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**ANALYSIS OF MEMBRANE TRANSPORTER SYSTEMS EXPRESSED DURING
SYMBIOTIC NITROGEN FIXATION IN THE MODEL LEGUME
*MEDICAGO TRUNCATULA***

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Dissertation submitted to
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Genetics and Developmental Biology

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Keywords: legume, *Medicago truncatula*, transporter, amino acid, copper

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ABSTRACT

ANALYSIS OF MEMBRANE TRANSPORTER SYSTEMS EXPRESSED DURING SYMBIOTIC NITROGEN FIXATION IN THE MODEL LEGUME *MEDICAGO TRUNCATULA*

Christina Laureen Wyman

As the second largest crop family, legumes provide a significant component to the human diet as well as livestock nutrition through forage species. Unique from most other plants groups, legumes are able to form a symbiosis with naturally occurring soil rhizobia, diazotrophs, which are able to reduce atmospheric nitrogen to usable forms of nitrogen for the plant. In return for providing reduced nitrogen, differentiated rhizobia are sustained by dicarboxylates, branched chain amino acids, and other nutrients from the plant. In order for the movement of nutrients to be translocated between symbionts and from the cytosol to cell organelles, other cells, and plant tissues, membrane transporters must be present to move substrates across lipid bilayers. Secondary transporters supply the necessary amino acids and additional nutrients to and from the nitrogen fixation zone of nodules to support symbiotic nitrogen fixation. While the biosynthesis pathways of amino acids to feed symbiosis is well understood, the involved transporters, including importers and exporters, of amino acids, as well as transporters of other nutrients to support infected cells are largely unknown and relatively few transporters have been functionally characterized in legumes. In order to assess potential amino acid transporters, I performed a genomic inventory of putative transporters belonging to the Amino Acid Polyamine-Organocation superfamily by compiling a list of transporters classified under the superfamily in the Transporter Classification Database, along with their protein size, transmembrane domains (TMDs), and compared them to characterized members of transporter families to determine confidence of functionality. I also compiled expression data to determine expression patterns to manually annotate candidate nodule specific transporters of interest for further study. Using the model species *Medicago truncatula* to study symbiotic nitrogen fixation, I genetically characterized the nodule specific membrane transporter, *MtAPC1* through *in silico* analysis of expression patterns, followed by transcriptional analysis of inoculation with rhizobial mutants to determine essential steps of infection necessary for gene expression. Transcriptional activity of *MtAPC1* was also fine-tuned to identify cell-type

within the nodule and the impact of the MtAPC1 knockdown in nodule development during symbiotic nitrogen fixation. I also characterized expression patterns of a copper transporter, MtCOPT1, regarding its transcriptional activity in nodules and its capability of importing copper into the cell cytosol. In addition, I preliminarily analyzed of two putative nodule-specific transporters, MtSEN1 and MtOPT1 for iron and short oligopeptides (3-8 amino acids in length) though *in silico* analysis with publicly available datasets, as well as determined expression patterns by RT-qPCR of nodulating root inoculated with rhizobial mutants and wild-type rhizobia.

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Chapter 1

OVERVIEW AND OBJECTIVES

Membrane transporters are ubiquitous in all biological membranes to translocate substrates essential to cell function. In legumes, during symbiotic nitrogen fixation, the nutritional flux between plant organs and rhizobia are sustained by substrate specific membrane transporters. The biosynthesis pathways of amino acids and other substrates feeding symbiosis are well understood, but the importers and exporters for amino acids, as well as some essential substrates of symbiotic nitrogen remain chiefly unknown. Given the central role of transporting substrates to bacteroids and also the return of nutrients to the plant cytosol, across plasma membranes to other cells, and the export of substrates to the xylem and sink tissues, our current lack of knowledge involving transporters needs to be addressed. Functional characterization of proteins involved in transport is required to fully understand the involved genes and essential components to support the dynamic symbiotic nitrogen fixation process.

The purpose of my dissertation work was to reanalyze putative transporters from the *Medicago truncatula* proteome and classify confidence levels of functionality based on transporter classification families (TCDB.org; Saier et al., 2016), as well as SwissProt annotation (Boeckmann et al., 2003), Gene Ontology (GO) classification (Ashburner et al., 2000; The Gene Ontology Consortium, 2017), and expected Pfam domains (Finn et al., 2016), as described in Benedito et al. (2010). This reannotation was performed to identify transporters of interest in the study of symbiotic nitrogen fixation and the Amino Acid Polyamine-Organocation (APC) Superfamily of transporters. In addition, this work was carried out to functionally characterize nodule specific transporters involved in the nitrogen fixation process to provide information about expression patterns, transcriptional activity, and impacts of gene knockdown of transporter proteins.

Specific Aim 1 - Genomic inventory of putative transporters belonging to the APC superfamily. In Chapter 3, I compiled a list of transporters classified under the APC Superfamily in the Transporter Classification Database and compared their protein size and TMDs to characterized members of transporter families to determine confidence of functionality. I also

compiled expression data to determine expression patterns to manually annotate candidate nodule specific transporters of interest for further study.

Specific Aim 2 – Genetic characterization of the nodule-specific MtAPC1 (*Medtr8g089360.1*) transporter during symbiotic nitrogen fixation. In Chapter 4, I further characterized a nodule-specific APC transporter by *in silico* analysis of expression patterns, followed by transcriptional analysis of inoculation with rhizobial mutants to determine essential steps of infection necessary for gene expression. I also fine-tuned the MtAPC1 transcriptional activity to cell-type within the nodule and determined the impact of the MtAPC1 knockdown in nodule development during symbiotic nitrogen fixation.

Specific Aim 3 - Functionally characterization of the nodule-specific MtCOPT1 (*Medtr4g019870.1*) transporter. In Chapter 5, I characterized expression patterns of a copper transporter regarding its transcriptional activity in nodules and its capability of importing copper into the cell cytosol.

Specific Aim 4 - Objective: Preliminarily analyses of two nodule-specific transporters, MtOPT1 (*Medtr7g092230.1*) and MtSEN1 (*Medtr4g094335.1*). In Chapter 6, I carried out analyses of gene expression for two nodule-specific putative transporters for iron and short oligopeptides (3-8 amino acids in length) through *in silico* analysis with publicly available datasets, as well as determined expression patterns by RT-qPCR of nodulating root inoculated with rhizobial mutants and wild-type rhizobia. This work will serve as a launchpad to further functional characterization of these transporters in order to find their roles to sustain symbiotic nitrogen fixation.

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Chapter 2

Symbiotic nitrogen fixation in legume crops are supported by membrane transporters enabling nutritional flux

Abstract

Worldwide, legumes provide a significant component of human diets and are essential to the industrial livestock production. Legumes (Fabaceae) are the second largest crop family, after cereals and the third botanical family, after Asteraceae and Orchidaceae, with over 20,000 species including the common bean, chickpea, soybean, peanuts and forage species. Unique from most other plants, many legume species can utilize atmospheric nitrogen, thus reducing the amount of synthetically-fixed nitrogen fertilizers applied to agricultural systems. The reduction of atmospheric nitrogen into usable forms to the plant is attained through a species-specific symbiosis with naturally occurring soil rhizobia in specialized root structures called nodules. The nutritional flux between rhizobia and plant organs is sustained by membrane transporters with affinity to specific molecules. The transporters that provide movement of nutrients are essential to the symbiotic nitrogen fixation process and the main focus of interest to our lab. In this chapter, I introduce legume biology and the importance of legumes for sustainable agriculture, make a case for using *Medicago truncatula* as a model system, explain why membrane transporters are central for symbiotic nitrogen fixation in the legume-rhizobial system, and present the outstanding scientific questions in the field.

INTRODUCTION

Importance of legumes and symbiotic nitrogen fixation to agriculture

Throughout the world, legumes (Fabaceae) encompass an important component of the human diet and account for approximately one third of the world's primary crop production (Smil, 1999; Vance et al., 2003). Legumes are the second largest agricultural family in the world, only behind cereals, providing both oils and protein to humans and animals. One hundred eighty million hectares of legumes are grown yearly, encompassing 12-15% of the Earth's arable surface (Graham and Vance, 2003; Reeves et al., 2016), which is larger than the total area of Alaska. Some well-known legumes include pulses (edible, dry beans) and other grains, such as common beans, soybean, peas, lentils, and peanuts, as well as important forage species, such as alfalfa, clover, vetch and stylo. In the United States, over 33 million hectares of soybean were grown in 2016, accounting for the area of New Mexico, the fifth largest state (<http://www.fao.org/faostat/en/#data/QC>). Indeed, legumes have been called the lynch pin of sustainable agriculture because of their ability to fix nitrogen when in symbiotic association with rhizobial bacteria (Udvardi et al., 2005). Being the third botanical family, with almost 20,000 species, legumes are found natively worldwide and three of them are used as biological models: *Medicago truncatula* (**Figure 1**), *Lotus japonicus*, and soybean (*Glycine max*).

