromising biosynthetic and hence defense capability in non-target tissues. Such approaches have created the foundation for the emerging field coined transport engineering.

Plant transport highways
Long distance transport of metabolites between source and sink tissues of plants is facilitated by transport pathways in two tissues: the xylem and the phloem. These consist of, respectively, dead xylem vessels facilitating upward movement of water and compounds from roots, and the sieve elements facilitating phloem movement from source to sink tissues. The source–sink transport route utilized predicts which membrane barriers metabolites must cross in order to access the long distance transport pathways and consequently the number and kind of transport proteins involved. Membrane barriers do not restrict the interface between the apoplast (extracellular space) and the xylem vessels, and therefore metabolites need only to be exported from cells into the apoplast adjacent to the xylem to access this transport pathway. In comparison, sieve elements and the associated companion cells are separated from the apoplast by a plasma membrane and entry into the phloem pathway typically requires the additional activity of a plasma membrane-localized importer (Figure 1). One may anticipate that transporters at the different barriers are not equally suitable for transport engineering approaches. However, transporters that reduce export out of source cells into the apoplast, or transporters at the entry point to the transport highway (in the case of phloem transport) are likely targets for engineering of long distance transport (Figure 1). Entry or exit from subcellular storage vacuoles in either source or sink tissues may constitute additional targets.

Model systems for studying long distance transport of specialized metabolites
Alkaloid model transport system
Thus far, nicotine, the alkaloid characteristic to Nicotiana species, has constituted a major model system for studying long distance transport of specialized metabolites from roots to leaves via the xylem, and 4 transporters
Figure 1

[Diagram showing transport of ions and energy molecules (ATP, ADP, H+) across plant tissues (Xylem, Phloem, Shoot, Root) with various transporters (Proton symporters, Primary transporters, Proton antiporters, Permeases) indicated.]
have been identified. NtMATE1, NtMATE2 and NjtAT1 were characterized as vacuole membrane-localized pyridine and tropane alkaloid/proton antiporters involved in sequestration of nicotine into vacuoles [11,12]. NUP1 was characterized as a plasma membrane-localized electroneutral, nicotine-specific nicotine/H⁺ importer [13**]. Expression studies showed NtMATE1, NtMATE2 and NUP1 to be mostly expressed in roots while NjtAT1 was expressed in all tissues [11].

Conclusive evidence for a transport protein being involved in long distance transport ideally requires that reduction of the transporter protein results in reduced accumulation of the transported metabolite in the sink tissue. RNAi lines with severely reduced NtMATE1 and NtMATE2 transcripts levels showed no significant difference in nicotine levels in roots and leaves, but had increased sensitivity to exogenous nicotine. This suggests a role for these transporters in protection of the root under inductive conditions where nicotine may accumulate to cytotoxic levels before being transported to leaves [11]. Conceptually, inhibiting vacuolar sequestration in a source tissue could have led to higher nicotine levels in sinks due to larger nicotine pools being available for long distance transport. Hence these results suggest that other transporters may contribute to vacuolar accumulation of nicotine in root cells. It is therefore not known whether modulation of vacuolar sequestration of nicotine in source tissues can influence long distance transport.

RNAi lines of NUP1 showed reduced content of nicotine in leaves [13**] which could have indicated a role for this protein in long distance transport of nicotine. However, nicotine content was decreased throughout the plant and transport capabilities from root to shoot of exogenously applied nicotine appeared unaffected [13**]. Combined with the root tip-specific expression of NUP1 and wild-type levels of nicotine biosynthesis transcripts it was suggested that this protein maintains a poorly understood root to rhizosphere nicotine homeostasis by enabling cellular uptake of nicotine into root tip cells. Thus, the reduced total nicotine levels in the NUP1 RNAi lines were owing to increased loss of nicotine to the soil [13**]. Consequently, proteins constituting targets for engineering long distance transport of nicotine have not yet been identified. These include plasma membrane-localized nicotine exporter and importer proteins, respectively, loading nicotine onto xylem in the roots and unloading nicotine from the xylem into the cytosol of leaf cells. It is, however, evident that metabolite levels can be manipulated by modulating transport proteins (such as NUP1) which may indirectly affect long distance transport by either increasing or decreasing the pool of metabolites available in the source tissue. Although the plasma membrane-localized NUP1 did not appear to be directly involved in loading or unloading to the xylem, the observations significantly contribute to the field. NUP1 belongs to the purine uptake permease (PUP) family and is the first example of a primary metabolite transporter with an additional physiological role in specialized metabolism [14]. Considering the widespread diversification of the PUP family in angiosperms [14], it is likely that other PUP members may transport specialized metabolites.

