

## Genomic inventory and transcriptional analysis of *Medicago truncatula* transporters

Vagner A. Benedito<sup>1,2</sup>, Haiquan Li<sup>1</sup>, Xinbin Dai<sup>1</sup>, Maren Wandrey<sup>3</sup>, Ji He<sup>1</sup>, Rakesh Kaundal<sup>1</sup>, Ivone Torres-Jerez<sup>1</sup>, S. Karen Gomez<sup>4</sup>, Maria J. Harrison<sup>4</sup>, Yuhong Tang<sup>1</sup>, Patrick X. Zhao<sup>1</sup>, Michael K. Udvardi<sup>1\*</sup>

<sup>1</sup>The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

<sup>2</sup>Genetics and Developmental Biology Program, Division of Plant and Soil Sciences, West Virginia University, 2090 Agricultural Sciences Building, PO Box 6108, Morgantown, WV 26506, USA

<sup>3</sup>Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany

<sup>4</sup>Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853, USA

\* Corresponding author. E-mail: [mudvardi@noble.org](mailto:mudvardi@noble.org)

### Summary

Transporters move hydrophilic substrates across hydrophobic biological membranes and play key roles in plant nutrition, metabolism, signaling, and consequently in plant growth, development, and responses to the environment. To initiate and support systematic characterization of transporters in the model legume, *Medicago truncatula*, we identified 3,830 transporters and classified 2,673 of these into 113 families and 146 sub-families. Analysis of gene expression data for 2,611 of these transporters identified 129 that are expressed in an organ-specific manner, including 50 that are nodule-specific and 36 specific to mycorrhizal roots. Further analysis uncovered 196 transporters that are induced at least five fold during nodule development and 44 in roots during arbuscular mycorrhizal (AM) symbiosis. Amongst the nodule- and mycorrhizal-induced transporter genes are many candidates for known transport activities in these beneficial symbioses. The data presented here are a unique resource for selection and functional characterization of legume transporters.

**Keywords:** legume, transporter, transcriptome, symbiosis, rhizobia, mycorrhiza

**Running title:** Membrane transporters and root symbioses in *Medicago*

## INTRODUCTION

Transporters are membrane-spanning proteins that selectively transport hydrophilic solutes across hydrophobic membranes. They are present and required in all cellular membranes, including the cell or plasma membrane that separates cellular contents from the external environment and membranes of the various sub-cellular organelles. By transporting metabolites and non-metabolites, such as inorganic ions, transporters play integral roles in cell metabolism, ion homeostasis, osmoregulation, signaling, and other processes. Transporters move solutes not only within cells, but also between cells, tissues, and organs of complex, multicellular organisms such as higher plants. Therefore, they help to coordinate metabolic, physiological, and developmental processes in higher plants and other organisms.

Transporter proteins/complexes contain multiple membrane-spanning domains that form an aqueous pore in the membrane, which enables movement of selected solutes from one side of the membrane to the other. Membrane-spanning domains are hydrophobic in nature, or at least partially so, which enables them to interact with the phospholipid bilayer of membranes. Many transporters contain hydrophobic  $\alpha$ -helical segments that span the membrane, while others contain  $\beta$ -barrel transmembrane domains. Computer programs have been developed to identify putative membrane-spanning  $\alpha$ -helices (Hoffman and Stoffel, 1993; Hirokawa et al., 1998) (Tusnady and Simon, 2001) and  $\beta$ -barrels (Koebnik et al., 2000; Valavanis et al., 2006), which facilitate *de novo* prediction of putative membrane proteins, including transporters. Databases of known, characterized transport proteins aid identification and classification of transporters in new species, via sequence similarity. Perhaps the most comprehensive of these is the Transporter Classification Database (TCDB) (Saier et al., 2006), which was created to serve as a repository of functionally characterized transporters. It also serves to categorize new transporters into families and subfamilies based on molecular, evolutionary, and functional properties. Presently, it consists of ~3,000 transporters classified in more than 500 families ([www.tcdb.org](http://www.tcdb.org)).

The legume family is second only to the grass family in importance to humans as a source of food, feed for livestock, and raw materials for industry (Graham and Vance, 2003). Legumes are the lynch pin of sustainable agriculture because they supply their own nitrogen by ‘fixing’ it (reducing  $N_2$  to  $NH_3$ ) in a symbiotic association with bacteria called rhizobia. This mutually-beneficial association provides legumes and subsequent crops with a free and renewable source of usable nitrogen (Udvardi and Day, 1997). Legumes also establish symbiosis with mycorrhizal fungi that help the plant mine phosphorous and other nutrients from the soil (Smith and Read, 2008)

Symbiotic nitrogen fixation (SNF) in root nodule cells of legumes is carried out by rhizobia that are completely surrounded by a plant membrane called the symbiosome membrane (SM), which forms a nitrogen fixing organelle, the symbiosome, within the plant cytoplasm. Infected cortical cells of nodules contain thousands of symbiosomes, each containing one or a few bacteria. 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 exchange for ammonium produced by bacterial nitrogenase and released to the plant, rhizobia receive reduced carbon (principally dicarboxylic acids such as malate) and every other nutrient required for bacterial cell growth and maintenance (Udvardi and Day, 1997). Exchange of nutrients between the plant cell cytoplasm and rhizobia is mediated by a variety of transporters in the SM, some of which are induced during nodule development (Benedito et al., 2008). 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.

In the arbuscular mycorrhizal (AM) symbiosis, the fungal symbionts inhabit the root cortex where they obtain carbon from the plant and in exchange they deliver mineral nutrients, particularly P and N, to the root. Mineral nutrient transfer between symbionts occurs at a

specialized symbiotic interface between branched hyphae, called arbuscules, and the cortical cells that they inhabit (Parniske, 2008). The interface is delimited by a plant-derived membrane called the periarbuscular membrane (PAM), which is continuous with the plasma membrane but contains some unique proteins including novel Pi transporters (Harrison et al., 2002; Paszkowski et al., 2002). These transporters are required to transfer Pi that is released from the arbuscule, into the cortical cell. It is assumed, but not yet shown directly, that N, and possibly other mineral nutrients such as zinc, is also transferred between the symbionts at this membrane interface (Smith and Read, 2008). However, the transport proteins involved are currently unknown. Likewise transporters involved in carbon transfer to the fungal symbiont have not been identified. While it is expected that the periarbuscular membrane will contain additional transport activities, currently, only a handful of transporters residing in this membrane have been identified.

Although in-roads have been made in the characterization of individual transporters in a variety of legume species, no systematic work has been done to identify and characterize all the transporters in any one species. Three legume species, *Medicago truncatula*, *Glycine max.* (soybean), and *Lotus japonicus* have been the subject of extensive cDNA and genomic DNA sequencing over the past few years (Young et al., 2003; Young et al., 2005; Sato et al., 2007; Sato et al., 2008), making them interesting model systems for whole-genome analysis of transporters. The genome sequence of *Medicago truncatula* is being annotated by the International Medicago Genome Annotation Group (IMGAG), which described 38,335 genes in its version 2.0 of the genome sequence (<http://www.medicago.org/genome/downloads/Mt2/>). Additional resources relevant to Medicago functional genomics include the Medicago Gene Expression Atlas (<http://bioinfo.noble.org/gene-atlas/v2>), which provides developmental expression data for the majority of Medicago genes (Benedito et al., 2008) and a *Tnt1* transposon-insertion mutant population with insertions in the majority of genes, which enables efficient forward and reverse genetics (Tadege et al., 2005; Tadege et al., 2008). To facilitate systematic functional analysis of transporters in Medicago, and especially those involved in nitrogen-fixing (NF) and AM symbioses, we have identified and categorized 2,673 transporter

genes and analyzed the expression pattern of 2,604 of these. The results of this work are presented here.

## RESULTS

### Identification of putative transporters

Initially, *Medicago truncatula* proteins predicted from genome sequence (IMGAG sequence release version 2.0) were analyzed for the presence of potential transmembrane domains (TMD) using three algorithms: HMMTOP 2.0 (Tusnady and Simon, 2001); TMPred (Hoffman and Stoffel, 1993); and SOSUI (Hirokawa et al., 1998). HMMTOP utilizes a machine-learning Hidden Markov Model (HMM) approach, whereas TMPred uses amino acid properties to identify hydrophobic stretches of amino acids that could interact with lipid membranes. SOSUI integrates multiple properties, including hydrophathy, amphiphilicity, amino acid charges, and sequence length to predict protein topology in the membrane.

Among the 38,335 IMGAG-annotated gene products (Figure 1A), 44% were predicted by TMPred to contain at least one TMD, while 32% and 21% were predicted to have one or more TMD by HMMTOP and SOSUI, respectively. In total, 18,684 proteins were predicted to contain at least one TMD by one or more of the three programs. 7,438 proteins were predicted to contain two or more TMD by at least one program, of which 2,405 were identified by all three programs (Figure 1B). Additionally, all 38,335 IMGAG proteins were compared by sequence homology to proteins of the Transporter Classification Database (TCDB) (Saier et al., 2006). This approach was used to identify potential transporters not recognized by any of the TMD prediction algorithms, as well as to guide transporter classification. Among the IMGAG-predicted proteins, 2,039 (5.3%) showed significant similarity to a TCDB sequence. Of these, 1,114 proteins were also found to contain at least two (2+) TMD by at least two prediction programs (Figure 1C).

Since *Medicago* genome sequencing is not yet complete, we also analyzed *Medicago* EST data (*Medicago truncatula* Gene Index (MTGI) version 8.0,

<http://compbio.dfci.harvard.edu/tgi/>), using tBlastX. Among the 36,850 Tentative Consensus (TC) sequences and singletons in the EST database, 2,051 (5.6%) encoded proteins similar to TCDB transporters, of which 1,249 did not match transporters predicted from IMGAG (genomic) sequences (Table 1, Figure 1D). TMD prediction was avoided as a method to select putative transporters encoded by ESTs because many of these sequences are incomplete.