A

B

Figure 2.1. *Medicago truncatula*. (A) Trifoliate leaf. Trichomes are visible on the leaves, petiole, and stem. (B) Twenty one day old plants growing in surface and vermiculite substrate.

Nitrogen and phosphorous are largely the most limiting mineral nutrients for plant growth in terrestrial ecosystems (Agren et al., 2012). Nitrogen fertilizer is produced for crop cultivation by the Haber-Bosch process, the main industrial process to generate ammonia. This process utilizes high pressure and temperature in order to convert atmospheric nitrogen and hydrogen into ammonia (Erisman et al., 2008). For each ton of ammonia produced in North America for agricultural purposes, 45.5 MJ of energy is used and produces 2.55 tons of carbon dioxide gas (Kool et al., 2012; Bicer et al., 2017). In 2016, whereas the United States used over 12 million tons of nitrogen fertilizer for agricultural use, the country produced less than 9 million tons (<http://www.fao.org/faostat/en/#data/RFN>).

The level of profit and sustainability of an agricultural system can increase as the use of applied fertilizers is reduced by relying mostly or exclusively on symbiotically fixed nitrogen. An estimated 40–60 million tons of nitrogen are fixed annually by cultivated legumes (Postgate, 1982; Smil, 1999). This accounts for an annual savings of about \$10 billion on nitrogen fertilizer produced by the Haber-Bosch process (Graham and Vance, 2003; Herridge et al., 2008). If given all of the necessary tools to foster a symbiotic relationship, this could reduce the necessity of applied fertilizer which averages 77.5 kg/hectare of agricultural land (<http://www.fao.org/faostat/en/#data/RFN>).

Together, the biological processes of lightening and symbiotic nitrogen fixation account for approximately 60 percent of the Earth's fixed nitrogen (Zahran, 1999). Fixed nitrogen in the biosphere is rapidly turned over and lost to the atmosphere as gaseous dinitrogen (N_2) due to microbial denitrification, thus requiring reduced nitrogen to frequently be replenished from the atmosphere (Mylona et al., 1995). This process is carried out by prokaryotes expressing a nitrogenase enzymatic system in order to reduce atmospheric nitrogen (N_2) into ammonia (NH_3). Through a symbiotic relationship, nitrogen fixation occurs most recurrently in species of the Fabaceae family and it must happen in mutualistic association with the soil bacteria *Rhizobia*.

However, symbiotic nitrogen fixation with rhizobia is not exclusive to legumes (Trinick, 1979; van Velzen et al., 2018). Species of few orders in the Rosids I clade (namely: Fabales, Fagales, Rosales, and Cucurbitales) establish this endosymbiotic relationship with rhizobial bacteria in unique root structures called nodules (Sprent, 2001; Oldroyd et al., 2011; Limpens et al., 2013). Recent transcriptomic comparisons between the nonlegume, nitrogen-fixing plant species *Parasponia andersonii* (Cannabaceae, Rosales) and the legume *Medicago truncatula*

indicate they may share a single origin for genes essential to nodule development and symbiotic nitrogen fixation. Also, comparison of *Parasponia* to closely related, nonnodulating species indicated parallel loss of genes essential to nodulation, which challenges the current paradigm of parallel evolution of nodulation in the Rosids I (van Velzen et al., 2018).

Concurrent model legumes and the uniqueness of *Medicago truncatula*

The legume family encompasses around 20,000 species recently regrouped into six subfamilies, of which the major clades are Caesalpinioideae (which now includes the mimosoid clade) and the Papilionoideae (Azani et al., 2017). All three established model legumes belong to the latter subfamily: Soybean (*Glycine max*), *Lotus japonicus* and *Medicago truncatula* (**Table 1**). They have similar generation times (~3 months, seed-to-seed) and are autogamous. They also had their genome sequenced and are well annotated (Sato et al., 2005; Grant et al., 2010; Young et al., 2011) and have transcriptome data publicly available (Lamblin et al., 2003; Severin et al., 2010; Verdier et al., 2013), in addition to plethora of genetic resources, such as mutant collections (Pislariu et al., 2012; Tsuda et al., 2015; Malolepszy et al., 2016).

Table 2.1. Comparison of the three model legume systems. *Medicago truncatula* is our model organism of choice due to small genome size, availability of genetic resources and indeterminate nodules (Mergaert et al., 2006).

	<i>Medicago truncatula</i>	<i>Lotus japonicus</i>	<i>Glycine max</i>
Clade	Hologalegina (IRLC)	Hologalegina	Phaseolid
Nodule growth	indeterminate	determinate	determinate
Bacteroid division after infection	Endoreduplication	No endoreduplication	No endoreduplication
Form of N exported	amides	amides	ureides
Genome size	390 Mb	470 Mb	1.1 Gb
Genome ploidy	Diploid (n=8)	Diploid (n=8)	Allotetraploid (n=20)

Medicago truncatula is unique among the legume models, in that it develops indeterminate nodules with a persistent meristem, a more primitive trait (Sprent, 2007; Ren, 2018). The Inverted Repeat-Lacking Clade (IRLC) of Hologalegina (galegoids), to which *Medicago* and most cultivated temperate legume crops belong (e.g., chickpea, fava bean, lentil), is characterized by the loss of a 25-kb inverted repeat region in the plastidial genome. In addition, however, and most

importantly, this clade (along with the Dalbergioid subfamily) controls bacteroid differentiation by a massive expression of nodule-specific cysteine-rich (NCR) peptides (Czernic et al., 2015; Wang et al., 2017a; Yang et al., 2017). Therefore, understanding nodulation and symbiotic nitrogen fixation in temperate legume crops can only be achieved by studying *Medicago truncatula* within the available model options.

Medicago truncatula is also closely related to the autotetraploid, perennial crop, alfalfa (*M. sativa*), the most cultivated forage legume worldwide (Yuegao and Cash, 2009). The alfalfa genome is highly syntenous to that of *M. truncatula* (Li et al., 2014), although its ploidy level and allogamous fertilization system preclude it as a model genetic system.

Medicago truncatula has many specific genetic databases and resources available for research. The Noble Research Institute (formerly, Samuel Roberts Noble Foundation) hosts the *Medicago truncatula* Gene Expression Atlas (MtGEA) (Benedito et al., 2008; He et al., 2009) that provides Affymetrix GeneChip data and thorough functional genomics tools for data analysis (<http://mtgea.noble.org/v3/>). The Noble Research Institute is also home to the *Medicago truncatula* mutant collection (<http://medicago-mutant.noble.org/mutant/>), which is the largest collection of DNA-insertion mutants of all legumes (Tadege et al., 2008; Pislariu et al., 2012). The tobacco retrotransposon *Tnt1* was used to tag insertion events in the *Medicago truncatula* genome, which are preferentially located in gene-rich regions (Tadege et al., 2008). The mutant database features over 19,000 *Tnt1* lines containing 470,000 random insertions in the genome, which covers ~90% of all genes in the *Medicago* genome.