Transport of the antimicrobial benzylisoquinoline alkaloid berberine in Coptis japonica from lateral roots to the rhizome [15] represents another system for studying transport of specialized metabolites. Here, three ABC transporters CjABC1-3 capable of transporting berberine have been identified [16,17]. CjABC1 and CjABC2 were both shown to be plasma membrane-localized and expressed in the rhizome. CjABC2 was specifically expressed in cells adjacent to the xylem in the rhizome. It is thus possible that CjABC1 and CjABC2 play a joint role in cellular uptake of berberine in rhizomal sinks [17], although it remains to be elucidated whether berberine levels can be modulated by inhibiting transport processes in sink tissues [18].

**Glucosinolate model transport system**

Aliphatic amino acid-derived glucosinolates represent a class of defense compounds, characteristic to the Brassicaceae order [19], which includes the agriculturally important Brassica napus and the model plant Arabidopsis. Glucosinolates are synthesized in both roots and vegetative tissues and accumulate to high levels in embryos in seeds, which do not perform de novo synthesis [20–22]. Glucosinolates must therefore be imported to seeds and may be utilizing both the xylem and phloem pathways en route from source to sink. Using a functional genomics screen of an Arabidopsis transporter library in Xenopus oocytes, we identified two members of the nitrate and peptide transport family (NRT/ PTR), GTR1 and GTR2, as high affinity plasma membrane-localized, glucosinolate-specific proton symporters [23**]. When seed glucosinolate levels in gtr mutants were compared to wildtype, gtr2 accumulated almost 50% less glucosinolates, whereas gtr1 displayed wildtype levels. Interestingly, the reduction in gtr2 seeds was accompanied by an over-accumulation in source tissues. Combined with the specific vasculature-associated expression, this suggests

(Figure 1 Legend) Potential targets for transport engineering of specialized metabolites in plants. Schematic representation of transport pathways for specialized metabolites in plants. For simplicity only root and shoot are included. Transporters are color coded according to transport mechanism. Only families which have been shown to be involved in specialized metabolism are indicated. Transport engineering aims to modify accumulation of specialized metabolites in tissues by targeting transport proteins. Likely candidates are involved in access to and from transport highways.
Box 1 NRT/PTR transporters in specialized metabolism

The NRT/PTRs constitute a large plant transporter family belonging to the ubiquitous proton-dependent oligopeptide transporter family (POT). In other organisms, the physiological role of the POTs is transport of dipptides and triptides and in humans two POT members have become a prime target for drug delivery owing to their central importance for delivery of peptidomimetic drugs to intestinal epithelial cells [25]. In plants, NRT/PTRs were until recently shown to transport either dipptides and triptides or nitrate [24]. Plant genomes, however, encode much larger numbers of NRT/PTR family members than other organisms (e.g. 53 in Arabidopsis and 81 in rice as opposed to e.g. six in humans). Accordingly, it has been speculated that such large family sizes indicate evolutionary diversification to serve new roles [24]. The ability of the GTRs to transport glucosinolates may have arisen through neo-functionalization of NRT/PTR family members. In contrast to the specificity of the NUP1 gene towards nicotine and not the purine substrates of the PUP family [19], both GTR1 and GTR2 have retained a nitrate transport activity. This seems to be a common property for other NRT/PTR members which recently have been assigned new substrate specificities. The well-studied dual affinity nitrate transporter [26] and sensor [27] NRT1.1 was shown to function as a nitrate-regulated auxin transporter. In the absence of NO$_3^-$, NRT1.1 lowered IAA accumulation in lateral root (LR) tips and thereby repressed LR growth [28]. NRT1.2, previously characterized as a low affinity nitrate transporter [29], and three other NRT/PTR were identified as putative ABA importers (AIT1-4) [30], possibly mediating previously reported NO$_3^-$–ABA signaling crosstalk [31,32]. In addition, AIT3 was able to transport GA$_3$. From an evolutionary perspective, this shows that members of the NRT/PTR family have evolved to transport defense compounds and plant hormones. The mechanism behind these dual substrate specificities is poorly understood. Biologically, these findings are of relevance beyond the immediate transport activities, specifically with respect to ADME (absorption, distribution, metabolism, excretion) in pharmacology, where understanding the evolution of transporter specificity and selectivity is of major importance for understanding xenobiotic and drug transport.