### **Transporter classification**

Medicago proteins with similarity to TCDB proteins were classified into families and, when sufficient evidence was available, into subfamilies. Many transporter families have distinctive characteristics, such as membrane topology (number of TMD), presence of conserved domains, and approximate protein size. These features were considered during transporter classification and used to determine a level of confidence (from 1 to 5) for our classification of each transporter. All putative membrane protein sequences (with 2+ TMD or a match to TCDB proteins) were further analyzed with respect to predicted length, presence of conserved domains (Pfam and InterPro), Gene Ontology (GO) annotation, and predicted subcellular localization (Suppl. Table S1). Medicago proteins with significant similarity to TCDB transporters, original annotations indicative of transporter activity, or with conserved domains characteristic of transporters were collected for manual curation and classification into TCDB families. To avoid false positives resulting from ‘forced’ matches to proteins in TCDB, which contains only 3,000 transporters, Medicago sequences were also compared to more comprehensive sequence databases: the well-annotated Swiss-Prot database (Boeckmann et al., 2003) and the comprehensive (Viridiplantae) Non-Redundant NCBI GenBank (Benson et al., 2008). Annotation based on TCDB analysis and other protein characteristics was checked against annotations of homologous proteins in the Swiss-Prot and NR-NCBI databases. Confidence level 1 in our classification indicates that all features of a protein are consistent with its membership in a particular TCDB transporter family/sub-family, while level 2 indicates some divergence from expected features. Level 3 means functionality is doubtful due to lack of key expected features (such as protein size, TMD absence or an unexpected number, or dubious TCDB homologies) or that classification is loose due to conflicting or weak pieces of evidence. Level 4 indicates that

the putative membrane protein did not match any TCDB transporter, although there was some evidence of transporter function such as a characteristic transporter domain, original annotation from IMGAG or EST, or GO annotation. TCDB contains some proteins, such as heat shock protein HSP70 family (TCDB 1.A.33) and group translocators, which transfer chemical groups from one molecule to another (TCDB category 4), that do not fit our conception of a transporter as a pore-forming membrane protein. We assigned such proteins level 5, although this does not necessarily indicate lack of confidence in their classification.

2,039 proteins predicted from the genome sequence had homology to TCDB proteins and an additional 543 proteins had features consistent with transporter function (such as the presence of conserved transporter domains). Of these, 1,681 proteins were classified into transporter families and subfamilies with confidence level from 1-3, 389 putative membrane transporters with no significant TCDB hit were assigned level 4, and 514 were assigned level 5 (Table S1). Likewise, among the 2,051 EST-encoded proteins with homology to TCDB proteins, 1,759 sequences were classified into transporter families and subfamilies with confidences 1-3 (Tables 1-2). A total of 2673 proteins predicted from genomic and EST sequences were classified into TCDB families and subfamilies. The largest *Medicago* transporter families are listed in Table 3 and a complete list is given in Table S2.

Our analysis of transporters encoded by genomic DNA was based on IMGAG version 2.0 annotation of the genome, which discarded seven IMGAG version 1.0 gene models encoding putative transporters represented by probesets on the Affymetrix *Medicago* GeneChip, including a putative ammonium transporter gene known to be expressed during mycorrhizal symbiosis (Gomez et al., 2009). Therefore, we included these seven putative transporter genes in our subsequent analyses of gene expression.

### **Analysis of transporter gene expression**

The Affymetrix *Medicago* GeneChip contains 50,900 *Medicago truncatula* probesets corresponding to most of the gene transcripts in this species. A script was written in Perl to map probesets to IMGAG version 2.0 gene sequences (see Materials and Methods). In this way,

Affymetrix probesets were assigned to 18,909 IMGAG-annotated genes and 21,284 ESTs (TCs and singlets) not represented by genomic sequence (Figure 1A). Of the 2,673 genes encoding TCDB-classified transporters, 2,604 were represented by probesets on the Affymetrix Medicago GeneChip (Table 2 and S3). Affymetrix probesets matched seven additional transporter genes predicted by IMGAG v.1 but not IMGAG v.2. We included these in our analyses and assigned them confidence level “X” to make them easy to identify in the tables.

Expression data for putative transporter genes were retrieved from the Medicago Gene Atlas (Benedito et al., 2008), including data from all major organ systems (Table S4), a nodule developmental series, and mycorrhizal roots (Table S5). Based on presence/absence calls (at least two present calls within three biological replicates) obtained from chip analysis, 94% of transporter genes were expressed in at least one organ, with each organ expressing about 60% of all transporter genes. Only 4.5% of transporter genes (129) were organ-specific (i.e. active in a single organ type), while 29% of genes (823) were expressed in more than one, but not all organ types. The majority of transporter genes (61%) were expressed in all organs analyzed, although at different levels (Table S4).

### **Transporter gene expression during root symbioses**

Regulation of transporter gene expression during development of two different root symbioses was investigated: nitrogen-fixing and arbuscular mycorrhizal symbioses.

Gene expression data for developing and nitrogen-fixing root nodules were obtained from two sets of experiments, one in which plants were grown in solid substrate (turf) and one in which plants were grown aeroponically (Benedito et al. 2008). In the first set of experiments, mature, nitrogen-fixing nodules were harvested 28 days after inoculation with *S. meliloti* and compared to non-inoculated, control roots of the same age. In the second set of experiments, plants were grown aeroponically and nodules were harvested at 4, 10, and 14 days after inoculation and compared to control, non-inoculated roots harvested immediately prior to inoculation of symbiotic plants. In both sets of experiments, three biological replicates were performed for both treated and control samples. Using a Bonferroni-corrected P-value cut-off of

1.14e-6 (Benedito et al., 2008) in pair-wise comparisons of nodules against control roots, 49-65% of transporter genes exhibited differential expression during nodule development. 196 genes showed >5 fold-increase in expression in nodules compared to root controls, with 25 genes showing >100 fold-change in expression (Table S5). Table 4 shows 37 genes that were induced >50-fold during nodule development. Figure 2 shows membrane transporters highly induced in, or specific to nodules.

Gene expression data for AM symbiosis were obtained from Medicago roots harvested 30 days after inoculation with *Glomus intraradices* and compared to data from non-inoculated control roots, with three biological replicates in both cases (Gomez et al., 2009). Changes in transporter gene expression during AM symbiosis were more subtle than during nodule development and NF symbiosis, as might be expected given the absence of new organ development during AM symbiosis. Nonetheless, 886 genes showed significantly altered expression during AM symbiosis, of which 44 genes were induced >5-fold compared to non-inoculated roots (Tables 5 and S5).

## **DISCUSSION**

Genome-wide identification and classification of transporters is an important first-step in the systematic analysis of transporters in model organisms. Manual curation of information collected for all Medicago proteins, including predictions of the number of TMD and homology to transporters in the TCDB, resulted in the identification and classification of 2,673 distinct transporters. This represents 4.4% of all predicted proteins in Medicago and is in line with what has been found in other plant species. For example, Arabidopsis has 1,269 transporter genes (4.6% of all genes, Bock et al., 2006) while transporter genes account for approximately 5% of all rice genes (Amrutha et al., 2007).

Classification into a specific family/subfamily was given a confidence score from 1-3, based on whether or not additional information supported the results of TCDB analysis, as described in the results section. Proteins with two or more TMD that did not match proteins in the TCDB, but for which additional information pointed to possible transporter function were given a confidence score of 4. Such additional information included the presence of conserved domains typical of transporters or annotation of homologous proteins in more extensive databases (Plant NR-NCBI and Swiss-Prot). Automatization of the classification process is underway (Li et al., 2008, 2009). A total of 389 putative transporters received a score of 4. Medicago proteins with similarity to TCDB proteins that we consider not to be involved in transport of solutes across lipid bilayers were given a score of 5 and not subjected to gene expression analysis. There were 768 such proteins. All putative Medicago transporters predicted from genomic and non-redundant cDNA/EST sequences are linked to Affymetrix probesets listed in the Medicago Gene Atlas database (<http://bioinfo.noble.org/gene-atlas>), which includes further links to gene expression and analysis tools. This database will be updated following release of IMGAG version 3 annotation of the Medicago genome.

The 3,062 putative transporters assigned confidence scores from 1 to 4 were represented by 2,886 non-redundant probesets on the Affymetrix Medicago GeneChip. Therefore, we were able to query published gene expression data (Benedito et al., 2008; Gomez et al., 2009) for 94% of all predicted Medicago transporters. While the majority of transporter genes were expressed in two or more organs, approximately 4.5% (129) were expressed in an organ-specific (Table S4). Presumably, these play specialized roles in organ development, differentiation, and/or function, and they represent interesting targets for future functional analysis. However, because of our interest in beneficial plant-microbe interactions, we focused most of our attention on genes induced during nodule development and SNF or during AM symbiosis. 87% of all transporter genes were differentially-expressed during nodule development and SNF, of which 196 were induced more than 5-fold compared to non-inoculated roots, and 25 were induced >100-fold. (Table S5). 886 genes were differentially-expressed during AM symbiosis, of which 44 were induced more than 5-fold compared to non-inoculated roots. In the following

paragraphs, we discuss some of these genes in the context of what is known about transport and transporters in the two types of symbiosis.

### **Transporters involved in N<sub>2</sub> fixation symbiosis**

A variety of complementary data (reviewed in Udvardi and Day, 1997) indicate 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. The high affinity, energy-dependent bacterial 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 plant counterpart of DctA on the SM is a lower affinity transporter that has been characterized biochemically (Udvardi et al., 1988), but not yet at the molecular level. However, the discovery of a dicarboxylate transporter, AgDCAT1 located on the SM in actinorrhizal nodules of the non-legume *Alnus glutinosa* (Jeong et al., 2004) suggests that related H<sup>+</sup>-dependent Oligopeptide Transporter Family (POT/PTR, TC 2.A.17) members may transport dicarboxylates across the SM of legume nodules. Interestingly, two *POT/PTR* genes are strongly induced during nodule development in *Medicago truncatula* (Table 4, Figure 2a) and *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<sub>4</sub><sup>+</sup>, via a cation channel that also transports K<sup>+</sup> (Tyerman et al., 1995; Roberts and Tyerman, 2002; Obermeyer and Tyerman, 2005), or as NH<sub>3</sub> (Niemiets and Tyerman, 2000), possibly via aquaglyceroporins of the NIP (Nodulin-like Intrinsic Protein) family (TC 1.A.8.12). The archetype of the NIP family is soybean nodulin 26, a nodule-specific protein of the SM. In *Lotus japonicus*, nodule-induced LIMP1 (a Tonoplast Intrinsic Protein, TIP family member) and LIMP2 (possibly an ortholog of Nod26) have been characterized, although their roles during SNF remain unclear (Guenther and Roberts, 2000). Two homologs of these proteins are also expressed more or less specifically in *Medicago* nodules (Table 4, Figure 2b-c). The molecular identity of the SM NH<sub>4</sub><sup>+</sup>/K<sup>+</sup> channel is unknown. However, it is unlikely to be a member of the

AMT family of  $\text{NH}_4^+$  transporters, which are relatively specific for  $\text{NH}_4^+$ , do not transport  $\text{K}^+$ , and seem to be located in the plasma membrane where they are likely to be involved in the recovery of ammonia lost from nodule cells by diffusion (Simon-Rosin et al., 2003; D'Apuzzo et al., 2004; Rogato et al., 2008). We identified nine AMT family (TC 1.A.11) members in *Medicago*, none of which were induced more than 5-fold during nodule development.