The process of nodulation and symbiotic nitrogen fixation

Due to the advances in genomics methods and significant investment in legume research, the processes of nodule development and symbiotic nitrogen fixation are being unveiled at the molecular genetic level since the last decade (Qin et al., 2012; Collier and Tegeder, 2012; Tejada-Jiménez et al., 2017; Kryvoruchko et al., 2018; Wang et al., 2017a; Wang et al., 2017b; Yang et al., 2017). Nodulation starts with a chemical communication between *Medicago truncatula* roots and the free-living soil rhizobia. When the nitrogen in the soil available is low, aromatic compounds known as flavonoids are secreted from *M. truncatula* roots into the rhizosphere, signaling the rhizobia to synthesize and secrete lipochitooligosaccharides called Nod factors

(Catoira et al., 2000). At the same time, rhizobia are attracted by chemotaxis, attach to the root hair, and become trapped in a curled structure in the tip of the root hair called a shepherd's crook (Esseling et al., 2003). Nod factors are perceived by receptors in the plasma membrane of root hair cells and induce a response in the infection pocket to produce invagination of the plasma membrane in order to form infection threads containing the rhizobia (Veereshlingam et al., 2004; Guan et al., 2013). Rhizobia travel through the infection threads towards the developing nodule primordia that is being concomitantly formed by cell divisions in the root cortex (Oldroyd and Downie, 2008). Upon arriving at the inner cortical tissues, these rhizobia are engulfed by the plasma membrane through endocytosis and differentiate into bacteroids contained within symbiosomes (Veereshlingam et al., 2004; Morieri et al., 2013). Infected root cells develop a new organ called the nodule, which provides the microaerobic environment necessary for nitrogenase activity (Jones et al., 2007). Rhizobia continue to produce Nod factors as the plant develops, allowing a continual perception during infection (Popp and Ott, 2011) and nodulation is produced by the simultaneous epidermal infection from rhizobia and cortical cell division (Guan et al., 2013). Indeterminate nodules, like those formed in *M. truncatula* (**Figure 2.2**) and pea roots, have five zones: meristem (I), invasion (II), interzone (II-III), nitrogen fixation (III), and senescence (IV). The meristem zone consists of a primordium-like structure composed of many dividing cells and a quiescent center (Maunoury et al., 2010). Bacteroids differentiate within the invasion and interzones. The nitrogen fixation zone contains mature bacteroids possessing the ability to fix atmospheric nitrogen. The senescence zone involves the proteolysis and degradation of the symbiosome (Van de Velde et al., 2006). Indeterminate nodules are produced through continuous growth from the apical meristem of the nodule (Györgyey et al., 2000), while determinate nodules are developed in species such as soybean and *Lotus japonicus*. It is worth noting that the interaction between rhizobia and legume species is highly specific. *Sinorhizobium meliloti* will form symbiosis with Medicago, Melilotus, and Trigonella (in the IRLC clade), while *Mesorhizobium loti* forms symbioses with Lotus species (Gibson et al., 2008). Efficiency of nitrogen fixation is greatly impacted by the interaction between the various strains of *S. meliloti* and ecotypes of *M. truncatula* (Kazmierczak et al., 2017; Wang et al., 2017a; Yang et al., 2017). For example, the association between the common ecotype 'A17' (the first *M. truncatula* genome sequenced) and *R. meliloti* strain 2011 leads to suboptimal nitrogen fixation rates for plant development (Moreau et al., 2008). More recently, the role of nodule-specific cystein-rich (NCR) peptides has been

linked with nitrogen fixation rates through legume-rhizobia interactions (Wang et al., 2017a; Yang et al., 2017).

Figure 2.2. *Medicago truncatula* cv. ‘A17’ nodules (28 days post inoculation with *Sinorhizobium meliloti* strain *Sm1021-LacZ*). The larger nodule is elongated and pink due to leghemoglobin and indicative of nitrogen fixation. The smaller, developing nodule still lacks leghemoglobin. Scale bar: 500 µm.

Membrane transporters and symbiotic nitrogen fixation in legumes

Transporters are ubiquitously present in all biological membranes – membrane transporters are a *sine qua non* for life to exist. Cell membranes are the most basic structure of a cell – they not only delimit the cell from the inorganic surrounding environment and its organelles within the cell, but they also function in signal perception and in establishing electrochemical gradients that allow coordinated transport of substances to occur in a very controlled manner. Some substrates need assistance because they are polar or too large to move across biological membranes by simple diffusion. Indeed, many different types of transporters are embedded in the phospholipid bilayer of the semi-permeable cellular membrane in order to facilitate this process.

Our understanding of the involvement of membrane transporters in integrating the legume physiology with symbiotic nitrogen fixation, as well as within the nodule cells, has been patchy

(Benedito et al., 2010; Udvardi and Poole, 2013). However, without fully identifying and characterizing membrane transporter genes involved in symbiotic nitrogen fixation, our understanding of the process will remain incomplete.

The several types of membrane transporters are presented in **Figure 2.3**. Those that require direct metabolic energy are pumps that hydrolyze ATP to move a substrate against the electrochemical gradient (**Figure 2.3A**). Channels do not utilize energy input directly, but they can only move ions and small molecules downhill (i.e., in favor of the electrochemical gradient) and use a gating system (open or closed states) to regulate their activity. Secondary transporters utilize energy stored as electrochemical gradient often created by a pump to couple the transport of a substrate against the gradient with the movement of another in favor of the gradient.

There are three types of secondary transporters (**Figure 2.3B**). Uniporters transport a substrate down its concentration gradient. Cotransporters can be divided into symporters and antiporters. Symporters move one molecule against its concentration gradient and is driven by the movement of an ion down the electrochemical gradient with both molecules moving in the same direction of the membrane. Antiporters also move molecules against the concentration gradient driven by a substrate moving down the electrochemical gradient, where the molecules or ions are moving in opposite orientation of each other.

Figure 2.3. Transport of large and polar molecules across lipid bilayers is mediated by specific transmembrane proteins or protein complexes called membrane transporters. (A) Pumps are primary active transporters that require the hydrolysis of ATP to move ions and molecules against the electrochemical gradient. Secondary transporters utilize the electrochemical gradient established by pumps to move molecules against the gradient. Channels allow ions to move across a membrane in favor of the electrical potential (downhill) through facilitated diffusion. (B) There are three types of secondary transporters. Uniporters move substrates in one direction across a membrane, antiporters move substrates in opposite directions, and symporters move substrates in the same direction across a membrane. Antiporters and symporters usually utilize the electrochemical potential established for one substrate to move it downhill while moving the other substrate uphill.

Plasma membrane transporters regulate movement of ions and substances from inside the cell to the extracellular environment and vice versa. Transporters also are ubiquitously present in

the membranes of subcellular organelles to regulate movement into and out of organelles. Transporters consist of proteins with multiple transmembrane domains encompassing stretches of in-tandem hydrophobic amino acids that span the lipid bilayer, along with hydrophilic amino acid residues that connect the transmembrane domains and may compose the N- and C-terminal tails.

The infected nodule cell is unique in that they confine the rhizobia within symbiosomes. Symbiosomes are organellar structures formed through endocytosis of bacteria in infected nodule cells, and therefore is of plasma membrane origin (**Figure 2.4**). They are comprised of the symbiosome membrane, the bacteroid (the nitrogen-fixing form of the endosymbiotic rhizobia), and the symbiosome or peribacteroid space (Catalano et al., 2007). The symbiosome space is highly acidic (pH~4.0), thus providing the electrical potential to enable secondary transport (Pierre et al., 2013). The symbiosome membrane is essential to mediate metabolite exchanges and provides a physical boundary between the symbionts (Catalano et al., 2004). Notwithstanding, only a few of the transporters that mediate these exchanges have been genetically identified and functionally characterized (**Table 2.2**).

Table 2.2. Functionally characterized membrane transporters in the model legumes, *Medicago truncatula*, *Lotus japonicus*, and *Glycine max* (soybean).

Species	Transporter	Gene ID	Substrate	References
<i>M. truncatula</i>	COPT1	<i>Medtr4g019870</i>	copper	Senovilla et al., 2018
<i>M. truncatula</i>	LATD/NIP/NPF1.7		nitrate	Bagchi et al., 2012
<i>M. truncatula</i>	MATE1		3'-O-Glucoside	Zhao et al., 2011
<i>M. truncatula</i>	MATE2	<i>Medtr1g100180</i>	flavonoid glucosides	Zhao et al., 2009
<i>M. truncatula</i>	MATE55	<i>Medtr5g067460</i>	iron	Wang et al., 2017b
<i>M. truncatula</i>	MATE66, MATE69	<i>Medtr2g097900</i> , <i>Medtr3g029510</i>	citric acid	Wang et al., 2017b
<i>M. truncatula</i>	MOT1.3	<i>Medtr3g464210</i>	molybdenum	Tejada-Jiménez et al., 2017
<i>M. truncatula</i>	MATE67	<i>Medtr8g037170</i>	citrate	Kryvoruchko et al., 2018
<i>M. truncatula</i>	NPF6.8/NRT1.3		nitrate	Pellizzaro et al., 2014
<i>M. truncatula</i>	SUT1-1, SUT4-1	<i>Medtr1g096910</i> , <i>Medtr5g067470</i>	sucrose	Doidy et al., 2012
<i>M. truncatula</i>	SWEET11	<i>Medtr3g098930</i>	sucrose	Kryvoruchko et al., 2016
<i>M. truncatula</i>	ZIP6	<i>Medtr4g083570</i>	zinc	Abreu et al., 2017
<i>G. max</i>	DMT1	<i>Glyma17G165200</i>	iron	Kaiser et al., 2003
<i>G. max</i>	N70	<i>Glyma18G018900</i>	anions	Vincill et al., 2005
<i>G. max</i>	PT5		phosphate	Qin et al., 2012
<i>G. max</i>	UPS1		ureide	Collier and Tegeder, 2012
<i>G. max</i>	ZIP1	<i>Glyma20G063100</i>	zinc	Moreau et al., 2002
<i>L. japonicus</i>	ALMT4		dicarboxylates	Takanashi et al., 2016
<i>L. japonicus</i>	AMT2;1		ammonium	Simon-Rosin et al., 2003
<i>L. japonicus</i>	KUP		potassium	Desbrosses et al., 2004
<i>L. japonicus</i>	MATE1		citrate	Takanashi et al., 2013
<i>L. japonicus</i>	N70		anions	Vincill et al., 2005
<i>L. japonicus</i>	SST1		sulfate	Krussel et al., 2005
<i>L. japonicus</i>	SUT4		sucrose	Flemetakis et al., 2003
<i>L. japonicus</i>	SWEET3		sucrose	Sugiyama et al., 2016