The 53 members of the Arabidopsis NRT/PTR family cluster into four phylogenetically distinct groups [24]. Interestingly, all AITs cluster together with NRT1.1 in subgroup 1, which suggests a functional diversification of the 11 members of this subgroup towards transport of NO$_3^-$ and hormones with vastly different chemical structures [30]. Functionally characterized members of subgroup 2 include low affinity nitrate transporters NRT1.5 [33] and NRT1.8 [34] which have been implicated in xylem loading and unloading of nitrate, respectively. In addition, this subgroup includes most of the characterized peptide transporters in the NRT/PTR family, namely PTR1, PTR2 and PTR5 [35–37] which have been shown to recognize dipptides and triptides with high affinity. Recently, PTR2 was shown to be tonoplast-localized as was two other closely related members named PTR4 and PTR6 [38,39]. Tonoplastic localization of these proteins indicates that members of the NRT/PTR family may play a role in facilitating export from the vacuole. Interestingly, neither PTR4 nor PTR6 showed peptide transport when expressed heterologously in yeast or Xenopus oocytes and it is speculated that they may transport other substrates [38]. GTR1 and GTR2 belong to the poorly characterized subgroup 4 which in Arabidopsis contains 19 members of which three have been characterized as low affinity nitrate transporters [40–42] and one as a passive nitrate effluxer [43]. Contemplating the wide range of specialized metabolites in plants and that phylogenetic analyses of the large NRT/PTR family show a high level of diversification, contraction and expansion [23**, we believe it is likely that members of this family may encode long-sought transporters of specialized metabolites.

The multidrug and toxin extrusion (MATE) family is universally present in all organisms and encodes transport proteins typically involved in detoxification processes [58]. As the transport activity of plant MATEs is dependent on a proton gradient in the opposite direction to that of which their substrate is transported [59,60*,61], the more acidic environments of the apoplast and vacuole lumen compared to the cytosol predict that MATEs could facilitate both export from plant cells and vacuolar sequestration of their substrates [62]. This is supported by a study on FRD3 which was characterized as a citrate efflux protein loading citrate onto the xylem [63,64], and the involvement of several MATE proteins in vacuolar sequestration of flavonoids [60*,61,65,66]. Lately, MATEs were shown to be implicated in transport across membranes of other organelles such as golgi and mitochondria [67,68]. In particular, MATE-mediated transport of flavonoids has provided important insights into the possible involvement of carriers and conjugation in vacuolar sequestration of specialized metabolites. Recently, a glutathione-S-transferase (TT19) in a role for GTR2 in loading glucosinolates onto phloem. In comparison, GTR1 was expressed both in vasculature and mesophyll cells indicating that GTR1 additionally may be involved in distributing glucosinolates within the leaf [23**]. Remarkably, knocking out both GTR proteins, led to a specific abolishment of seed glucosinolates in gtr1 gtr2 double mutants [23**]. This result constitutes a highly welcome proof-of-concept for transport engineering being a viable technology for eliminating antinutritional metabolites from edible parts through the modulation of a discrete number of transporters involved in loading metabolites onto a long distance transport highway.

Members of the plant NRT/PTR family had until recently been shown to transport either dipptides and triptides or nitrate [24]. The ability of the GTRs to transport glucosinolates assigns a new role in specialized metabolism to yet another primary metabolite transporter family (see Box 1).

Transport engineering in synthetic biology

Synthetic biology aims at engineering new biological processes for specific industrial applications such as, for example, microbial production of valuable plant specialized metabolites. In general, low yield can be caused by toxicity and feedback inhibition of the final products [53,54], and implementation of transport engineering to export specialized metabolites out of the cell or to enable subcellular sequestration in storage compartments may enable higher yield production [53,55,56]. There are only very few reports on implementation of transport engineering in synthetic biology [57], which possibly reflects our limited molecular knowledge on intracellular transport and cellular excretion of specialized metabolites.