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 nutrients are obtained from the plant. Although sulfate transport into symbiosomes has not been studied directly, map-based cloning of *LjSST1* identified a nodule-induced sulfate transporter gene in *L. japonicus* that is essential for nodule function (Krusell et al., 2005). Its reported location on the SM (Wienkoop and Saalbach, 2003) suggests that SST1 is essential for sulfur supply to the bacteroids. Thirteen homologs of *LjSST1* in the SulP/SULTR family (TC2.A.53) were induced more than two-fold during nodule development in *Medicago*, and two were essentially nodule-specific (Table 4, Figure 2d-e). Some members of the SulP family transport substrates other than sulfate, including nitrate, bicarbonate, chloride, and molybdate (Tejada-Jimenez et al., 2007; Tomatsu et al., 2007) so it will be interesting to determine the substrates of the various nodule-induced SulP transporters in *Medicago*.

Lotus LjN70 and soybean GmN70, two nodulins of the Nitrate/Nitrite Porter family (NPP, TC 2.A.1.8), were shown to be anion channels with ion selectivity similar to a soybean SM transporter characterized biochemically earlier (Udvardi et al., 1991; Vincill et al., 2005). Two homologs of these NPP transporters, together with 32 other members of the Major Facilitator Superfamily (MFS, TC 2.A.1), were induced during nodule development in *Medicago* (Table 4, Figure 2f-g).

Iron transport across the SM and bacteroid membranes of soybean nodules have been characterized biochemically (Moreau et al., 1995; LeVier et al., 1996; Moreau et al., 1998), and a nodule-induced divalent metal transporter, GmDMT1 capable of ferrous iron transport has been cloned and characterized (Kaiser et al., 2003). GmDMT1 was localized 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). A homolog of GmDMT1 (NRAMP, TC 2.A.55 – *not to be confused with the DMT superfamily, TC 2.A.7*) is expressed in a nodule-specific manner in Medicago (Table 4). An unrelated, nodule-specific SM protein of soybean called GmZIP1 (TC 2.A.5), which transports zinc has also been characterized (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). We identified 23 ZIP family members in Medicago, six of which were induced more than 2-fold during nodule development (Table S5).

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  $\text{NH}_4^+/\text{K}^+$  channel described above, and possibly others, but the identity of these proteins is unknown. A nodule-induced potassium transporter of the KUP family (TC 2.A.72), LjKUP1 from Lotus was cloned and characterized, but its location on the plasma membrane of plant cells suggests that it plays a role in plant rather than bacteroid potassium nutrition/homeostasis (Desbrosses et al., 2004). Of the 25 putative potassium transporters identified in Medicago, one gene (represented by two probesets) showed a massive induction during the onset of nitrogen fixation and in mature nodules (Table 4, Figure 2i).

Calcium uptake into symbiosomes has been documented for yellow lupin (Andreev et al., 1998) and broad bean (Andreev et al., 1999), driven by a  $\text{Ca}^{2+}$ -ATPase (TC 3.A.3.2) in the latter case, but the corresponding proteins remain to be discovered. Interestingly, the  $\text{NH}_4^+/\text{K}^+$  channel of the Lotus SM also appears to transport  $\text{Ca}^{2+}$  (Roberts and Tyerman, 2002). Among the 15  $\text{Ca}^{2+}$ -ATPases found in Medicago, one EST showed >150-fold change during late states of nodule development and nitrogen fixation (Table 4).

A P-type  $\text{H}^+$ -ATPase (TC 3.A.3) and possibly other proton pumps energize the SM, which drives many secondary transport processes on this membrane (Udvardi and Day, 1989; Fedorova et al., 1999). However, the specific isoforms responsible remain to be identified. We found a nodule-specific P-type ATPase in Medicago, which is an interesting candidate for this activity (Table 4).

ATP for plant membrane energization and metabolism in nodule cells is provided by mitochondria, which undergo both morphological (Werner and Mörschel, 1978) and biochemical differentiation (Suganuma and Yamamoto, 1987) during nodule development. We found that several mitochondrial transporter genes are induced during *Medicago* nodule development, including a Mitochondrial Protein Translocase (MPT, TC 3.A.8; Table 4), which generally transports preproteins into mitochondria (Lister et al., 2007), and two Mitochondrial Carriers (MC, 2.A.29), which may transport TCA cycle intermediates like  $\alpha$ -oxoglutarate to the cytoplasm for ammonium assimilation/ amino acid biosynthesis (Picault et al., 2002; Palmieri et al., 2008).

The ultimate source of energy and carbon skeletons for nodule metabolism and ammonium assimilation is sucrose, which is imported into nodules from photosynthetic organs (Vance et al., 1997; Gordon et al., 1999). LjSUT4 is a sucrose transporter in *Lotus japonicus* that is up-regulated during nodule development and presumably plays a role in sucrose uptake into nodule cells (Flemetakis et al., 2003). Three members of the sucrose/proton symporter subfamily (TC 2.A.2.4) of the Glycoside-Pentoside-Hexuronide:Cation Symporter family (GPH, TC 2.A.2) were identified in our analysis of *Medicago* transporters, but none of them were induced during nodulation.

Nodules of temperate legumes, such as *Medicago*, typically export fixed nitrogen to the root and shoot in the form of amides (glutamine and asparagine; Temple et al., 1998; Lodwig and Poole, 2003). Putative amino acid transporters from different families were identified in our analysis as strongly induced during nodule development (>5-fold change over control roots): 5 members of the Amino Acid-Polyamine-Organocation family (APC, TCDB 2.A.3); 17 Drug/Metabolite Transporters (DMT, 2.A.7); 12 Multidrug/Oligosaccharidyl-lipid/Polysaccharide Flippases (MOP/MATE, 2.A.66); 4 Oligopeptide Transporters (OPT, 2.A.67); and 1 Aromatic Acid Exporter (ArAE, 2.A.85) (Table S5), making them interesting targets for future work aimed at identifying transporters with key roles in amino acid export from nodules.

Nodule development is subject to hormonal regulation (Mabood et al., 2006; Prayitno et al., 2006; Murray et al., 2007; Ding et al., 2008; Oldroyd and Downie, 2008), which in the case

of auxin appears to involve changes in auxin transport (de Billy et al., 2001; Wasson et al., 2006). An auxin influx carrier, CgAUX1 (Amino Acid/Auxin Permease Family, AAAP, TC 2.A.18) was proposed to be active during actinorrhizal nodule formation in *Casuarina glauca* (Péret et al., 2007). We found one AAAP transporter that is essentially nodule-specific in Medicago (Table 4), although it is not the most similar in sequence to the CgAUX1.

Apart from interesting candidates for known transport functions in nodules, our analysis of Medicago transporter gene expression identified many nodule-induced or nodule-specific transporters that presumably carry out novel transport functions in nodules. Clearly, both sets of transporters warrant further work in future to characterize their biochemical and physiological roles in nodules.

### **Transporters involved in mycorrhizal symbiosis**

The number of differentially induced transporters in mycorrhizal roots in comparison to control roots was much smaller than that observed during nodulation, with only 33 transporters showing > 5 fold-change induction in mycorrhizal roots. This was probably due to a dilution effect, since whole root systems were sampled, in contrast to nodule samples, which were excised from nodulated roots and compared to non-nodulated control roots. Nonetheless, some transporters showed a massive induction (>100 fold-change) in mycorrhizal roots. Statistical test revealed 658 transporters differentially expressed in mycorrhizal roots (Table S5).

One of the greatest benefits of mycorrhizal symbiosis for the plants is an increased phosphate uptake, mediated by the AM fungi, which deliver Pi directly to the root cortex. Plant Pi transporters responsible for acquiring Pi delivered by the fungus have been identified previously and MtPT4, a transporter belonging to the Phosphate: H<sup>+</sup> Symporter Family (PHS, 2.A.1.9) is expressed specifically in mycorrhizal roots (Table 4; (Harrison et al., 2002; Javot et al., 2007; Pumplin and Harrison, 2009). We found one member of the Sulfate Permease family (SP, 2.A.53) up-regulated 4.5 fold in mycorrhizal roots, indicating a possible symbiotic role in mycorrhizal roots. Moreover, this sulfate permease is different from the ones up-regulated in nodules. It is important to emphasize that members of this family have also been implicated in

transport of other anions as well, such as molybdate (Fitzpatrick et al., 2008), bicarbonate and heavy metals. Likewise, the solute flippase (MOP/MATE family, 2.A.66), aforementioned as nodule induced, also showed induction (6-fold change) in mycorrhizal roots. Three members of the H<sup>+</sup>-dependent Oligopeptide Transporter (POT/PTR, 2.A.17), also within the MFS, were found to be induced in mycorrhizal roots. Although most members of this family transport peptides, some of them transport other substrates, such as nitrate and dicarboxylates and, therefore, their roles in mycorrhizal roots remain uncertain.

The high energetic requirement of AM symbiosis likely influences mitochondrial metabolism. Our data show two putative Mitochondrial Carriers (MC, 2.A.29), one of them induced more than 100 times in mycorrhizal roots, indicating a possible function in this symbiosis. Many members of this family are ATP/ADP carriers whereas others transport TCA-derived compounds. There was also high expression of a proton ATPase (3.A.3) (50-fold change) which polarizes the plasma membrane through proton extrusion at the expense of ATP hydrolysis. Interestingly, the same gene was also induced in nodules, although to a lesser extent. Proton ATPases induced in AM symbiosis have been reported previously (Rosewarne et al., 2007) including this particular gene, which was named *Mtha1* (Krajinski et al., 2002). *Mtha1* was shown to be expressed exclusively in cortical cells containing arbuscules, where it is assumed to maintain the proton gradient across the peri-arbuscular membrane (Krajinski et al., 2002). Ultimately, this provides the energy to drive proton-coupled symport activities such as that of MtPT4. A second gene classified in this family was found induced nine-fold in mycorrhizal roots (and not expressed in nodules) and may play a complementary role.

Many eukaryotic ATP-binding Cassette Transporters (ABC, 3.A.1) function as extruders of diverse solutes, including organic acids and secondary metabolites, at the expense of ATP. We identified four ABC transporters induced >20 times in mycorrhizal roots. This classification cannot predict whether these transporters import or export solutes, or the nature of their substrates, although the expression levels indicate an important function during AM symbiosis.