Figure 2.4. Infected nodule cells in the nitrogen fixation zone. Each infected cell encloses numerous bacteroids. Green autofluorescence shows elongated and differentiated bacteroids that are capable of fixing nitrogen. Red signal from DsRed shows the roots transformed with the vector pUBIcGFP that contains the DsRed gene constitutively expressed as visual marker (Kryvoruchko et al., 2016). Scale bar: 10 μ m. Image taken on a Zeiss Violet confocal LSM 510.

Figure 2.5. Simplified diagram of an infected nodule cell. Infected cells can hold up to 10,000 bacteroids in each cell (Bailey, 1986). In *Medicago truncatula*, each symbiosome contains a single bacteroid that is terminally differentiated (Mergaert et al., 2006). Membrane transporters are found on the plasma membrane as well as on the symbiosome membrane, vacuole and other membranes and are coded for by the plant genome. Those found on the bacteroid membrane are coded for by the rhizobia. As ammonia moves through the symbiosome space it is protonated into ammonium.

In the nodule, membrane transporters mediate nutrient exchanges during nodule development and symbiotic nitrogen fixation (Udvardi et al., 1988; Benedito et al., 2006, Benedito et al., 2008, Benedito et al., 2010) and are key targets to improve the plants' ability to take up and utilize nutrients (Schroeder et al., 2013) as well as integrate the whole physiology of the plant by directing the translocation of diverse metabolites and signals.

Recent developments and outstanding questions in legume biology, symbiotic nitrogen fixation and membrane transport

The recent elucidation of mechanisms responsible for compatible and incompatible genotypes of rhizobia and plants, raises the question of other unknown interactions involved in making a successful symbiosis, since plant and rhizobial symbioses are complex and intricate

interactions which have yet to be fully described (Wang et al., 2017a; Yang et al., 2017). However, our focus on nutritional flux between symbionts also has many unanswered questions. In nodules, the movement of substrates and products created by and consumed by symbiotic nitrogen fixation must be transported between the rhizobia and plant and across membranes including the symbiosome membrane and the plasma membrane. Amino acid transporters, among others, must be involved in symbiosis but have not been molecularly characterized. An aspartate/glutamate shuttle has been proposed 15 years ago, asserting a transporter must be present in the bacteroid membrane to import glutamate into the bacteroid for utilization as a transamination donor during asparagine synthesis and this hypothesis has not yet been confirmed (Lodwig et al., 2003). Another outstanding question involves transporters of branched-chain amino acids (BCAA: leucine, isoleucine, and valine). BCAAs must be supplied to the bacteroids by the plant, as under symbiotic conditions bacteroids become auxotrophic for these three amino acids (Rastogi and Watson, 1991; Lodwig et al., 2003; Prell et al., 2009, 2010). Alanine has also been proposed to be an alternative product of nitrogen fixation in the bacteroid that is exported to the plant cytoplasm, although this hypothesis has not been assessed to date (Day et al., 2001). The transporters at the symbiosome membrane supplying those amino acids have not been functionally characterized so far. Through the functional characterization of legume genes necessary for successful symbiosis, greater understanding of the intricate balance and transport supporting the energy intensive process of nitrogen fixation can be understood and built upon.

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Chapter 3

Identification of Membrane Transporters of the Amino Acid Polyamine-Organocation (APC) Superfamily in *Medicago truncatula* Reveals Key Candidates for Amino Acid Transporter in Root Nodules

Abstract

Symbiotic nitrogen fixation in legumes relies profoundly on the transport of nutrients, including amino acids, to infected cells of the nodules. Secondary transporters supply the necessary amino acids to and from the nitrogen fixation zone of nodules to support symbiotic nitrogen fixation. The biosynthesis pathways of amino acids to feed symbiosis is well understood, but the involved transporters, including importers and exporters, of amino acids to support infected cells are largely unknown. Transporters mediate passage of amino acids across membranes within cell organelles, from cell to cell, and from organ to organ. Relatively few amino acid transporters have been functionally characterized in legumes. The assimilation of ammonium during nitrogen fixation by the cytosol of the infected cell in temperate legumes requires amino acids to be exported to the xylem in order to travel to sink tissues for translocation of fixed organic nitrogen throughout the plant. The Amino Acid Polyamine-Organocation (APC) superfamily consists of 18 families and is the second largest superfamily of secondary carriers behind the Major Facilitator Superfamily (MFS) characterized in the Transporter Classification Database. The APC family proper (TCDB 2.A.3) is categorized within the APC superfamily, and consists of 15 subfamilies, two of which are present in plants. Manual curation of APC superfamily members in the *Medicago truncatula* proteome was performed leading to the identification of amino acid transporters potentially involved in the flux of amino acids during the symbiotic nitrogen fixation within nodules and an ideal candidate was found within the Cationic Amino Acid Transporter (CAT) Subfamily.

INTRODUCTION

Centrality of Amino Acids in Legume Metabolism

Amino acids are vital to support plant growth and function through transport of organic nitrogen throughout the plant as precursors to nucleic acids, secondary metabolites, and hormones, in addition to the essential role they play as building blocks of proteins. Recent and historical research proposed that amino acids are among the first metabolites created by living organisms (Miller 1953; Hernandez-Montes et al., 2008). Recent studies predict biosynthetic routes of amino acids have multiple origins and evolved to biosynthetic network branches converging to the same end product (Hernandez-Montes et al., 2008). This could explain the integral involvement of amino acids in metabolic and cellular processes in plants and why amino acids are used to shuttle organic nitrogen within a plant (Pratelli and Pilot, 2014). Depending on environmental context and structure, amino acids can be anionic, cationic, or zwitterionic. Side chains can be aromatic, aliphatic, hydrophobic, semipolar, or strongly hydrophilic (Saier et al., 2000). Because of the size and structure of amino acids, movement within plants requires the transport across many membranes both across membranes within the plant as well as through organellar membranes within the cell. In the legume-*Rhizobium* symbiosis, amino acids move across membranes present in infected cells of the nodule in support of symbiotic nitrogen fixation.

In the legume nodule, amino acid transporters are essential to feed the nitrogen fixation process, as well as to desiminate the fixed nitrogen throughout the plant. During symbiotic nitrogen fixation, plants provide dicarboxylic acids in the form of succinate or malate to the bacteroids as a carbon source and, in return, receive ammonia. Ammonia moves across the bacteroid membrane and is protonated in the acidic symbiosome space before being transported across the symbiosome membrane into the cytoplasm of the infected cell. There, ammonium and 2-oxoglutarate, also called α -ketoglutarate, form glutamate through an amination reaction catalyzed by glutamate dehydrogenase (GDH). A condensation reaction of ammonia and glutamate by the enzyme glutamine synthetase (GS) forms glutamine, which can then be exported to sink tissues via the xylem of temperate legume species (**Figure 3.1**). Oxaloacetate originating from sucrose through the TCA cycle is converted to aspartate and 2-oxoglutarate by aspartate aminotransferase (AAT). Glutamine and aspartate can be converted to glutamate and asparagine

In plants, amino acids are carriers of assimilated nitrogen between organs by both the xylem and phloem (Pratelli and Pilot, 2014). Glutamine and asparagine exported to the xylem are the primary amino acids used to shuttle organic nitrogen around the plant, and especially in temperate (galeoid) legumes, such as *Medicago truncatula*, pea, lentil, and chickpea. On the other hand, in tropical legumes, transport of nitrogen is done with the ureides allantoin and allantoic acid (Lea and Miflin, 1980; Peoples and Gifford, 1993; Lodwig et al., 2003; Pélissier et al., 2004, 2007).

