CSIRO PUBLISHING

www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2006, **33**, 731–736

***Review*:**

Functional genomics of plant transporters in legume nodules

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This paper originates from a presentation at the Third International Conference on Legume Genomics and Genetics, Brisbane, Queensland, Australia, April 2006.

Abstract. Over the past few decades, a combination of physiology, biochemistry, molecular and cell biology, and genetics has given us a basic understanding of some of the key transport processes at work in nitrogen-fixing legume nodules, especially those involved in nutrient exchange between infected plant cells and their endosymbiotic rhizobia. However, our knowledge in this area remains patchy and dispersed over numerous legume species. Recent progress in the areas of genomics and functional genomics of the two model legumes, *Medicago truncatula* and *Lotus japonicus* is rapidly filling the gap in knowledge about which plant transporter genes are expressed constitutively in nodules and other organs, and which are induced or expressed specifically in nodules. The latter class in particular is the focus of current efforts to understand specialised, nodule-specific roles of transporters. This article briefly reviews past work on the biochemistry and molecular biology of plant transporters in nodules, before describing recent work in the areas of transcriptomics and bioinformatics. Finally, we consider where functional genomics together with more classical approaches are likely to lead us in this area of research in the future.

Keywords: functional genomics, legume nodule, symbiotic nitrogen fixation, transporter.

Introduction

Symbiotic nitrogen fixation (SNF) in legume root nodules is a process of global importance that injects tens of millions of tonnes of nitrogen (N) into agricultural and natural ecosystems each year (Smil 1999). SNF in legume nodules is carried out by the nitrogenase enzyme-complex of gram-negative bacteria called rhizobia, within a specialised organelle called the symbiosome that is delimited from the surrounding plant cytoplasm by a membrane of plant origin called the symbiosome membrane (SM). Symbiosomes each contain one or a few nitrogen-fixing bacteroids, and infected plant cells typically contain thousands of these organelles, which fulfill not only the N-requirements of the host cell, but also of the plant as a whole. Infected plant cells, interspersed with non-infected cells constitute the central tissue of nodules, which is surrounded by uninfected tissue that restricts gas exchange with the soil, and phloem and xylem, which import and export nutrients from the nodule, respectively. In return for reduced nitrogen from the bacteroids, the plant provides its microsymbionts with a source of reduced carbon derived from photosynthesis in the shoot, and other essential nutrients. 'Bartering' of nutrients between plant and bacteria is controlled by transporters in the bacteroid inner and outer membranes and the SM (Udvardi and Day 1997). However, transporters perform many other important roles in nodules, such as short- and long-distance transport of nutrients between plant cells and tissues, and between the nodule and other organs, processes facilitated by proteins of the plant cell plasma membrane. On the other hand, transporters on the membranes of organelles such as mitochondria, plastids, and peroxisomes facilitate the movement of metabolites between cellular compartments, which is crucial for nodule metabolism and SNF.

Knowledge about nodule transport processes has come from a variety of experimental disciplines, including plant and bacterial physiology, biochemistry and molecular biology, and genetics. Invariably, these traditional approaches have focused on one or a few specific transport steps, and have left us far from a complete picture of the full repertoire of transporters that are active in legume nodules, and of how

Abbreviations used: EST, expressed sequence tag; N, nitrogen; NIP, nodulin-like intrinsic protein; SM, symbiosome membrane; SNF, symbiotic nitrogen fixation; TC, tentative consensus sequence; VDAC, voltage-dependent anion channel.

 10.1071/FP06085 1445-4408/06/080731

they support nodule metabolism and SNF. Recent advances in genomics and functional genomics are beginning to fill this void, so that in the foreseeable future we can expect not only a complete catalogue of transporters that are expressed in nodules, but also information about where they act and what roles they fulfill. This paper briefly summarises what we have learned from traditional approaches, before describing in more detail the functional genomic approaches that are beginning to shed new light on this area of research.