Nitrogen transfer from fungi to plants, potentially as ammonium, can occur in both endo- and ectomycorrhizal symbioses (Govindarajulu et al., 2005; Chalot et al., 2006). In poplar, among 14 ammonium transporters (AMT) identified in the genome, *PtrAMT1;2* was induced in

ectomycorrhizal roots, whereas no expression was detected in mock roots (Couturier et al., 2007). Its ortholog, *PttAMT1;2* was also induced in ectomycorrhizal roots (Selle et al., 2005). However, among ten *Medicago* AMT genes, only one was found up-regulated more than 5-fold in mycorrhizal roots and this was recently shown to be expressed in cortical cells containing arbuscules (Gomez et al., 2009). *LjAMT2;2* was recently identified in *Lotus* and found to be expressed exclusively in mycorrhizal roots (Guether et al., 2009).

Surprisingly, a Voltage-dependent Anion Selective Channel (VDAC, 1.B.8.1) was induced almost 10-fold in mycorrhizal roots. VDAC porins belong to the Mitochondrial and Plastid Porin Family (MPP, 1.B.8), and have been implicated in organellar  $\text{Ca}^{2+}$ -regulated homeostasis of ATP and other small molecules (Bathori et al., 2006). Additionally, a Transient Receptor Potential  $\text{Ca}^{2+}$  Channel (TRP-CC, 1.A.4) was induced >3-fold in mycorrhizal roots. The same gene was also induced (>20 fold) in nodules. So far, this TCDB family of channels and sensors does not include any plant proteins, and its members are mostly from animals, which resulted in low confidence scores for putative members of this family during manual curation. Many members of this family have an ankyrin-repeat domain, and consequently are classified in the Ankyrin Family (8.A.28). Functional analysis of these transporters would help better resolve this classification, as well as their symbiotic roles.

Plant defensins (PD, 1.C.45) are cysteine-rich polypeptides proposed to transport small molecules, such as ions, by forming channels in the membrane (Kagan et al., 1990). Isolated proteins presented antimicrobial properties (Thomma et al., 2002; Finkina et al., 2008) and *in vivo* they may regulate microsymbiont differentiation. We noticed four mycorrhizal-induced genes classified in this family, with transcription induction ranging from 14 to 145-fold. Whether they have a defensive function, or alternatively control development of the fungal symbiont remains to be determined. With four very similar proteins, the potential for functional redundancy is high and this makes analysis of their roles in symbiosis a challenge.

Three aquaporins (Major Intrinsic Proteins, MIP, 1.A.8) were induced in mycorrhizal roots (up to 30 times) relative to non-mycorrhizal controls. Interestingly, the most up-regulated aquaporin did not show differential expression during nodulation, indicating a specific role in

mycorrhizal symbiosis, whereas other members induced in nodules did not show variation in mycorrhizal roots.

Gomez et al. (2009) reported 49 *Medicago* EST-derived probesets on the Affymetrix GeneChip of probable fungal origin. The mycorrhizal root cDNA libraries have the potential to contain transcripts from both plant and AM fungal symbionts. Five of these were classified as putative transporters (supplemental tables indicate these) and were induced between 3 and 10-fold in mycorrhizal roots (Table S5). In our study, we found evidence for an additional fungal-derived transporter belonging to the Iron/Lead Transporter Superfamily (ILT, 9.A.10) that is induced 33-fold in mycorrhizal roots (Table 4).

## **Perspectives**

Our search for transporters in *Medicago* uncovered and classified 2,673 putative transporter genes, many of which are induced during symbiosis with nitrogen-fixing rhizobia or AM fungi. Given the importance of these symbioses to plant nutrition and sustainable agriculture, it will be interesting to characterize the function of many of the symbiosis-induced transporters. Systematic analysis of transporters in *Medicago* will be aided not only by the results presented here, but also by the availability of *Tnt1* insertion lines of *Medicago* (Tadege et al., 2008).

## **MATERIALS AND METHODS**

### **Transporter identification and sequence analyses**

Predicted protein sequences of *Medicago truncatula* from IMGAG v.2 were retrieved ([ftp://ftpmips.gsf.de/plants/medicago/MT\\_2\\_0/](ftp://ftpmips.gsf.de/plants/medicago/MT_2_0/)) and analysed for the presence of transmembrane domains (TMD) using two algorithms: HMMTOP 2.0 (Tusnady and Simon, 2001) and TMPred (Hoffman and Stoffel, 1993). The resulting putative TMD proteins were analyzed further using a

more conservative TMD identification algorithm, SOSUI (Hirokawa et al., 1998). In parallel, all IMGAG v.2 predicted proteins were analyzed for sequence similarity to transporters of the TC database (TCDB, <http://www.tcdb.org/index.php>), using BLASTP with an e-value cutoff  $\leq e^{-3}$ . All sequences with two or more predicted TMD or with significant similarity to TCDB proteins were selected for manual curation.

Expressed sequence tags (tentative consensus and singlets) of the *Medicago truncatula* Gene Index v.8 (MTGI, <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago>) were retrieved and compared to TCDB proteins through tBLASTX with e-value  $\leq e^{-3}$ . Since most ESTs are not full-length cDNA sequences, TMD analysis, although performed, was not taken into account for selection. Sequences with significant similarity to TCDB proteins were selected for further analyses.

Medicago proteins predicted from genomic and cDNA (mostly EST) sequences were screened for conserved domains by Pfam (<http://pfam.sanger.ac.uk/>) and InterProScan (<http://www.ebi.ac.uk/Tools/InterProScan/>) with e-value  $\leq e^{-3}$ , and submitted to sequence similarity analysis against broader databases: the curated Swiss-Prot database (evalue  $\leq e^{-3}$ ; <http://expasy.org/sprot/>) and the Viridiplantae subset of the comprehensive NR-NCBI GenBank (evalue  $\leq e^{-3}$ ; <http://www.ncbi.nlm.nih.gov/>). Gene Ontology classification was carried out through protein homology to sub-terms of GO: 0022857 (transmembrane transporter activity; <http://www.geneontology.org/>). Additionally, TMD proteins without TCDB homologs but with annotation (IMGAG, Medicago Gene Index, Gene Ontology, Swiss-Prot or NCBI) indicating transporter functions (such as transpor\*, \*porter, carrier, channel, translocase, permease, ATPase, extrusion and exchanger) were retrieved for further analyses.

### **Curation and classification of transporters**

Selected sequences were analyzed with respect to the presence of TMD, predicted protein size, presence of typical conserved domains, annotation of best matches in comprehensive databases, and homology to classified TCDB transporters. Medicago proteins were classified according to TCDB transporter homology at the family, or subfamily level, and a confidence level was assigned to each categorization, according to a variety of evidence. Confidence level 1

indicates that all features of a protein are consistent with its membership in a particular TCDB transporter family/sub-family, while level 2 indicates some divergence from expected features. Level 3 means functionality is doubtful due to lack of key expected features (such as protein size, TMD absence or an unexpected number, or dubious TCDB homologies). Level 4 designates proteins with no TCDB homology but with some indications of transporter activity (such as conserved transporter domain, IMGAG annotation, transporter as best hit in broader databases). Level 5 indicates proteins classified into TCDB families that do not conform to our strict definition of a transporter, such as molecular chaperones and plasmodesmata proteins (potentially kinases). Transporter classification, confidence levels and additional features are provided in the Supplementary Table 1. The *Medicago truncatula* transporter classification resulting from this study has been incorporated into Medicago Gene Expression Atlas (<http://bioinfo.noble.org/gene-atlas/v2>) (Benedito et al., 2008).

### **Mapping of IMGAG v.2 predicted genes onto the Medicago Affymetrix GeneChip**

Affymetrix GeneChip Array (<http://www.affymetrix.com>) probesets comprise 11 perfect-match 25-mer antisense probes designed to hybridize to each gene transcript. Because the current Medicago GeneChip was designed, in part, on a previous version of IMGAG gene annotations, we reanalyzed probesets by mapping them onto the current IMGAG sequence release. Predicted coding sequences (CDS) derived from IMGAG v.2 were matched to probesets using ProbeMatch from the NetAffx package of Affymetrix ([https://www.affymetrix.com/analysis/netaffx/probematch/probe\\_match.affx](https://www.affymetrix.com/analysis/netaffx/probematch/probe_match.affx)). This software post-processes BLASTn results among probes and transcript sequences, and scores the alignments using the position mismatch penalty matrix [1 1 1 1 1 2 2 2 2 2 3 3 3 3 2 2 2 2 2 1 1 1 1], which corresponds to the 25 nucleotides of each probe on chip. Thus, the maximum score is 45 and a score of at least 43 implies two mismatches in the margin or one mismatch in the middle locations, but no mismatches in the central 5 positions. Each probeset on the Affymetrix Medicago GeneChip was designed to have 11 perfect-match probes (as well as 11 mismatched probes, disregarded in this analysis). We set a threshold of 8 out the 11 probes with a score of  $\geq 43$  as the minimum requirement to match a gene to a probeset. Probeset mapping information for all identified transporters is provided in Supplementary Table 2. The complete mapping (all

probesets on the Medicago GeneChip to all IMGAG v.2 genes and MTGI v.8 transcripts) can be downloaded at [http://bioinfo.noble.org/gateway/index.php?option=com\\_wrapper&Itemid=65](http://bioinfo.noble.org/gateway/index.php?option=com_wrapper&Itemid=65).

### **Expression analyses of identified Medicago transporters**

The expression of IMGAG genes and MTGI transcripts mapped onto the Affymetrix GeneChip was analyzed for all organs of mature (4-week old) plants, during nodule development, and in mycorrhizal roots. Expression data were retrieved from the Medicago Gene Atlas version 1 (<http://bioinfo.noble.org/gene-atlas/>), normalized and analyzed according to Benedito et al. (2008). Probesets of the identified transporters are shown in Supplementary Tables 4 and 5. Statistical analyses for differential expression between control roots and nodules or mycorrhizal roots, and hierarchical cluster analyses were also carried out as described by Benedito et al. (2008).

### **ACKNOWLEDGMENTS**

We thank the Samuel Roberts Noble Foundation and the Max Planck Society for supporting this work.