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Arabidopsis was shown to function as a carrier that transports anthocyanin (without glutathionation) from the production site in ER-associated cytosol to the tonoplast [69]. Previously, in Medicago trunculata it was shown that malonylated flavonoid glucosides had increased affinity for MtMATE2 [60], and it was hypothesized that upon malonylation of anthocyanins, the modified anthocyanins were released from TT19 at the tonoplast, and subsequently taken up by tonoplast-localized MATE transporters [67]. Although various conjugations appear to be a prerequisite for vacuolar uptake of flavonoids (reviewed in [65]), this does not appear to be a general property of specialized metabolites as exemplified with alkaloids [11,12].

Another transport family implicated in cellular efflux and vacuolar sequestration is the ATP binding cassette (ABC) protein family which is a ubiquitous and diverse group of proteins, whose direct utilization of ATP for energizing transport processes allow transport of substrates across a multitude of biological membranes (for review see [44]). Similar to the MATE family, ABC transporters are often associated with detoxification processes. The impressive list of substrates that can be transported by ABC transporters includes peptides, carbohydrates, lipids, heavy metal chelates, inorganic acids, steroids, cell wall monomers and xenobiotics [44,70]. The diverse functions and substrate specificities assigned to MATE and ABC transporters and the large size of these families in the plant kingdom (e.g. ~56 MATEs and ~130 ABCs in Arabidopsis) create a rich gene pool, which may provide transport proteins of relevance for synthetic biology approaches.

Unloading defense compounds to the apoplast

Future applications of transport engineering may include increased resistance to fungal pathogens as there is evidence that members of the pleiotropic drug resistance (PDR/ABCG) subfamily of the large ABC transporter family are involved in pathogen defense and/or crosstalk between plant and microorganisms [44]. In Arabidopsis, PEN3/PDR8/ABCG36 which has been shown to play a role in cadmium and Na⁺ tolerance [45,46] is also important for resistance to fungus barley powdery mildew [47]. This protein is localized to the plasma membrane of epidermal cells, where it concentrates at sites of infection, supposedly to export yet-unidentified toxic compounds to the apoplast near the attempted invasion sites [47,48]. In wheat, LR34, another member of the ABCG subfamily controls durable disease resistance against some of the most devastating fungal pathogens such as leaf rust and stripe rust, potentially by exporting antifungal metabolites similar to the proposed role for PEN3 although the mechanism is currently not understood [49]. Additional ABCG transporters suggested to be involved in basal defense include the NpPDR1 of Nicotiana plumbagifolia proposed to have the antifungal terpenoid sclareol as in vivo substrate [50].

For beneficial interactions between plant and microorganisms, export of specialized metabolites to function as attractants has been reported. For example, a yet unidentified ABC transporter was involved in exporting the isoflavonoid genistein for establishment of legume–rhizobia symbiosis [51]. Similarly, indirect evidence suggests that the Medicago trunculata ABCG transporters MtSTR1 and MtSTR2, of which close homologues are found in almost all plants but Arabidopsis (that does not form mycorrhiza), are involved in mycorrhization, although the substrate is unknown [52].

Better understanding of the molecular mechanism and functional roles (including knowledge of native substrates) is required to evaluate whether transport engineering approaches can be developed to improve disease resistance towards pathogens or beneficial interactions with microorganisms.

Identification of transporters for specialized metabolites

Several difficulties are associated with identifying transporters of specialized metabolites. Firstly, researchers often confine their homology searches to already identified transporter families such as the MATEs and ABCs. However, as outlined in this review, transporters of specialized metabolites may extend into many more transporter families. Secondly, specialized metabolites are often not directly amenable for yeast functional complementation which has otherwise enabled efficient screening of plant cDNA libraries for transporters of primary metabolites. Thirdly, characterization of transporters of specialized metabolites is revealing some surprising dual substrate affinities which may mislead scientists. These challenges have stimulated development of innovative approaches in the transport field. Co-expression of libraries of unknown plant transporters together with optical glucose or sucrose sensors in human cell lines enabled the identification of sugar efflux transporters, the identity of which had remained elusive [71]. In another example, employment of receptor complexes to drive expression of selection markers enabled functional screening of a plant cDNA library to identify transporters of phytohormones in yeast [30]. Other studies have taken in vivo approaches and performed metabolite profiling on root exudates from transporter mutants to identify possible substrates [73]. Although effective, the low throughput of these approaches remains an inherent bottleneck for identification of transporters for specialized metabolites. In our opinion, breakthroughs in transport engineering await the development of platforms for high-throughput functional screening of transport proteins. The functional genomics approach used to identify glucosinolate transporters.
involved screening of a normalized library consisting of Arabidopsis transporter cDNAs in Xenopus oocytes for uptake of glucosinolates [23]. As the library was screened in pools and uptake was detected by LC–MS analyses, this approach allows for repeated screening of the entire transporter library for uptake activities against libraries of compounds or even Arabidopsis extracts in a semi high throughput manner.