In the galeoid clade, supplying branched-chain amino acids (BCAA: valine, leucine, and isoleucine) by the plant to bacteroids is essential for bacteroid development and persistence during symbiotic nitrogen fixation, since under symbiotic conditions bacteroids become auxotrophs for these amino acids. The specific transporters carrying out this essential function at the symbiosome membrane have not been identified (Rastogi and Watson, 1991; Lodwig et al., 2003; Prell et al., 2009, 2010).

We further explore the *Medicago truncatula* genome to identify genes coding for membrane proteins potentially related to amino acid transport activity. We aimed to create and curate an inventory of the APC superfamily, which encompasses important amino acid transporters in living organisms, and especially in plants. Then, we explore the transcriptional activity of these transporter genes to identify those highly or uniquely expressed in the nodule during active symbiotic nitrogen fixation. This catalogue is a resource to identify genes for further functional characterization in the search to better understand amino acid transport in the legume plant, and particularly, the role of amino acids in nodules.

The APC Superfamily

As with the Enzyme Commission (E.C.) numerical classification established for enzymes by the International Union of Biochemistry and Molecular Biology (IUBMB: <http://www.sbcs.qmul.ac.uk/iubmb/>), transporters are hierarchically classified according to their function and phylogenetic relationships accordingly with the IUBMB-ratified Transporter Classification system (TCDB: <http://www.tcdb.org>). The Amino Acid-Polyamine-Organocation (APC) superfamily is the second largest superfamily of electrochemical potential-driven secondary transporters (Wong et al., 2012; Vastermark et al., 2014), after only the Major Facilitator Superfamily (MFS), and includes 162 members in the TCDB. Since it was first described in 2000

(Jack et al., 2000), the APC superfamily has expanded multiple times due to new phylogenetic relationships found with several other transporter families (**Figure 3.2**). The current composition of this superfamily consists of 18 families (**Table 3.1**), amongst which is the APC family proper (Jack et al., 2000; Wong et al., 2012; Vastermark et al., 2014).

Figure 3.2. The APC Superfamily shows evolutionary relationships among several transporter families. Families identified in the *Medicago truncatula* genome are shown in blue, whereas those families with plant members in the TCDB database are shown in boxes. Adapted from Vastermark et al. (2014).

Transporters in the APC superfamily show an archetype with 4-7 transmembrane domains (TMD) that evolved into duplicated repeat sequences with some transporters exhibiting additional TMDs at the N- and C-termini (Wong et al., 2012; Vastermark et al., 2014). As a result, most families of the APC superfamily exhibit a 5+5 topology, with some showing a 7+7 topology

structure, including SulP, BenE, AE, and NCS2 (**Figure 3.2**). The APC family proper (TCDB# 2.A.3) is classified with a 5+5+2 topology (12 TMD), the same as NSS (2.A.22), LIVCS (2.A.26), NRAMP (2.A.55), and KUP (2.A.72) (Vastermark et al., 2014).

The APC Family proper

The Amino acid-Polyamine-Organocation (APC, 2.A.3) family of membrane transporters contains solute:cation symporters and solute:solute antiporters (Schweikhard and Ziegler, 2012). APC transporters are present in all kingdoms of life, with polypeptide chains varying in lengths between 350 and 850 amino acid residues (Reig et al., 2007). Most members of the family have 12 transmembrane domains, but some have 10 or 14 TMDs.

The founding member of the APC family, the *Arabidopsis thaliana* amino acid permease AAP1, was first reported to have broad specificity of amino acid transport (Frommer et al., 1993; Kwart et al., 1993). However, another group reported it to rather be a histidine and alanine symporter (Hsu et al., 1993). AAP1 was later established to have affinity for glutamate, histidine, phenylalanine, and alanine and localize to the plasma membrane of root epidermal cells, root hairs, and the root tip for the uptake of organic nitrogen from the soil (Lee et al., 2007).

Okumoto and Pilot (2011) noted earlier that very few exporters have so far been identified due to inefficient screening techniques. Despite the fact that amino acid export activity is central for plant physiology, this statement still holds true to today. The *Arabidopsis thaliana* Bidirectional Amino acid Transporter 1 (BAT1) is expressed in vascular tissues and exports lysine and glutamic acid across the mitochondrial membrane as well as imports GABA, alanine, arginine in vascular tissues, therefore potentially being involved in phloem loading and unloading (Dündar and Bush, 2009; Michaeli et al., 2011). The *Arabidopsis*, Siliques are Red1 (SiAR1) is another bidirectional amino acid transporter at the plasma membrane capable of exporting glutamine and valine and importing aspartate (Ladwig et al., 2012). Bidirectional amino acid transport is also carried out by AtUMAMIT14, which imports glutamate and citrulline in seeds and exports glutamine, proline, alanine, leucine, and lysine by unloading from phloem in roots (Müller et al., 2015; Besnard et al., 2016).

In the Transporter Classification Database (TCDB), 15 subfamilies are recognized under the APC family. TCDB refers to them as families, but for clarity here we will refer to them as

subfamilies. So far, only two of these subfamilies have characterized plant members classified in the TCDB database: CAT and PHS (**Table 3.1**).

Table 3.1. The APC family proper consists of 15 subfamilies. Bolded subfamilies represent those in the Transporter Classification Database with functionally characterized plant members.

APC subfamilies		
Acronym	Subfamily	TCDB #
AAT	Amino Acid Transporter	2.A.3.1
APA	Basic Amino Acid/Polyamine Antiporter	2.A.3.2
CAT	Cationic Amino Acid Transporter	2.A.3.3
ACT	Amino Acid/Choline Transporter	2.A.3.4
EAT	Ethanolamine Transporter	2.A.3.5
ABT	Archaeal/Bacterial Transporter	2.A.3.6
GGA	Glutamate:GABA Antiporter	2.A.3.7
LAT	L-type Amino Acid Transporter	2.A.3.8
SGP	Spore Germination Protein	2.A.3.9
YAT	Yeast Amino Acid Transporter	2.A.3.10
AGT	Aspartate/Glutamate Transporter	2.A.3.11
PHS	Polyamine:H⁺ Symporter	2.A.3.12
AAE	Amino Acid Efflux	2.A.3.13
U-APC1	Unknown APC-1	2.A.3.14
U-APC2	Unknown APC-2	2.A.3.15

Identification of APC Superfamily members in the *Medicago truncatula* genome

To facilitate characterization of membrane transporters, we analyzed the *Medicago truncatula* proteome Mt4.0v2 annotation (Krishnakumar et al., 2014) using the TransportTP Prediction Server (Li et al., 2009). Of the 57,585 genes annotated in the genome, we found 1774 putative transporters. With amino acid transport in mind, we further analyzed more closely and manually curated putative transporters within the 18 families encompassing the APC superfamily (**Table 3.1**). The TransportTP output rendered 212 putative transporters within 10 of the APC families. The transporters were curated for family and subfamily feature consistencies with functionally characterized transporters in the TCDB, membrane topology (number of TMDs), expected size of the protein, similarity to proteins in the SwissProt database (Boeckmann et al.,

2003), Gene Ontology (GO) classification (Ashburner et al., 2000; The Gene Ontology Consortium, 2017), and identification of expected Pfam domains (Finn et al., 2016).

Table 3.2. Transporter identification of the *Medicago truncatula* proteome. Sequences were analyzed by the prediction server TransporterTP. Proteins belonging to the APC Superfamily were further manually curated for potential functionality according to key characteristics of their respective families.

Protein Sequences	Number
Number of proteins annotated in the genome	57,585
Proteins with TCDB homologs	1774
APC Superfamily transporters (10 families)	212
Curated APC superfamily proteins consistent with transporter function	130

Transporter families have distinguishing characteristics including protein length, number of transmembrane domains, and conserved domains. These characteristics were used to classify transporters and to determine the level of confidence for that classification and functionality of the transporter, as described in Benedito et al. (2010). A confidence ranking of level 1 was used for putative transporters consistent with all features of the family of proteins. A confidence level 2 was used when the protein deviated marginally from expected features or did not find a hit in annotated databases. A confidence level 3 was assigned when the function of a protein is doubtful due to major deviations of key features of the family (e.g., unexpected number of TMDs, inconsistent protein size, lack of a key conserved domain, similarity to well-characterized non-transporter proteins).