## LITERATURE CITED

- Amrutha RN, Sekhar PN, Varshney RK, Kishor PBK** (2007) Genome-wide analysis and identification of genes related to potassium transporter families in rice (*Oryza sativa* L.). *Plant Sci* **172**: 708-721
- Andreev I, Krylova V, Dubrovo P, Izmailov S** (2005) Passive potassium transport by symbiosomes from broad bean root nodules. *Plant Sci* **168**: 1005-1010
- Andreev IM, Dubrovo PN, Krylova VV, Izmailov SF** (1998) Calcium uptake by symbiosomes and the peribacteroid membrane vesicles isolated from yellow lupin root nodules. *J Plant Physiol* **153**: 610-614
- Andreev IM, Dubrovo PN, Krylova VV, Izmailov SF** (1999) Functional identification of ATP-driven Ca<sup>2+</sup> pump in the peribacteroid membrane of broad bean root nodules. *FEBS Letters* **447**: 49-52
- Bathori G, Csordas G, Garcia-Perez C, Davies E, Hajnoczky G** (2006) Ca<sup>2+</sup>-dependent Control of the Permeability Properties of the Mitochondrial Outer Membrane and Voltage-dependent Anion-selective Channel (VDAC). *J Biol Chem* **281**: 17347-17358
- Benedito VA, Torres-Jerez I, Murray JD, Andriankaja A, Allen S, Kakar K, Wandrey M, Verdier J, Zuber H, Ott T, Moreau S, Niebel A, Frickey T, Weiller G, He J, Dai X, Zhao PX, Tang Y, Udvardi MK** (2008) A gene expression atlas of the model legume *Medicago truncatula*. *Plant J* **55**: 504-513
- Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL** (2008) GenBank. *Nucl Acids Res* **36**: D25-30
- Bock KW, Honys D, Ward JM, Padmanaban S, Nawrocki EP, Hirschi KD, Twell D, Sze H** (2006) Integrating membrane transport with male gametophyte development and function through transcriptomics. *Plant Physiol* **140**: 1151-1168
- Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, Pilbout S, Schneider M** (2003) The SWISS-PROT protein knowledge base and its supplement TrEMBL in 2003. *Nucl Acids Res* **31**: 365-370
- Chalot M, Blaudez D, Brun A** (2006) Ammonia: as candidate for nitrogen transfer at the mycorrhizal interface. *Trends Plant Sci* **11**: 263-266
- Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK** (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant J* **39**: 487-512
- Couturier J, Montanini B, Martin F, Brun A, Blaudez D, Chalot M** (2007) The expanded family of ammonium transporters in the perennial poplar plant. *New Phytol* **174**: 137-150
- D'Apuzzo E, Rogato A, Simon-Rosin U, El-Alaoui H, Barbulova A, Betti M, Dimou M, Katinakis P, Marquez A, Marini AM, Udvardi MK, Chiurazzi M** (2004) Characterization of three functional high-affinity ammonium transporters in *Lotus japonicus* with differential transcriptional regulation and spatial expression. *Plant Physiol* **134**: 1763-1774
- de Billy F, Grosjean C, May S, Bennett M, Cullimore JV** (2001) Expression studies on AUX1-like genes in *Medicago truncatula* suggest that auxin is required at two steps in early nodule development. *Mol Plant-Microbe Interact* **14**: 267-277

- Desbrosses G, Kopka C, Ott T, Udvardi MK** (2004) *Lotus japonicus* LjKUP is induced late during nodule development and encodes a potassium transporter of the plasma membrane. *Mol Plant-Microbe Interact* **17**: 789-797
- Ding Y, Kalo P, Yendrek C, Sun J, Liang Y, Marsh JF, Harris JM, Oldroyd GE** (2008) Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. *Plant Cell* **20**: 2681-2695
- Fedorova E, Thomson R, Whitehead LF, Maudoux O, Udvardi MK, Day DA** (1999) Localization of H<sup>+</sup>-ATPases in root nodules. *Planta* **209**: 25-32
- Finkina EI, Shramova EI, Tagaev AA, Ovchinnikova TV** (2008) A novel defensin from the lentil *Lens culinaris* seeds. *Biochem Biophys Res Communications* **371**: 860-865
- Fitzpatrick KL, Tyerman SD, Kaiser BN** (2008) Molybdate transport through the plant sulfate transporter SHST1. *FEBS letters* **582**: 1508-1513
- Flemetakis E, Dimou M, Cotzur D, Efroze RC, Aivalakis G, Colebatch G, Udvardi M, Katinakis P** (2003) A sucrose transporter, LjSUT4, is up-regulated during *Lotus japonicus* nodule development. *J Exp Bot* **54**: 1789-1791
- Gomez SK, Javot H, Deewatthanawong P, Torres-Jerez I, Tang Y, Blancaflor EB, Udvardi MK, Harrison MJ** (2009) *Medicago truncatula* and *Glomus intraradices* gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. *BMC Plant Biol* **9**: 10
- Gordon AJ, Minchin FR, James CL, Komina O** (1999) Sucrose synthase in legume nodules is essential for nitrogen fixation. *Plant Physiol* **120**: 867-878
- Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bücking H, Lammers PJ, Shachar-Hill Y** (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435**: 819-823
- Graham PH, Vance CP** (2003) Legumes: importance and constraints to greater use. *Plant Physiol* **131**: 872-877
- Guenther JF, Roberts DM** (2000) Water-selective and multifunctional aquaporins from *Lotus japonicus* nodules. *Planta* **210**: 741-748
- Harrison MJ, Dewbre GR, Liu J** (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* **14**: 2413-2429
- Hirokawa T, Boon-Chieng S, Mitaku S** (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**: 378-379
- Hoffman K, Stoffel W** (1993) TMbase - A database of membrane spanning proteins segments. *Biol Chem Hoppe-Seyler* **374**: 166
- Javot H, Penmetza RV, Terzaghi N, Cook DR, Harrison MJ** (2007) A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc Natl Acad Sci U S A* **104**: 1720-1725
- Jeong J, Suh S, Guan C, Tsay Y-F, Moran N, Oh CJ, An CS, Demchenko KN, Pawlowski K, Lee Y** (2004) A nodule-specific dicarboxylate transporter from alder is a member of the peptide transporter family. *Plant Physiol* **134**: 969-978
- Kagan BL, Selsted ME, Ganz T, Lehrer RI** (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc Natl Acad Sci U S A* **87**: 210-214

- Kaiser BN, Moreau S, Castelli J, Thomson R, Lambert A, Bogliolo S, Puppo A, Day DA** (2003) The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. *Plant J* **35**: 295-304
- Koebnik R, Locher KP, Gelder Pv** (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**: 239-253
- Krajinski F, Hause B, Gianinazzi-Pearson V, Franken P** (2002) *Mth1*, a plasma membrane H<sup>+</sup>-ATPase gene from *Medicago truncatula*, shows arbuscule-specific induced expression in mycorrhizal tissue. *Plant Biol* **4**: 754-761
- Krusell L, Krause K, Ott T, Desbrosses G, Krämer U, Sato S, Nakamura Y, Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Miyamoto A, Suganuma N, Udvardi MK** (2005) The sulfate transporter SST1 is crucial for symbiotic nitrogen fixation in *Lotus japonicus* root nodules. *Plant Cell* **17**: 1625-1636
- LeVier K, Day DA, Guerinot ML** (1996) Iron uptake by symbiosomes from soybean root nodules. *Plant Physiol* **111**: 893-900
- Li H, Dai X, Zhao X** (2008) A Nearest Neighbor Approach for Automated Transporter Prediction and Categorization from Protein Sequences. *Bioinformatics* **24**: 1129-1136
- Li H, Benedito VA, Udvardi MK, Zhao PX** (2009) TransportTP: A two-phase classification approach for membrane transporter prediction and characterization. *BMC Bioinformatics* **10**: 418
- Lister R, Carrie C, Duncan O, Ho LHM, Howell KA, Murcha MW, Whelan J** (2007) Functional definition of Outer Membrane Proteins involved in preprotein import into mitochondria. *Plant Cell* **19**: 3739-3759
- Lodwig E, Poole P** (2003) Metabolism of *Rhizobium* bacteroids. *CRC Crit Rev Plant Sci* **22**: 37-78
- Mabood F, Souleimanov A, Khan W, Smith DL** (2006) Jasmonates induce Nod factor production by *Bradyrhizobium japonicum*. *Plant Physiol Biochem* **44**: 759-765
- Moreau S, Day DA, Puppo A** (1998) Ferrous iron is transported across the peribacteroid membrane of soybean nodules. *Planta* **207**: 83-87
- Moreau S, Meyer JM, Puppo A** (1995) Uptake of iron by symbiosomes and bacteroids from soybean nodules. *FEBS Letters* **361**: 225-228
- Moreau S, Thomson RM, Kaiser BN, Trevaskis B, Guerinot ML, Udvardi MK, Puppo A, Day DA** (2002) GmZIP1 encodes a symbiosis-specific zinc transporter in soybean. *J Biol Chem* **277**: 4738-4746
- Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K** (2007) A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. *Science* **315**: 101-104
- Niemietz CM, Tyerman SD** (2000) Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. *FEBS Letters* **465**: 110-114
- Obermeyer G, Tyerman SD** (2005) NH<sup>4+</sup> currents across the peribacteroid membrane of soybean. Macroscopic and microscopic properties, inhibition by Mg<sup>2+</sup>, and temperature dependence indicate a subpicoSiemens channel finely regulated by divalent cations. *Plant Physiol* **139**: 1015-1029
- Oldroyd GE, Downie JA** (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. *Ann Rev Plant Biol* **59**: 519-546

- Palmieri L, Picault N, Arrigoni R, Besin E, Palmieri F, Hodges M** (2008) Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization. *Biochem J* **410**: 621-629
- Parniske M** (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Rev Microbiol* **6**: 763-775
- Paszkowski U, Kroken S, Roux C, Briggs SP** (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proc Natl Acad Sci U S A* **99**: 13324-13329
- Péret B, Swarup R, Jansen L, Devos G, Auguy F, Collin M, Santi C, Hocher V, Franche C, Bogusz D, Bennett M, Laplaze L** (2007) Auxin influx activity is associated with Frankia infection during actinorhizal nodule formation in *Casuarina glauca*. *Plant Physiol* **144**: 1852-1862
- Picault N, Palmieri L, Pisano I, Hodges M, Palmieri F** (2002) Identification of a novel transporter for dicarboxylates and tricarboxylates in plant mitochondria: bacterial expression, reconstitution, functional characterization, and tissue distribution. *J Biol Chem* **277**: 24204-24211
- Prayitno J, Rolfe BG, Mathesius U** (2006) The Ethylene-insensitive sickle mutant of *Medicago truncatula* shows altered auxin transport regulation during nodulation. *Plant Physiol* **142**: 168-180
- Pumplin N, Harrison MJ** (2009) Live-cell imaging reveals periarbuscular membrane domains and organelle location in *Medicago truncatula* roots during arbuscular mycorrhizal symbiosis. *Plant Physiol* (*in press*)
- Roberts DM, Tyerman SD** (2002) Voltage-dependent cation channels permeable to  $\text{NH}_4^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  in the symbiosome membrane of the model legume *Lotus japonicus*. *Plant Physiol* **128**: 370-378
- Rogato A, D'Apuzzo E, Barbulova A, Omrane S, Stedel C, Simon-Rosin U, Katinakis P, Fletmetakis M, Udvardi M, Chiurazzi M** (2008) Tissue-specific down-regulation of *LjAMT1;1* compromises nodule function and enhances nodulation in *Lotus japonicus*. *Plant Mol Biol* **68**
- Ronson CW, Lyttleton P, Robertson JG** (1981)  $\text{C}_4$ -dicarboxylate transport mutants of *Rhizobium trifoli* form ineffective nodules on *Trifolium repens*. *Proc Natl Acad Sci U S A* **78**: 4284-4288
- Rosendahl L, Glenn AR, Dilworth MJ** (1991) Organic and inorganic inputs into legume root nodule nitrogen fixation. In MJ Dilworth, AR Glenn, eds, *Biology and Biochemistry of Nitrogen Fixation.*, pp 259-291
- Rosewarne GM, Smith FA, Schachtman DP, Smith SE** (2007) Localization of proton-ATPase genes expressed in arbuscular mycorrhizal tomato plants. *Mycorrhiza* **17**: 249-258
- Saier MJ, Tran C, Barabote R** (2006) TCDB: the Transporter Classification Database for membrane transport protein analyses and information. *Nucl Acids Res* **1**: D181-186
- Sato S, Nakamura Y, Asamizu E, Isobe S, Tabata S** (2007) Genome sequencing and genome resources in model legumes. *Plant Physiol* **144**: 588-593
- Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, Fujishiro T, Katoh M, Kohara M, Kishida Y, Minami C,**