Biochemistry and molecular biology of nodule transport

A variety of complementary data (reviewed by Udvardi and Day 1997) indicates that sucrose translocated into nodules from the shoot is converted to dicarboxylic acids, such as malate, before being transported from the cytoplasm of infected cells to nitrogen fixing bacteroids. Bacterial genetics has shown that dicarboxylate transport and metabolism, but not that of sugars for instance, is crucial for SNF, supporting the conclusion that dicarboxylic acids are the primary source of carbon and energy for bacteroid metabolism. The high affinity, energy-dependent dicarboxylate transporter, DctA is mainly responsible for dicarboxylate uptake by nitrogen fixing bacteroids, and is indispensable for SNF (Ronson *et al*. 1981; Udvardi and Day 1997). The counterpart of DctA on the SM is a lower-affinity transporter that has been characterised biochemically (Udvardi *et al*. 1988), but not yet at the molecular level. However, the recent discovery of a dicarboxylate transporter, AgDCAT1 located on the SM in actinorrhizal nodules of the non-legume *Alnus glutinosa* (alder, Betulaceae — birch family; Jeong *et al*. 2004) suggests that related PTR-family transporters may transport dicarboxylates across the SM of legume nodules. Interestingly, PTR-family genes are among those that are strongly induced during nodule development in the legume, *Lotus japonicus* (Colebatch *et al*. 2004).

Ammonia produced by nitrogen-fixing bacteroids appears to be transported out across the bacteroid membranes by simple diffusion, before being transported across the SM as either NH_4^+, via a cation channel that also transports K^+ (Tyerman *et al*. 1995; Roberts and Tyerman 2002; Obermeyer and Tyerman 2005), or as NH_3 (Niemietz and Tyerman 2000), possibly via aquaglyceroporins of the NIP (Nodulin-like Intrinsic Protein) family. The archetype of the NIP family is soybean nodulin 26, a nodule-specific protein of the SM. The molecular identity of the SM NH_4^+ / K^+ channel is unknown. However, it is unlikely to be a member of the AMT family of NH_4^+ transporters, which are relatively specific for NH_4^+, do not transport K^+, and seem to be located in the plasma membrane where they are likely to be involved in recovering ammonia lost from nodule cells by diffusion (Simon-Rosin *et al*. 2003; D'Apuzzo *et al*. 2004). While the relative importance of NH_4^+ *v.* NH_3 transport across the SM *in vivo* remains to be determined, there is some debate about whether or not the principal role of the SM NH_4^+ / K^+ channel is nutrient transport. An alternative hypothesis is that it plays a role as a buffer for trans-membrane voltage or pH generation via the SM H^+-ATPase.

A range of inorganic nutrients, including phosphorus, sulfur, potassium, sodium, calcium, vanadium, iron, molybdenum, nickel and cobalt are required by rhizobia for multiplication and maintenance (Rosendahl *et al*. 1991), but little is known about how most of these substances are obtained from the plant. Although sulfate transport into symbiosomes has not been studied directly, recent map-based cloning of *LjSST1*, which encodes a nodule-induced sulfate transporter in *Lotus*, revealed it to be essential for nodule function (Krusell *et al*. 2005). Its supposed location on the SM (Wienkoop and Saalbach 2003) suggests that SST1 is essential for sulfur supply to the bacteroids. Another nodule-induced transporter in *Lotus*, LjN70, together with its likely orthologue in soybean, GmN70 were recently shown to be anion channels with ion selectivity similar to a soybean SM transporter characterised biochemically earlier (Udvardi *et al*. 1991; Vincill *et al*. 2005). Iron transport across the SM and bacteroid membranes of soybean nodules has been characterised biochemically (LeVier *et al*. 1996; Moreau *et al*. 1995, 1998), and a nodule-induced divalent metal transporter, GmDMT1, capable of ferrous iron transport has been cloned and characterised (Kaiser *et al*. 2003). GmDMT1 was localised to the SM and also appears to transport zinc, copper and manganese, so it may play a role in supplying a variety of metal ions to bacteroids (Kaiser *et al*. 2003). An unrelated, nodule-specific SM protein of soybean called GmZIP1, which transports zinc has also been characterised (Moreau *et al*. 2002). Inhibition of zinc transport into isolated symbiosomes by an antibody to GmZIP1 implicates the transporter in zinc supply to the bacteroids (Moreau *et al*. 2002). Six related members of the ZIP family that transport zinc, manganese or iron have been identified in *M. truncatula* but their location and roles, if any, in nodules remain to be determined (López-Millán *et al*. 2004). Thus, apart from SST1 in *Lotus*, none of the cloned transporters have been shown to be essential for nutrient supply to bacteroids or for SNF. Clearly, these and other nodule-induced transporters are good targets for reverse-genetics experiments in the future.