Of the 212 proteins found in the APC superfamily, 130 are consistent with potential function demonstrating typical protein length, typical number of TMDs for the family, and conserved protein domains predicted by Pfam (Finn et al., 2016), Gene Ontology (GO) classification (<http://www.geneontology.org/>), and significant similarity with proteins in the curated Swiss-Prot database (Boeckmann et al., 2003). (**Supplemental Table 1**).

Transporter gene expression shows two nodule specific members in the APC Superfamily

Fourteen transporters were expressed in an organ-specific manner across five APC families (APC, AAAP, AE, NCS2, Nramp). Three transporters were expressed in only roots and nodules:

Medtr8g089320.1 and *Medtr8g089340.1* (2.A.3.3), and *Medtr1g093030.1* (2.A.18). Two genes were specific to nodules, with one very lowly expressed (AAAP, 2.A.18.2: *Medtr4g103890.1*) and the highly expressed *Medtr8g089360.1* (**Supplemental Table 1**) (Young et al., 2011). Indeed, *Medtr8g089360.1* (MtAPC1 – cf. Chapter 4 of this dissertation) is expressed in a nodule-specific manner (**Figure 3.3**) and belongs to the cationic amino acid transporter (CAT) subfamily (TCDB #2.A.3.3), which is conserved in both animal and plant kingdoms (Tegeder and Ward, 2012).

Interestingly, two of the three genes expressed exclusively in roots and nodules are closely related to and clustered in tandem with *Medtr8g089360.1* on chromosome 8. Given the limited number of nodule-specific transporters in the APC Superfamily, it is reasonable to propose that this specific expression is indicative of an essential function to symbiotic nitrogen fixation in the transport of crucial amino acids to the process.

Figure 3.3. Expression of APC Superfamily members with expression in blade, bud, flower, root and nodule organs that were classified with either confidence level 1 or 2 constructed using MeV (Howe et al., 2011) from RNA-seq data based on TPM (transcripts per million reads) (Young et al., 2011). White shows basal expression with higher expression shown in red.

Figure 3.4. Expression of APC Superfamily members with expression in the nodule zones that were classified with either confidence level of one or two constructed using MeV (Howe et al., 2011) from RNA-seq data based on TPM (transcripts per million reads) (Roux et al., 2014). FI, meristem; FIId, distal to the interzone; FIIP, proximal to the interzone; IZ, interzone; ZIII, nitrogen fixation zone. White shows basal expression with higher expression shown in red.

The Cationic Amino acid Transporter (CAT) subfamily in plants

The most highly expressed nodule specific gene found during our transporter analysis of the APC superfamily belongs to the Cationic Amino acid Transporter subfamily. The CAT subfamily was so named because the first transporters characterized from mammalian species had preferential affinity to cationic amino acids including arginine, leucine, and lysine (Van Winkle et al., 1985; Van Winkle et al., 1988; Kim et al., 1991; Wang et al., 1991). However, only some plant members show preferential transport of cationic amino acids. Eukaryotic CAT transporters show a unique topology with 14 TMDs while prokaryotic CAT members consistently have 12 TMDs. Moreover, most other transporters within the APC superfamily also have 12 TMDs regardless of whether they are prokaryotic or eukaryotic, excluding those in the Spore Germination Protein Subfamily, which typically have 10 TMDs (2.A.3.9) (Jack et al., 2000). Arabidopsis CAT proteins localize to several types of membranes including the plasma membrane, tonoplast and endoplasmic reticulum and do not show specificity for individual amino acids, but rather groups of amino acids (**Table 3.3**) (Su et al., 2004; Hammes et al., 2006; Yang et al., 2010; Yang et al., 2014).

Table 3.3. Functionally characterized CAT transporters in *Arabidopsis thaliana*.

Gene	Gene ID	Substrate Affinity	Subcellular Localization
CAT1	<i>At4g21120</i>	basic, acidic, neutral	PM
CAT2	<i>At1g58030</i>	n.d.	VM
CAT3	<i>At5g36940</i>	neutral or acidic	ER
CAT4	<i>At3g03720</i>	n.d.	VM
CAT5	<i>At2g34960</i>	basic	PM
CAT6	<i>At5g04770</i>	neutral or acidic	PM
CAT7	<i>At3g10600</i>	n.d.	n.d.
CAT8	<i>At1g17120</i>	neutral or acidic	VM
CAT9	<i>At1g05940</i>	n.d.	VM

PM, plasma membrane; VM, vacuolar membrane; ER, endoplasmic reticulum. (Su et al., 2004; Hammes et al., 2006; Yang et al., 2010; Yang et al., 2014). n.d.: not determined.

The CAT subfamily in the model legume, *Medicago truncatula*

In the *Medicago truncatula* genome, we identified thirteen CAT transporter genes. The nodule-specific MtAPC1 transporter (*Medtr8g089360.1*) is closely related to Arabidopsis CAT1 (**Figure 3.5**), which transports basic amino acids with high affinity (Fisher et al., 1995) as well as acidic and neutral amino acids to a lesser extent (Frommer et al., 1995).

Figure 3.5. Maximum likelihood phylogenetic analysis of all prokaryotic and eukaryotic CAT subfamily transporters present in the Transporter Classification Database (www.TCDB.org) along with all *Arabidopsis thaliana* members (shown with blue dots) and all *Medicago truncatula* family members (shown with green dots and nodule specific *Medtr8g089360.1* shown with a black dot). Human subfamily members are shown with red dots, mouse (*Mus musculus*) are shown with pink dots, and *Saccharomyces cerevisiae* is shown with a light blue dot. Branch length represents average residue substitution per site.

Medtr8g089360.1, MtAPC1, is closely related to *Medtr8g089340.1* and *Medtr8g089320.1*. This local triplication was possibly created by asymmetrical recombinations during the evolution of the *Medicago* genome and may account for potential functional redundancies (**Figure 3.5**). Also closely related is the *Arabidopsis* CAT1, which has been shown to transport basic amino acids and positively charged amino acids and localize to the plasma membrane (Frommer et al., 1995; Su et al., 2004). The *Arabidopsis* CAT5 transports basic amino acids and localized to the plasma membrane (Su et al., 2004).

Herein, we identified 212 genes potentially coding for membrane transporters belonging to the APC Superfamily. Amongst these, we found that 130 present features consistent with functional activity. 106 were expressed in nodules, with 2 being nodule-specific. We have selected *Medtr8g089360.1* (MtAPC1) for further functional characterization, which is presented in the next chapter of this dissertation.

MATERIALS AND METHODS

Transporter analysis: *Medicago truncatula* genome Mt4.0v2 protein annotation from (<http://www.medicago genome.org/downloads>) was uploaded to the TransportTP Transporter Prediction server (<http://bioinfo3.noble.org/transporter/>; Li et al., 2009). Reference organism used was *Arabidopsis thaliana* with an E-value threshold of 0.05. Amino acid length was added as well as gene expression data from Young et al., 2011. Levels of classification were annotated based on protein characteristics of families classified in TCDB.org (protein length, number of TMDs) and through Pfam (<https://pfam.xfam.org/>; Finn et al., 2016), Gene Ontology (GO) (<http://www.geneontology.org/>), and the well annotated Swiss-Prot database (<http://www.uniprot.org/>; Boeckmann et al., 2003). Level 1 confidence indicated consistency to family characteristics, level 2 indicated slight divergence from family characteristics, and level 3 indicated doubtful functionality of a protein due to (small or large protein size and absence of or unexpected TMDs not characteristic of the family).

Heatmap analysis: The heatmap showing gene expression in mature plant organs growing under optimal conditions was constructed using MeV (<http://mev.tm4.org/>; Howe et al., 2011) from RNA-seq data based on TPM (transcript per million total reads) (Young et al., 2011; Roux et al., 2014).

Phylogenetic Analyses and membrane topology prediction: Phylogenetic trees were produced using MEGA v.7 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2012) by the maximum likelihood method with 1,000 bootstraps. Protein sequences were aligned using

muscle and imported into construct/test maximum parsimony tree with the following parameters: Ten initial trees, MP search level 1, and complete deletion of Gaps/Missing data. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Medicago truncatula* sequences were obtained from <http://bioinfo3.noble.org/getseq/>.