- Nakayama S, Nakazaki N, Shimizu Y, Shinpo S, Takahashi C, Wada T, Yamada M, Ohmido N, Hayashi M, Fukui K, Baba T, Nakamichi T, Mori H, Tabata S** (2008) Genome structure of the legume, *Lotus japonicus*. *DNA Res* **15**: 227-239
- Simon-Rosin U, Wood C, Udvardi MK** (2003) Molecular and cellular characterisation of *LjAMT2;1*, an ammonium transporter from the model legume *Lotus japonicus*. *Plant Mol Biol* **51**: 99-108
- Smith SE, Read DJ** (2008) Mycorrhizal Symbiosis. Academic Press, Inc., San Diego, CA
- Suganuma N, Yamamoto Y** (1987) Respiratory metabolism of mitochondria in soybean root nodules. *Soil Sci Plant Nutr* **33**: 93-101
- Tadege M, Ratet P, Mysore KS** (2005) Insertional mutagenesis: a Swiss army knife for functional genomics of *Medicago truncatula*. *Trend Plant Sci* **10**: 229-235
- Tadege M, Wen JQ, He J, Tu HD, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX, Chabaud M, Ratet P, Mysore KS** (2008) Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. *Plant J* **54**: 335-347
- Tchieu JH, Fana F, Fink JL, Harper J, Nair TM, Niedner RH, Smith DW, Steube K, Tam TM, Veretnik S, Wang D, Gribskov M** (2003) The PlantsP and PlantsT Functional Genomics Databases. *Nucl Acids Res* **31**: 342-344
- Tejada-Jimenez M, Llamas A, Sanz-Luque E, Galvan A, Fernandez E** (2007) A high-affinity molybdate transporter in eukaryotes. *Proc Natl Acad Sci* **104**: 20126-20130
- Temple SJ, Vance CP, Gantt JS** (1998) Glutamate synthase and nitrogen assimilation. *Trend Plant Sci* **3**: 51-56
- Thomma BP, Cammue BP, Thevissen K** (2002) Plant defensins. *Planta* **216**: 193-202
- Tomatsu H, Takano J, Takahashi H, Watanabe-Takahashi A, Shibagaki N, Fujiwara T** (2007) An *Arabidopsis thaliana* high-affinity molybdate transporter required for efficient uptake of molybdate from soil. *Proc Natl Acad Sci U S A* **104**: 18807-18812
- Tusnady GE, Simon I** (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**: 849-850
- Tyerman SD, Whitehead LF, Day DA** (1995) A channel-like transporter for  $\text{NH}_4^+$  on the symbiotic interface of  $\text{N}_2$ -fixing plants. *Nature* **378**: 629-632
- Udvardi MK, Day DA** (1989) Electrogenic ATPase activity on the peribacteroid membrane of soybean (*Glycine max* L.) root nodules. *Plant Physiol* **90**: 982-987
- Udvardi MK, Day DA** (1997) Metabolite transport across symbiotic membranes of legume nodules. *Ann Rev Plant Physiol Plant Mol Biol* **48**: 493-523
- Udvardi MK, Lister DL, Day DA** (1991) ATPase activity and anion transport across the peribacteroid membrane of isolated soybean symbiosomes. *Arch Microbiol* **156**: 362-366
- Udvardi MK, Price GD, Gresshoff PM, Day DA** (1988) A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. *FEBS Letters* **231**: 36-40
- Valavanis IK, Bagos PG, Emiris IZ** (2006)  $\beta$ -barrel transmembrane proteins: Geometric modelling, detection of transmembrane region, and structural properties. *Comput Biol Chem* **30**: 416-424
- Vance CP, Miller SS, Driscoll BT, Robinson DL, Trepp G, Gantt JS, Samas DA** (1997) Nodule carbon metabolism: organic acids for  $\text{N}_2$  fixation. In CE Elmerich, A Kondorosi, WE Newton, eds, *Biological Nitrogen Fixation for the 21st Century*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 443-448

- Vincill ED, Szczyglowski K, Roberts DM** (2005) GmN70 and LjN70. Anion transporters of the symbiosome membrane of nodules with a transport preference for nitrate. *Plant Physiol* **137**: 1435-1444
- Wasson AP, Pellerone FI, Mathesius U** (2006) Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. *Plant Cell* **18**: 1617-1629
- Werner D, Mörschel E** (1978) Differentiation of nodules of *Glycine max*. *Planta* **141**: 169-177
- Wienkoop S, Saalbach G** (2003) Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. *Plant Physiol* **131**: 1080-1090
- Young ND, Cannon SB, Sato S, Kim D, Cook DR, Town CD, Roe BA, Tabata S** (2005) Sequencing the genespaces of *Medicago truncatula* and *Lotus japonicus*. *Plant Physiol* **137**: 1174-1181
- Young ND, Mudge J, Ellis THN** (2003) Legume genomes: more than peas in a pod. *Curr Opin Plant Biol* **6**: 199-204

**Table 1.** Genomic analysis of *Medicago truncatula* membrane transporters.

	IMGAG <sup>a</sup> v.2	MTGI <sup>b</sup> v.8	Total <sup>c</sup>
number of sequences	38,335	36,850	60,823
proteins with TMD <sup>d</sup>	18,684	-	-
proteins with TCDB homologues	2,039	2,051	3,830
classified transporters	1,681	1,759	2,673

<sup>a</sup> IMGAG: International Medicago Genome Annotation Group  
(<http://www.medicago.org/genome/downloads/Mt2/>);

<sup>b</sup> MTGI: *Medicago truncatula* Gene Index (<http://compbio.dfci.harvard.edu/tgi/>).

<sup>c</sup> non-redundant number of genes.

<sup>d</sup> proteins with transmembrane domains identified by at least one algorithm.

**Table 2.** Membrane transporter classification according to TCDB classes and mapping onto the Affymetrix Medicago GeneChip.

Transporter Class	IMGAG v.2	MTGI v.8	Total <sup>a</sup>	probesets
Class 1. Channels and pores	223	244	365	379
Class 2. Secondary transporters	755	756	1181	1132
Class 3. Primary active transporters	452	536	791	734
Class 8. Accessory factors	35	20	47	41
Class 9. Incompletely characterized	213	203	289	318
Total (confidence levels 1-3)	1,681	1,759	2673	2,611
Not classified (level 4) <sup>b</sup>	389	-	389	275
Excluded from analysis (level 5) <sup>c</sup>	514	292	768	715

<sup>a</sup> non-redundant number of genes.

<sup>b</sup> Genes with no significant TCDB homology but with indications of transport function.

<sup>c</sup> TCDB families that were not considered further or potentially false positives (see discussion).

**Table 3.** The 30 most abundant membrane transporter families in *Medicago truncatula*<sup>a</sup>.

TCDB number	Family name (acronym)	IMGAG genes	MTGI transcripts	non-redundant sequences	Affymetrix probesets
1.A.1	Voltage-gated Ion Channel (VIC) Superfamily	30	13	33	23
1.A.4	Transient Receptor Potential Ca <sup>2+</sup> Channel (TRP-CC)	73	65	116	113
1.A.8	Major Intrinsic Protein (MIP)	32	53	64	72
1.A.20	gp91phox Phagocyte NADPH Oxidase-associated Cytochrome b558 H <sup>+</sup> channel (CytB)	15	34	34	43
1.A.31	Annexin (Annexin)	19	14	25	22
1.C.45	Plant Defensin (PD)	9	14	21	20
2.A.1	Major Facilitator Superfamily (MFS)	110	116	175	163
2.A.3	Amino Acid-Polyamine-Organocation (APC)	8	27	29	33
2.A.5	Zinc (Zn <sup>2+</sup> )-Iron (Fe <sup>2+</sup> ) Permease (ZIP)	11	15	19	23
2.A.6	Resistance-Nodulation-Cell Division (RND) Superfamily	13	18	23	27
2.A.7	Drug/Metabolite Transporter (DMT) Superfamily	119	93	168	159
2.A.17	H <sup>+</sup> -dependent Oligopeptide Transporter (POT)	71	79	111	101
2.A.18	Amino Acid/Auxin Permease (AAP)	57	61	96	87
2.A.19	Ca <sup>2+</sup> :Cation Antiporter (CaCA)	11	14	18	26
2.A.29	Mitochondrial Carrier (MC)	91	86	134	119
2.A.37	Monovalent Cation-Proton Antiporter-2 (CPA2)	29	6	30	29
2.A.40	Nucleobase:Cation Symporter-2 (NCS2)	10	14	19	18
2.A.49	Chloride Channel (CIC)	26	14	32	38
2.A.53	Sulfate Permease (SulP)	12	29	33	34
2.A.66	Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily	53	48	73	79
2.A.67	Oligopeptide Transporter (OPT)	23	17	30	26
2.A.69	Auxin Efflux Carrier (AEC)	22	9	27	23
2.A.72	K <sup>+</sup> Uptake Permease (KUP)	15	22	30	25
3.A.1	ATP-binding Cassette (ABC) Superfamily	160	160	254	219
3.A.2	H <sup>+</sup> /Na <sup>+</sup> -translocating F-, V- and A-type ATPase (F-ATPase) Superfamily	34	49	64	77
3.A.3	P-type ATPase (P-ATPase) Superfamily	37	71	85	84
3.A.5	General Secretory Pathway (Sec)	63	82	108	114
3.A.8	Mitochondrial Protein Translocase (MPT)	11	26	30	34
3.A.9	Chloroplast Envelope Protein Translocase (CEPT or Tic-Toc)	40	51	73	75
3.A.16	Endoplasmic Reticular Retrotranslocon (ER-RT)	72	67	109	114

<sup>a</sup> The complete list is given in Table S2.