Potassium is transported across the SM of soybean, broad bean, and *Lotus* (Tyerman *et al*. 1995; Roberts and Tyerman 2002; Andreev *et al*. 2005) via the NH_4^+ / K^+ channel described above, and possibly others, but the identity of these proteins is unknown. A nodule-induced potassium transporter of the KUP family, LjKUP1 from *Lotus*, was recently cloned and characterised, but its location on the plasma membrane of plant cells suggests that it plays a role in plant rather than bacteroid potassium nutrition (Desbrosses *et al*. 2004). Calcium uptake into symbiosomes has been documented for yellow lupin (Andreev *et al*. 1998) and broad bean (Andreev *et al*. 1999), driven by a Ca^{2+}-ATPase in the latter case, but the corresponding

proteins remain to be discovered. Interestingly, the NH_4^+/K^+ channel of *Lotus* SM also appears to transport Ca^{2+} (Roberts and Tyerman 2002). A P-type H^+-ATPase and possibly other proton pumps energise the SM, which drives many secondary transport processes of this membrane (Udvardi and Day 1989; Fedorova *et al*. 1999), but again the specific isoforms responsible remain to be identified.

Few other transporters of legume nodules have been characterised at either the biochemical or molecular levels. Uninfected cells of *Vicia faba* nodules are able to take up apoplastic glucose and sucrose, apparently via H^+-sugar symport, while infected cells lack this activity suggesting that they are reliant on sugars supplied symplastically from neighbouring uninfected cells (Peiter and Schubert 2003). A nodule-induced sucrose transporter, LjSUT4 of *Lotus* has been cloned that may be involved in distributing incoming sugars from the phloem to cells throughout the nodule (Flemetakis *et al*. 2003). On the flip side, nodules typically export fixed nitrogen to the root and shoot principally in the form of amides (glutamine and asparagine) in the case of temperate legumes, or ureides (allantoin or allantoic acid) in the case of tropical legumes. An allantoin transporter of French bean, PvUPS1 was recently identified, which is expressed predominantly in endodermal and vascular cells of nodules and presumably plays a role in ureide export from nodules in this species (Pélissier *et al*. 2004). Finally, five voltage-dependent anion channels (VDAC) of *Lotus japonicus* have been cloned, functionally characterised, and localised to the mitochondrial membrane (Wandrey *et al*. 2004), but none were found on the SM, as inferred from proteomic analysis of partially-purified SM (Wienkoop and Saalbach 2003).

Functional genomics of nodule transport

The past few years have seen a tremendous increase in the amount of sequence information for legume genes in public databases. Initially, most of the sequence information came from partially sequenced cDNA clones, known as expressed sequence tags (ESTs). At present there are ~330 000 ESTs from soybean (*Glycine max*), 227 000 from *Medicago truncatula*, and 110 000 from *Lotus japonicus* in the TIGR database (http://www.tigr.org/tdb/tgi/plant.shtml; verified 26 May 2006). Genome projects for *Medicago* and *Lotus* are well underway (Young *et al*. 2005) and planned for soybean, so that in the next few years we can expect to have access to nearly complete genome sequences for each of these legumes. Rapid progress in legume genomics has spurred activity in the area of functional genomics, which aims to assign function to genes discovered by sequencing, and includes high-throughput analysis of gene transcripts (transcriptomics) and proteins (proteomics). High-density arrays of cDNA or oligonucleotides representing thousands or tens of thousands of genes from soybean, *Medicago*, and *Lotus* have been generated and used to profile gene expression in these species (Oldroyd *et al*. 2005; Udvardi *et al*. 2005). This work resulted in the identification of a host of new transporter genes that are expressed in nodules, many of which are induced during nodule development. Colebatch *et al*. (2004) identified ~860 genes that are induced in mature 6-week-old nodules relative roots in *Lotus*, of which 5% (46 genes) encode transporters, including putative transporters of sulfate, phosphate, nitrate, amino acids or peptides, sugars, and purines. Kouchi *et al*. (2004) identified 65 putative membrane transporters among 1076 genes that are up-regulated during the establishment of symbiosis in *Lotus*, including sulfate, sugar, metal, peptide, and mannitol transporters.