**Conclusion and prospects**

Transport engineering of plant specialized metabolites is an emerging field with potential for improving nutritional value and disease resistance in crops and for increasing yield in synthetic biology strategies. The number of identified transporters of specialized metabolites is rapidly increasing owing to new identification strategies. The involvement of members from primary metabolite transport families, such as the PUP and NRT/Ptr families, not only broadens the scope for these transporter families but also for the entire field of transport of specialized metabolites as it indicates that the hitherto focus on ABC and MATEs as candidates for transporters of specialized metabolites is too narrow. We lack fundamental knowledge about exporters, which are likely to be excellent transport engineering targets for blocking export from synthesizing cells or from storage compartments. In addition, export proteins are expected to be of importance for synthetic biology where yield increase and easier extraction can be achieved by exporting end products out of host organisms. Given the occurrence of glucosinolates in the model plant Arabidopsis with its excess of molecular tools combined with the lack of pleiotropic effects upon glucosinolate redistribution in the glr1 glr2 double mutant, suggests the long distance transport pathway of glucosinolates as an excellent model system for studying transport of specialized metabolites [22]. A successful transport engineering strategy that eliminates glucosinolates from the seeds of Arabidopsis has been reported [23]. The close synteny between Arabidopsis and B. napus (rapeseed) enables rapid translational biology strategies to improve the nutritional value of a globally important crop which will function as case study for development of novel generic approaches for specifically eliminating anti-nutritional specialized metabolites from edible parts of crops.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


Through co-expression NUP1, a member of the PUP family of purine transporters is shown to transport nicotine across plasma membranes in tobacco roots with high specificity. NUP1 is involved in maintaining a root to rhizosphere exchange of nicotine.


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In this paper, two transporters from the NRT1/PTR family are identified as specific high affinity glucosinolate/H+ symporters with an essential role for translocation of glucosinolates to seeds. Transporters were identified by a functional genomics approach in Xenopus oocytes. Accumulated expression of glucosinolates in seeds provide strong proof of concept for transporters and peptide transporters. FEBS Lett 2007, 581:2290-2300.


This paper shows that NRT1.1 has substrate-specificity towards both nitrate and the plant hormone auxin, which links soil nitrate availability with auxin-regulated lateral root development.


In this paper employment of receptor complexes enabled functional screening of a plant cDNA library in yeast to identify transporters of phytohormones, and the AIT/NRT1.2 was identified as an ABA importer in addition to being a low affinity nitrate transporter.


This paper shows that the half-sized Medicago truncatula ABC transporters MISTR1 and MISTR2 are involved in mycorrhization, although the substrate is unknown.


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69. Sun Y, Li H, Huang JR: Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. Mol Plant 2011 http://dx.doi.org/10.1093/mp/ssr110. TT19, a glutathione S-transferase is shown to be a flavonoid binding protein which increase water solubility and which have different affinities towards different flavonoids. TT19 is proposed to bind cyanidin and anthocyanin derivatives in the cytosolic side of ER in a binary complex which is then recruited to the tonoplast. Bound cargo may be azylated before release from TT19.


71. Chen LQ, Xu QO, Hou BH, Sossos D, Osorio S, Fernie AR, Frommer WB: Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science 2012, 335:207-211. In this paper optical sensors are used to screen a plant transporter library for sucrose transporters in Mamalian cell lines. The identified transporters are shown to be the long sought sucrose effluxers, responsible for effluxing sucrose from phloem parenchyma proximal to the companion cells in which sucrose is taken up for long distance transport through the phloem.