**Table 4.** Membrane transporters induced in nodules in comparison to non-nodulating roots (>50 FC)<sup>a</sup>.

probesets	family	cert	Superfamily/Family name	IMGAG locus	MTGI	Nod0 <sup>b</sup>	Nod4	Nod10	Nod14	Root <sup>c</sup>	Nod28	max ratio
Mtr.2246.1.S1_at	1.A.8	1	Major Intrinsic Protein (MIP)		BG582951	13	103	1830	1866	13	529	149
Mtr.37525.1.S1_at	1.A.8	1	Major Intrinsic Protein (MIP)	AC168148_28.4	TC100851	175	1701	16032	14866	187	9459	92
Mtr.32104.1.S1_s_at	1.A.20	3	gp91phox Phagocyte NADPH Oxidase-associated Cytochrome b558 (CytB) H+ channel	AC161241_8.5	AW329243 TC105131	12	28	1447	1051	276	379	121
Mtr.39812.1.S1_s_at	1.A.20	3	gp91phox Phagocyte NADPH Oxidase-associated Cytochrome b558 (CytB) H+ channel	AC161241_8.5	TC105783	26	39	2028	1966	275	519	78
Mtr.52057.1.S1_at	1.A.20	3	gp91phox Phagocyte NADPH Oxidase-associated Cytochrome b558 (CytB) H+ channel	AC161241_18.5		17	24	1476	1341	163	368	85
Mtr.9266.1.S1_at	1.C.45	1	Plant Defensin (PD)	CT963132_15.4	TC102850	14	16	4896	3661	13	2971	340
Mtr.433.1.S1_at	2.A.1	1	Major Facilitator Superfamily (MFS)	AC137838_17.4	TC107287	131	1424	7902	7662	180	6272	60
Mtr.21610.1.S1_at	2.A.1	1	Major Facilitator Superfamily (MFS)	AC147178_32.4		10	10	2868	1753	10	928	277
Mtr.37205.1.S1_at	2.A.1	1	Major Facilitator Superfamily (MFS)	AC167034_32.4	TC100124	11	133	4620	2789	11	3197	404
Mtr.4676.1.S1_at	2.A.3	1	Amino Acid-Polyamine-Organocation (APC)	AC154036_21.4	AL375006	10	2288	4790	4052	11	3099	457
Mtr.42325.1.S1_at	2.A.3	1	Amino Acid-Polyamine-Organocation (APC)	AC154036_21.4	TC111233	14	1203	2952	2168	15	2149	205
Mtr.12845.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily		TC96179	8	1080	881	965	8	610	137
Mtr.28141.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily		BG583743	15	41	1173	760	15	458	79
Mtr.28851.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily	AC150779_27.4	BQ124404	26	2004	597	1225	46	2684	59
Mtr.37389.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily		TC100560	21	1202	1950	1712	35	2498	71
Mtr.40650.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily		TC107753	15	133	9430	8067	18	7357	629
Mtr.41092.1.S1_at	2.A.7	1	Drug/Metabolite Transporter (DMT) Superfamily		TC108704	8	712	2387	1667	9	2106	291
Mtr.27727.1.S1_at	2.A.17	1	H+ -dependent Oligopeptide Transporter (POT)	AC149471_42.5	BE997589	12	124	439	332	13	724	58
Mtr.31737.1.S1_at	2.A.17	1	H+ -dependent Oligopeptide Transporter (POT)	AC175049_14.4	AL377677	8	766	1866	1650	11	1450	225
Mtr.50491.1.S1_at	2.A.18	1	Amino Acid/Auxin Permease (AAP)	AC140027_4.5		20	1039	401	808	16	1472	95
Mtr.1587.1.S1_at	2.A.29	3	Mitochondrial Carrier (MC)		AW573662	9	405	1792	1259	19	1200	194
Mtr.43719.1.S1_at	2.A.29	3	Mitochondrial Carrier (MC)	CT573353_28.4	TC95911	9	9	1359	1070	9	689	154
Mtr.19388.1.S1_at	2.A.49	1	Chloride Channel (ClC)	AC140549_27.5		14	14	866	403	12	83	63
Mtr.37708.1.S1_at	2.A.53	1	Sulfate Permease (SulP)	AC139842_4.5	TC101252	12	730	8943	6309	237	4474	776
Mtr.49248.1.S1_at	2.A.53	1	Sulfate Permease (SulP)		TC100150 TC100173	36	14336	8140	8895	35	10186	397

Mtr.27423.1.S1_at	<b>2.A.66</b>	1	Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily		AW980583	20	280	423	651	20	1801	89
Mtr.28858.1.S1_at	<b>2.A.66</b>	1	Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily	AC165438_46.5	BQ124842	14	1586	219	575	16	1783	108
Mtr.49406.1.S1_at	<b>2.A.66</b>	1	Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase Superfamily	AC144375_31.4		10	32	3064	2463	12	1947	303
Mtr.49182.1.S1_x_at	<b>2.A.67</b>	1	Oligopeptide Transporter (OPT)	AC143341_26.4		21	1149	430	649	17	1061	55
Mtr.20006.1.S1_at	<b>2.A.85</b>	1	Aromatic Acid Exporter (ArAE)	AC151522_26.4		12	22	828	1162	11	972	95
Mtr.49882.1.S1_at	<b>3.A.1</b>	1	ATP-binding Cassette (ABC) Superfamily	AC146548_55.5		21	85	428	625	15	1191	81
Mtr.3724.1.S1_at	<b>3.A.2</b>	1	H <sup>+</sup> - or Na <sup>+</sup> translocating F <sub>0</sub> F <sub>1</sub> -V- and A-type ATPase (F <sub>0</sub> F <sub>1</sub> -ATPase) Superfamily		BG583840	14	31	5652	4130	14	1608	403
Mtr.42937.1.S1_s_at	<b>3.A.2</b>	2	H <sup>+</sup> - or Na <sup>+</sup> translocating F <sub>0</sub> F <sub>1</sub> -V- and A-type ATPase (F <sub>0</sub> F <sub>1</sub> -ATPase) Superfamily		TC93945 TC94148	17	40	7945	7575	20	8828	451
Mtr.42017.1.S1_at	<b>3.A.3</b>	1	P-type ATPase (P-ATPase) Superfamily		TC110571	13	12	2003	1390	10	1427	152
Mtr.40309.1.S1_s_at	<b>3.A.8</b>	3	Mitochondrial Protein Translocase (MPT)	CT573353_23.4	TC98311 TC107014 TC100596	14	7079	10753	11381	69	10337	803
Mtr.9479.1.S1_at	<b>9.B.24</b>	3	Testis-Enhanced Gene Transfer (TEGT)		TC103475	20	245	2814	1845	22	2077	139
Mtr.636.1.S1_at	<b>unclass</b>	4	(Not defined)	AC148995_45.5		15	13	741	470	11	546	51

<sup>a</sup> Data was retrieved from the Medicago Gene Expression Atlas (Benedito et al., 2008).

<sup>b</sup> Days after rhizobia inoculation (Nod0-Nod14 comprise a time-course of nodule development, numbers indicate days after inoculation of rhizobia).

<sup>c</sup> Control for the Nod28 sample (mature nodule belonging to the organ series).

**Table 5.** Membrane transporters induced in mycorrhizal roots in comparison to control roots (>10 FC)<sup>a</sup>.

probesets	family	certainty	Superfamily/Family name	IMGAG locus	MTGI	myc <sup>-b</sup>	myc <sup>+</sup>	ratio
Mtr.7596.1.S1_at	<b>1.A.8.</b>	1	Major Intrinsic Protein (MIP)	AC174468_10.4	AJ499650	11	318	30
Mtr.37525.1.S1_at	<b>1.A.8</b>	1	Major Intrinsic Protein (MIP) - MtNIP I	AC168148_28.4	TC100851	93 <sup>c</sup>	1822	20
Mtr.7210.1.S1_at	<b>1.C.45</b>	1	Plant Defensin (PD)		TC101060	13 <sup>c</sup>	1863	146
Mtr.31214.1.S1_s_at	<b>1.C.45</b>	1	Plant Defensin (PD)		AL385826 AJ499425	8	164	20
Mtr.35854.1.S1_at	<b>1.C.45</b>	1	Plant Defensin (PD)		TC98064	12	495	40
Mtr.35484.1.S1_at	<b>1.C.45</b>	1	Plant Defensin (PD)		TC104515	10	147	14
Mtr.43062.1.S1_at	<b>2.A.1.9</b>	1	Phosphate:H <sup>+</sup> Symporter Family (PHS) - MtPT4		TC94453	14	1902	136
Mtr.36985.1.S1_at	<b>2.A.17</b>	1	H <sup>+</sup> -dependent Oligopeptide Transporter (POT)	AC140547_5.4	TC105446	7	490	68
Mtr.36985.1.S1_s_at	<b>2.A.17</b>	1	H <sup>+</sup> -dependent Oligopeptide Transporter (POT)	AC140547_5.4	TC105446	13	531	40
Mtr.50194.1.S1_at	<b>2.A.17</b>	1	H <sup>+</sup> -dependent Oligopeptide Transporter (POT)	AC140547_5.4		11	516	45
Mtr.39705.1.S1_at	<b>2.A.29</b>	1	Mitochondrial Carrier (MC)		TC105531	11	454	42
Mtr.1103.1.S1_at	<b>3.A.1</b>	1	ATP-binding Cassette (ABC) Superfamily		AC141435_35.5	10	214	22

Mtr.44070.1.S1_at	<b>3.A.1</b>	<b>1</b>	ATP-binding Cassette (ABC) Superfamily	AC202319_10.4	TC96634	20	544	28
Mtr.46524.1.S1_at	<b>3.A.1</b>	<b>1</b>	ATP-binding Cassette (ABC) Superfamily		AC152057_1.5	21	888	42
Mtr.51195.1.S1_at	<b>3.A.1</b>	<b>1</b>	ATP-binding Cassette (ABC) Superfamily	AC126010_13.4		10	249	24
Mtr.43470.1.S1_at	<b>3.A.3</b>	<b>1</b>	P-type ATPase (P-ATPase) Superfamily		TC95400	13	610	48
Mtr.31910.1.S1_at	<b>3.A.16</b>	<b>2</b>	Endoplasmic Reticular Retrotranslocon (ER-RT)		AL385223	9	99	11
Mtr.4771.1.S1_at <sup>d</sup>	<b>9.A.10</b>	<b>1</b>	Iron/Lead Transporter (ILT) Superfamily		AL382013	11	369	33
Mtr.37110.1.S1_at	<b>9.A.12</b>	<b>1</b>	Copper Transporter (Ctr)		TC97522	12 <sup>c</sup>	552	47

<sup>a</sup> Data was retrieved from Gomez et al. (2009) and is publicly available in the Medicago Gene Expression Atlas v. 2 (<http://bioinfo.noble.org/gene-atlas/v2/>).