Several different studies have identified transporter genes that are induced during nodule development in *Medicago*. Bioinformatic analysis of *Medicago* tentative consensus sequences (TCs), which consist of two or more overlapping EST sequences, identified 34 transporter TCs derived from ESTs exclusively found in nodules. These included TCs encoding putative transporters for purine, copper, hexoses, sulfate, amino acids, as well as ABC- and MATE-type transporters of unknown substrate specificity (Fedorova *et al*. 2002). Nodule-specificity of a putative purine permease was confirmed in 4-week-old nodules by RNA gel-blot analysis (Fedorova *et al*. 2002). El Yahyaoui *et al*. (2004) reported the activation during *Medicago* nodule development of genes encoding hexose and amino acid transporters, and a NIP homologous to soybean Nodulin 26, among other transporters. Manthey *et al*. (2004) confirmed through microarray and real-time RT–PCR approaches the induction in 4-week-old nodules of two genes encoding Nodulin 26-like proteins, a phosphate, and a cationic amino acid transporter as well as an NRAMP homologous to GmDMT1 of soybean (see above). An hexose transporter and a Nodulin 26 homologue were among the 40 most-strongly induced genes in *Medicago* nodules harvested 10 d post-inoculation (Küster *et al*. 2004).

Table 1 summarises and compares data on nodule-induced transporters from *Lotus* and *Medicago* obtained in the studies described above. Comparisons were facilitated by using the tentative orthologous groups of TIGR.

Bioinformatics of membrane proteins in *Medicago truncatula*

International sequencing efforts have already yielded genomic sequences for more than half of all genes in both *Lotus* and *Medicago*. Annotation of *Medicago* genes is being performed by the International Medicago Gene Annotation Group (IMGAG: www.medicago.org/genome/IMGAG/; verified 26 May 2006), which as of 9 February 2006 had annotated 24 156 putative genes (ftp site: ftp://ftpmips.gsf.de/plants/medicago). We have taken the IMGAG-annotated genes and used two different programs, Tmpred (Hofmann and Stoffel 1993), a hydropathy-based method, and HMMtop (Tusnady and Simon 2001), a machine learning-based prediction method trained on characteristics

Table 1. Nodule-induced transporters of *Medicago* and *Lotus*
TCs were updated from original references to the latest TIGR release (Medicago, Mt: release 8.0; Lotus, Lj: release 3.0)

Substrate class	Annotation	TIGR orthologous group	TIGR TC identifier with (nodule / root expression ratio)
Carbon	Triose-phosphate / phosphate translocator	895864	LjTC14096 (2.9[A])
	Purine permease	900298	MtTC107753 (7.0[B]), LjTC16969 (6.2[A])
	Sugar transporter	896734	MtTC107287 (8.5[E], 21.9[D])
		–	LjTC8113 (10.6[A])
		–	LjTC8114 (8.6[A])
Nitrogen	Oligopeptide transporter	895217	LjTC9160 (9.3[A])
	Nitrate transporter	896444	LjTC18424 (19.4[A])
	Amino acid transporter	905054	MtTC96926 (5.2[E])
	Peptide transporter	900924	LjTC14389 (47.5[A])
		906699	LjTC14955 (297.8[A])
Other macronutrients	Phosphate transporter	910898	LjTC10841 (4.2[A])
	Sulfate transporter	896297	LjTC8045 (65.4[A])
		899390	LjTC9746 (8.4[A])
		899805	LjTC8880 (146.0[A])
Micronutrients	Ferrous iron transporter	894446	MtTC106427 (37.5[A])
	Zinc transporter	913501	LjTC11999 (4.3[F])
	Sodium / proton antiporter	902030	LjTC12985 (174.6[A])
Energisers	Proton–ATPase (mitochondrial)	895183	LjTC16976 (4.3[A])
		895380	LjTC7877 (2.5[A])
	Adenosine triphosphatase subunit H (vacuolar)	895481	LjTC9615 (2.4[A])
	Proton–ATP synthase	896442	LjTC7822 (2.2[A])
	Adenosine triphosphatase	897279	LjTC11159 (6.1[A])
	Adenosine triphosphate synthase proton pump (vacuolar)	922970	LjTC15203 (5.2[A])
	ABC-type transporter	–	Lj singleton AV408313 (1.9[C])
Porins	VDAC porin	894497	LjTC7918 (2.8[A])
	OEP24-like pore protein	899734	LjTC8709 (3.3[A])
Aquaporins	PIP aquaporin	894466	LjTC8724 (2.0[A])
	NIP aquaporin	900316	MtTC100851 (5.9[E]), MtTC94970 (33.6[D])
		901730	LjTC14463 (2.6[A])
Other or unknown functions	Membrane transporter	897709	LjTC14492 (3.7[A])
		898316	LjTC14419 (2.5[A])
		907082	LjTC10527 (3.7[A])
		928914	LjTC14887 (81.1[A])
		–	LjTC18667 (12.8[A])