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Chapter 4

Functional characterization of the *Medicago truncatula* nodule-specific amino acid transporter *MtAPC1*

Amino acid transport in legumes is crucial to shuttle organic nitrogen throughout the plant and aid in cellular and metabolic processes. During symbiotic nitrogen fixation, when atmospheric nitrogen is reduced and assimilated into amino acids in the nodule, amino acids must cross membranes to feed bacteroids, allow nitrogen assimilation, and be exported to other parts of the plant. Little is known about the genetic identities of transporters involved in moving amino acids across membranes in the root nodule. In an attempt to better understand the molecular mechanisms involved in the symbiotic nitrogen fixation process, we aimed to study Amino Acid Polyamine-Organocation (APC) transporters within the model organism *Medicago truncatula*. APC transporters generally have affinity for groups of amino acids rather than to a specific amino acid. In plants, the Cationic Amino acid Transporter (CAT) (sub)family encompassed by the APC family shows affinity to basic, acidic, and neutral amino acids. In the *Medicago truncatula* genome, we identified thirteen CAT genes. *Medtr8g089360.1*, here called *MtAPC1* is nodule specific and highly expressed in the nitrogen fixation zone. RNAi analysis showed *MtAPC1* is non-essential to symbiotic nitrogen fixation, potentially due to gene redundancy with another CAT gene that co-expresses strongly, but non-specifically in nodules. *MtAPC1* is localized to the plasma membrane in the *Nicotiana benthamiana* leaf infiltration heterologous transient expression system. *MtAPC1* did not show amino acid import activity in yeast. Altogether, our results suggest that *MtAPC1* may be associated with, but not essential for amino acid trafficking within the infected cells in the nitrogen fixation zone, or the translocation of amino acids to the rest of the plant.

INTRODUCTION

The essentiality of amino acid transporters in legumes to enable symbiotic nitrogen fixation

Plants are capable of taking up nitrogen from the soil in the form of ammonium or nitrate and assimilating it into amino acids for plant utilization (Andrews et al., 2013). Remarkably, legumes can also assimilate into amino acids the ammonium produced via symbiotic nitrogen fixation resulting from the reduction of atmospheric dinitrogen. After assimilation by the plant, amino acids serve many critical roles for plant function, ranging from shuttling organic nitrogen throughout the plant, to acting as precursors of hormones, nucleic acids and secondary metabolites, to providing the building blocks of proteins (Wink, 2013; Tegeder, 2014). Transport of amino acids is essential to allow translocation from the point of assimilation in the cytosol of the nodule infected cells after nitrogen fixation to elsewhere in the plant (Day et al., 2001; Tegeder, 2012). In order for polar or large substances, such as amino acids, to be translocated in the plant or moved within organelles in the cell, appropriate transporters embedded in biological membranes must be present. Currently, despite its essentiality to the process, very little is known about the molecular identifies of amino acid transporters that sustain symbiotic nitrogen fixation in legumes. This missing information is, however, key for our full understanding and further biotechnological manipulations of this important agricultural trait.

Figure 4.1. Nitrogen assimilation cycle in legumes. 2-oxoglutarate is aminated by GDH to form glutamate. Glutamate can interchange to glutamine and back via the GS/GOGAT cycle. AAT interconverts oxaloacetate plus glutamate into aspartate plus 2-oxoglutarate. Glutamine can be used to transaminate aspartate in order to produce glutamate and asparagine by ASN. In temperate legumes like *Medicago truncatula*, the assimilated forms of symbiotically fixed nitrogen translocated from nodules to other parts of the plants via xylem are glutamine and asparagine. GDH, Glutamate Dehydrogenase; GOGAT, Glutamate Synthase; GS, Glutamine Synthetase; AAT, Aspartate Aminotransferase; ASN, Asparagine Synthetase. Adapted from Mériçout et al., 2008.

It is estimated that in higher plants 95 percent of ammonium is assimilated via the GS/GOGAT pathway. Glutamate is formed by 2-oxoglutarate, also called α -ketoglutarate, via amination by Glutamate Dehydrogenase (GDH) (**Figure 4.1**). Glutamine Synthetase (GS) catalyzes the first step of nitrogen assimilation via a condensation reaction of ammonia and glutamate to form glutamine. Glutamate Synthase (GOGAT) acts opposite to GS, synthesizing glutamate from glutamine. Oxaloacetate can be converted to aspartate by the enzyme Aspartate Aminotransferase (AAT). Asparagine Synthetase (ASN) converts aspartate and glutamine to asparagine and glutamate (Lea and Mifflin, 1974; Masclaux-Daubresse et al., 2005; Mériçout et al., 2008; Seabra and Carvalho, 2015; Zhang et al., 2017). The amides asparagine and glutamine carrying the assimilated nitrogen are transported out of the cell to the xylem and throughout the

plant (Lodwig et al., 2003; Pratelli and Pilot, 2014). Glutamine can be used for the synthesis of all other essential nitrogenous compounds in the cell (Kaiser et al., 1998; Seabra and Carvalho, 2015).

In *Medicago*, as well as in peas, supplying branched-chain amino acids (BCAA: valine, leucine, and isoleucine) from the infected nodule cell to bacteroids is essential for bacteroid differentiation into the nitrogen-fixing form and persistence during symbiotic nitrogen fixation, since the rhizobia shuts down the synthesis of BCAA under symbiotic conditions. Therefore, bacteroids become symbiotic auxotrophs for these amino acids providing the plant with greater control over the symbiotic balance of power. However, the genetic identity of transporter or transporters for branched chain amino acids have not yet been elucidated. This information may lead to better understanding of molecular mechanisms responsible for feeding symbiotic nitrogen fixation (Rastogi and Watson, 1991; Lodwig et al., 2003; Prell et al., 2009, 2010).

During symbiotic nitrogen fixation, plants provide dicarboxylic acids in the form of succinate or malate to the bacteroids as a carbon source (Ronson et al., 1981; Finan et al., 1983; Udvardi et al., 1988). In addition to the supply of dicarboxylic acids to the bacteroid, Lodwig et al. (2003) proposed the additional transport of glutamate, or a glutamate precursor, to be supplied to the bacteroid to act as a transamination donor for the production of aspartate or possibly alanine in the bacteroid cytosol. They also proposed the export of aspartate to the cytosol of the infected plant cell, which would then be converted to asparagine by ASN. Exported alanine could be converted to glutamate by Glutamate Pyruvate Transaminase or to aspartate by ASN. Similarly to the mitochondrial system, the aspartate-glutamate shuttle in the bacteroid membrane would move glutamate into the bacteroid and transport the aspartate back out towards the cytosol (Lodwig et al., 2003). Although already 15 years old, this hypothesis has not yet been proven, leaving unanswered questions about the transport and involved transporters of amino acids intimately integrated in supporting symbiotic nitrogen fixation. Herein, we identified potential amino acid transporters expressed in the legume nodule and carried out functional analyses aiming to shed light to these questions.

RESULTS AND DISCUSSION

Transporter families involved in amino acid transport and the nodule-specific MtAPC1

In the *Medicago truncatula* genome, thirteen family members of the Cationic Amino Acid Transporter family (TCDB# 2.A.3.3) were identified. Gene expression analysis of these putative transporters revealed a single gene showing nodule specific expression: *Medtr8g089360.1*, herein called *MtAPC1*, which could potentially be involvement in amino acid transport to enable symbiotic nitrogen fixation.

Eighteen transporter families are categorized under the Amino Acid Polyamine-Organocation (APC) superfamily in the Transporter Classification Database (TCDB: www.tcdb.org), the second largest superfamily of transporters. Within the APC superfamily, is the APC family proper (2.A.3), which encompasses fifteen transporter families and, among those is the Cationic Amino Acid (CAT) (sub)family of transporters (TCDB# 2.A.3.3) (Vastermark et al., 2014; Saier et al., 2016). Within the CAT family, relatively few members of the CAT family have been functionally characterized in plants.

In *Arabidopsis thaliana*, CAT transporters have been shown to have affinity to basic, acidic, and neutral amino acids and to localize to several membranes in the cell including the plasma membrane, endoplasmic reticulum, and tonoplast (Su et al., 2004; Hammes et al., 2006; Yang et al., 2010; Yang et al., 2014). In poplar, CAT11 was shown to have affinity to glutamine and arginine in senescent cells (Couturier et al., 2010). In *Camellia sinensis*, CAT transporters have affinity to glutamate, glutamine and theanine, a non-protein amino acid found in tea plants (Feng et al., 2017) and the tomato SlCAT9 was shown to be a glutamate, aspartate and GABA exchanger at the tonoplast (Snowden et al., 2015).