<sup>b</sup> Expression level in control roots (*myc*<sup>-</sup>) and mycorrhizal roots (*myc*<sup>+</sup>) as mean values of three biological replicates of Affymetrix chip expression.

<sup>c</sup> Expression induction in root cells with arbuscules was confirmed by RT-PCR (Gomez et al., 2009).

<sup>d</sup> This transcript is probably derived from AM fungal RNA. cDNA libraries generated from mycorrhizal roots contain transcripts from both plant and fungal symbionts.

## **SUPPLEMENTARY MATERIAL:**

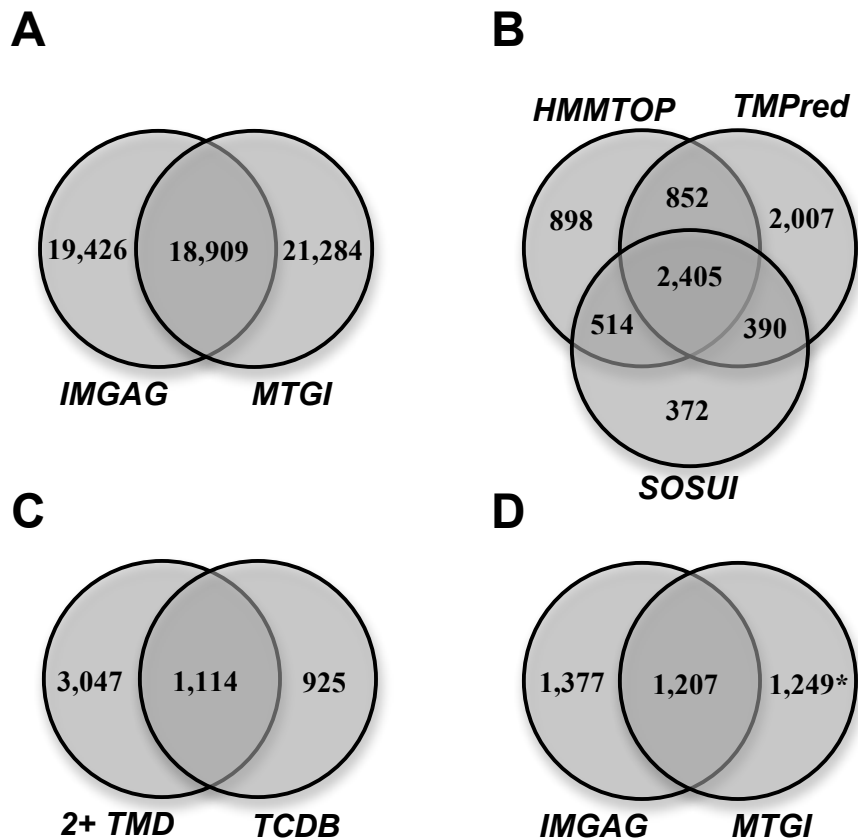
**Table S1.** Classification of *Medicago truncatula* membrane transporters.

**Table S2.** Number of members of each *Medicago* membrane transporter family.

**Table S3.** Correspondence of Affymetrix *Medicago* Gene Chip membrane transporter probesets to IMGAG v.2 coding sequences and MTGI transcripts.

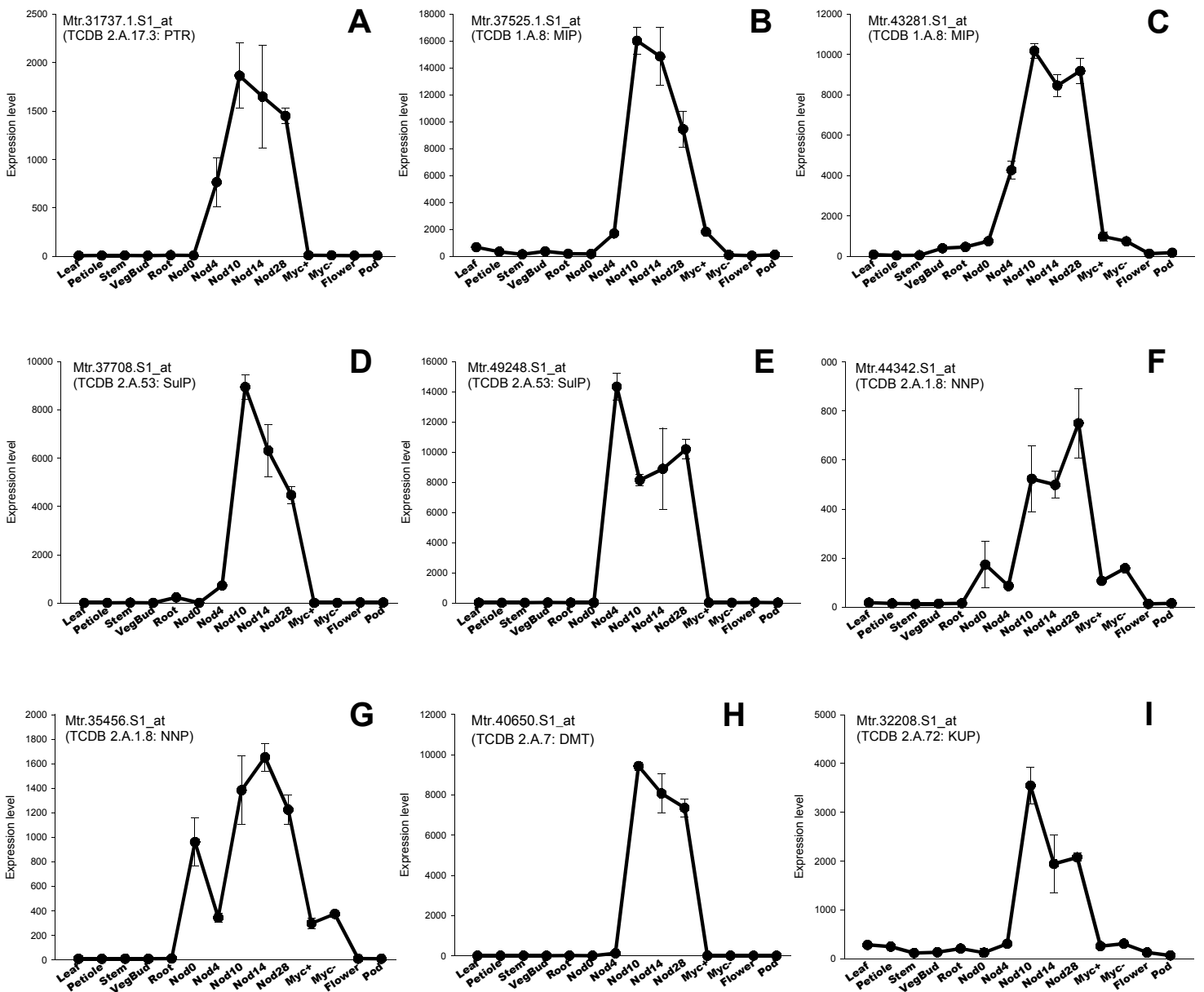
**Table S4.** Expression profile of each identified *Medicago truncatula* membrane transporters across vegetative and reproductive organs.

**Table S5.** Differential gene expression and statistical analyses of *Medicago truncatula* membrane transporters during nodule development and in mycorrhizal roots. Nod0-Nod14 comprise a time-course of nodule development, numbers indicate days after inoculation of rhizobia. The sample Root is a non-inoculated organ that serves as a control for the Nod28, a mature organ sample harvested along with the mature vegetative and reproductive organs (Benedito et al., 2008). Myc<sup>-</sup> is a non-inoculated root control sample to the mycorrhizal root sample myc<sup>+</sup> (Gomez et al., 2009).



**Figure 1.** Genomic analysis of *Medicago truncatula* membrane transporters. **(A)** Number of genome-predicted genes (IMGAG v2) and transcripts (MTGI), and overlap between databases. **(B)** Number of IMGAG-predicted proteins with at least two transmembrane domains (2+ TMD) according to different algorithms. **(C)** Overlap between the 2,039 IMGAG-predicted proteins with significant similarity (e-value < e-3) to TCDB members and the 4,161 proteins with 2+ TMD predicted by at least two topology algorithms. **(D)** Number of identified membrane transporters derived from IMGAG v. 2.0 or MTGI v. 8 databases.

\*Note that the total number of MTGI sequences is higher than shown in Table 1 due to gene redundancy or multiple probeset mapping.



**Figure 2.** Nodule-enhanced and nodule-specific membrane transporters in *Medicago truncatula*. Gene expression represents signal strength of *Medicago* GeneChip identifiers from the *Medicago* Gene Expression Atlas (Benedito et al., 2008) and Gomez et al. (2009). Samples for the mature organ series were taken from non-inoculated 28-day old plants (except nodules) growing at optimal conditions (Benedito et al., 2008). For nodule samples (Nod), numbers indicate days post-inoculation with rhizobia. Myc indicate root samples with (+) or without (-) mycorrhizal association. Bars indicate standard error of three biological replicates. Affymetrix probesets and their respective Transporter Classification (TC; Saier et al., 2006) numbers and family acronyms are shown for each transcript. **(A)** Member of the H<sup>+</sup>-dependent Oligopeptide Transporter Family (POT/PTR). **(B)** and **(C)** Members of the Major Intrinsic Protein family (MIP, aquaporins). **(D)** and **(E)** members of the Sulfate Permease Family (SulP/SULTR). **(F)** and **(G)** Members of the Nitrate/Nitrite Porter family (NNP), which belong to the Major Facilitator Superfamily (MFS). **(H)** Member of the Drug/Metabolite Transporter Superfamily. **(I)** A member of the K<sup>+</sup> Uptake Permease (KUP) family, homolog to LjKUP1 from *Lotus japonicus*.