[A]Colebatch *et al*. (2004). [B]Fedorova *et al*. (2002). [C]Kouchi *et al*. (2004). [D]Küster *et al*. (2004).
[E]Manthey *et al*. (2004). [F]Suganuma *et al*. (2004).

of transmembrane segments, including properties of amino acids, localisation, and sequential information of amino acid and signal peptide, to identify putative membrane proteins in *Medicago* (Fig. 1). Between 29 and 49% of IMGAG proteins are predicted to contain at least one transmembrane domain, depending upon which of the two programs is used, while 11–22% contain two or more putative transmembrane domains. The latter category is expected to include many transporters, and we are currently classifying these based on functional categories defined by the Transport Commission (www.chem.qmul.ac.uk/iubmb/mtp/; verified 26 May 2006). In preliminary work, a simple keyword search of the IMGAG annotations, using the terms Transporter, Channel, Permease, Symporter, Antiporter, Major Facilitator Superfamily, Porin, Carrier, and Exchanger found 307 proteins that are likely to be involved in membrane transport. Most of these (85–88%) are predicted by our analyses (Fig. 1) to contain two or more transmembrane domains. Finally, ~88% of all proteins with two or more predicted transmembrane domains are represented by corresponding gene probe sets on the Affymetrix Medicago GeneChip, suggesting that the Genechip contains probe sets for the majority (88%) of all *Medicago* genes. Similar calculations reveal that the Affymetrix Lotus GeneChip, which has recently been

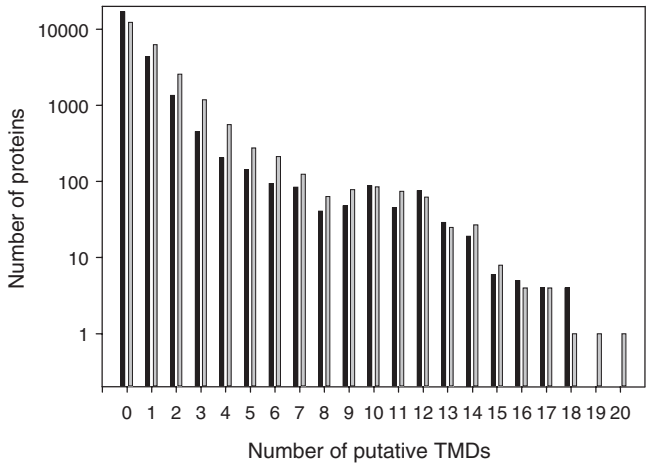

Fig. 1. Predicted number of transmembrane domains (TMDs) for IMGAG-annotated proteins of *Medicago* (9 February 2006 release), using HMMtop 2.1 (Tusnady and Simon 2001; black bars) or Tmpred with score >500 (Hofmann and Stoffel 1993, grey bars).

commissioned by an international consortium (M Udvardi, J Stougaard, L Schauser, S Sato, S Tabata unpubl. data), contains probe sets for ~90% of all *Lotus* genes.

Outlook

With the *Medicago* and *Lotus* Affymetrix GeneChips now being used in several laboratories, we can soon expect an almost complete list of the transporters that are expressed during nodule development and differentiation. It will take a few more years, depending on when genome sequencing of these species is completed, before a full parts-list of all transporters at work in nodules can be assembled. In the meantime, systematic approaches to determine the tissue, cell type, and intracellular location of each transporter, their substrate specificity, and their function within the context of the nodule will begin, using existing sequence and transcriptomics data. The cell biology, biochemistry, and biophysics of nodule transporters will be facilitated by high-throughput approaches to clone the open reading frame (ORF) of each nodule-expressed transporter gene, and to transfer these ORFs to a variety of existing expression vectors, using GATEWAY cloning technology for instance (Curtis and Grossniklaus 2003; Earley *et al*. 2006). Finally, reverse genetics approaches, using growing populations of *Medicago* and *Lotus* mutants (Tadege *et al*. 2005; Udvardi *et al*. 2005), will enable the *in vivo* role of nodule transporters to be addressed in a systematic manner, opening up the possibility of optimising the performance of symbiotic nitrogen fixation in legume nodules by manipulating key transporter genes.

Acknowledgments

We thank the Samuel Roberts Noble Foundation and the Max Planck Society for generous support of research in our laboratories, and the German DFG for specific funding related to legume nodule transport.

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Manuscript received 11 April 2006, accepted 25 May 2006