The phylogenetic relationships of the thirteen *Medicago* CAT transporters were examined along with their expression patterns in mature organs of *Medicago* (**Figure 4.2**). Three closely related genes are displayed in tandem on chromosome 8, and another more distantly related is located on the same chromosome. The predicted architectures (i.e., exon-intron structure) among the *Medicago* CAT genes vary, and distinct patterns were noticed. Interestingly, the 3'-UTR of *Medtr1g030860.1* is split into the first and second exons, which is uncommon. *Medtr8g089360.1* is expressed in the nodule, and the closely related *Medtr8g089340.1* is expressed in the root and

nodule. This local gene duplication and possible redundancy may have implications on the essentiality of *Medtr8g089360.1* in nodules. Two alternative splicing variants of *Medtr8g089360* are predicted, as well as four alternative splice variants for another gene, *Medtr7g007850*. Expression patterns for both *Medtr8g089360.1* and *Medtr8g089360.2* forms show high expression in nodules over other organs, while none of the four variants of *Medtr7g007850* show organ specific expression (Young et al., 2011).

Divergence of gene structure in *Medicago truncatula* CAT family members is mirrored with diverse organ expression

Figure 4.2. Phylogenetic tree of the thirteen *Medicago truncatula* CAT transporters. The tree was constructed using the Maximum Likelihood method with 1,000 bootstraps in MEGA 7. The heatmap showing gene expression in mature plant organs growing under optimal conditions was constructed using MeV from RNA-seq data based on TPM (transcripts per million reads) (Young et al., 2011). Expression level is denoted by red scale, with white denoting no expression. MtAPC1 is the only nodule-specific CAT member in the *Medicago* genome. Gene architectures show the evolution of introns and exons of *Medicago* CAT genes. Dark blue boxes represent UTRs, light blue boxes represent exons, and black lines represent introns.

The MtAPC1 membrane topology is representative of the CAT family

MtAPC1 is predicted to have three exons and two introns (**Figure 4.3**), a structure somehow distinct from its two in-tandem duplicates, *Medtr8g089340.1* and *Medtr8g098320.1*, which have one exon and two exons, respectively (**Figure 4.2**).

Figure 4.3. Map of chromosome 8 with expanded view of *MtAPC1* (*Medtr8g089360.1*). Predicted architecture of the nodule-specific MtAPC1 transporter gene with three exons (dark gray boxes) and two introns (black lines). Untranslated regions (UTRs) are shown as light gray boxes. Chromosomal coordinates of the transcribed region as well as the start and stop codons are indicated (genome annotation IMGAG v.4.0).

The protein structures of *Medtr8g089320.1*, *Medtr8g089340.1*, and *Medtr8g089360.1* show the predicted amino acids of the putative transporters as well as the transmembrane domains relative to *Medtr8g089360.1* (**Figure 4.4**). Together, their phylogenetic relationships, gene architectures, and protein sequences indicate that *Medtr8g089340* is the ancestral gene, with the nodule-specific *Medtr8g089360* being the last one to appear in the cluster.

Figure 4.4. Protein alignment of the CAT membrane transporters in *Medicago truncatula* that are located in tandem with the nodule-specific *Medtr8g089360.1*. According to TMHMM v.2 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), the *Medtr8g089360.1* gene product spans 14 transmembrane domains (TMD, indicated by red underlines). The three neighboring CAT transporters show highly conserved protein sequences.

Despite their similar protein structures (**Table 4.1**), we noticed distinct transcription patterns. *Medtr8g089340.1* showed expression levels closer to *Medtr8g089360.1* in nodules in addition to high expression in roots, while *Medtr8g089320.1* showed overall only a low, basal expression level in all plant organs. Unless the *Medtr8g089320* gene is induced in a very special circumstance not captured in the sampling, it is possible that it is undergoing pseudogenization.

Table 4.1. Identity and similarity matrix of *Medtr8g089320.1*, *Medtr8g089340.1*, and *Medtr8g089360.1* protein sequences.

	<i>Medtr8g089320.1</i>	<i>Medtr8g089340.1</i>	<i>Medtr8g089360.1</i>
<i>Medtr8g089320.1</i>		94% similarity	85% similarity
<i>Medtr8g089340.1</i>	91% identity		82% similarity
<i>Medtr8g089360.1</i>	74% identity	73% identity	

Both TMHMM and Protter predicted MtAPC1 to have 14 transmembrane domains which is typical of eukaryotic APC family members as well as CAT family members (Saier, 2000; Omasits et al., 2014) (**Figure 4.5**).

Figure 4.5. Membrane topology of MtAPC1 (*Medtr8g089360.1*). (A) Fourteen predicted transmembrane α -helices were found using the TMHMM v.2 (Sonnhammer et al., 1998; Krogh et al., 2001; <http://www.cbs.dtu.dk/services/TMHMM-2.0/>). Eukaryotic APC transporters and CAT family members typically have 14 putative α -helical transmembrane domains (TMDs) (Jack et al., 2000; Fotiadis et al., 2013). (B) *Medicago truncatula* APC1 protein topology analysis in Protter (Omasits et al., 2014; <http://wlab.ethz.ch/protter>) confirmed the 14 predicted TMDs.

Transcriptional analysis showed high expression of MtAPC1 in the fixation zone

The MtAPC1 transporter is expressed specifically in nodules in the mature organs of the plant, with the maximum level of expression found during later nodule development after 10 days post inoculation, and especially in the nitrogen fixation zone (**Figure 4.6**).

Figure 4.6. Expression profile of the nodule-specific MtAPC1 gene (*Medtr8g089360.1*). (A) MtAPC1 is expressed exclusively in the nodule. (B) MtAPC1 expression starts early during nodule development (4 days post inoculation, dpi), peaking in young, mature nodules at 10 dpi. The expression of MtAPC1 is inhibited by nitrate, a known inhibitor of nodulation and symbiotic nitrogen fixation. (C) In mature nodules, MtAPC1 expression is highest in the nitrogen fixation zone. Data were collected from public gene expression databases: (A, B) Medicago Gene Atlas (probeset number Mtr.42325.1.S1_at; <http://mtgea.noble.org/v3/>; Benedito et al., 2008, 2010; He et al., 2009); (C) Roux et al., 2014 (gene number Mt0009_00215)

Real time analysis was conducted on Medicago genotype R108 infected with a functional *lacZ* *S. meliloti* rhizobia strain or non-functional mutants that fail in proceeding in specific stages of symbiosis establishment. *exoA* rhizobia cannot infect root cells, *bacA* rhizobia are unable to differentiate into bacteroids, and the *fixJ* strain proceeds through bacteroid differentiation, but is inefficient to fix nitrogen (Mitra and Long, 2004; Griffitts and Long, 2008). Rhizobial infection is necessary for gene expression of MtAPC1 to occur, but not bacteroid differentiation or nitrogen fixation *per se* (**Figure 4.7**). MtAPC1 expression was not detected at 3 dpi but it is perceived at 10 dpi (young, functional nodules) until later in mature nodules at 28 dpi. These results indicate that MtAPC1 has a role after the initial infection and invasion of rhizobia.

Figure 4.7. Analysis of expression of MtAPC1 by real-time PCR analysis. (A) Expression analysis of nodulating roots infected with rhizobial mutants and the functional *LacZ* rhizobia in *Medicago truncatula* genotype R108. (B) Time-course of MtAPC1 gene expression in of in *Medicago truncatula* genotype R108 roots infected with the functional *LacZ* rhizobia. Relative expression analysis used non-inoculated root as control. Error bars represent standard error. Statistical significance of $p < 0.05$ using Tukey-Kramer.

RNAi knockdown of MtAPC1 shows no phenotypical change in the nodule

Hairpin-based RNAi was used to knockdown the expression of MtAPC1 in root nodules. Expression of MtAPC1 in RNAi nodulating roots was less than 4% of that of the WT nodulating roots (**Figure 4.8**). No phenotypic differences were observed between the wild-type (WT) and MtAPC1-RNAi transgenic nodules (**Figure 4.8**). Both WT and RNAi nodules have normal size and shape and show pink fixation zones indicative of active nitrogen fixation. Despite the fact that expression of MtAPC1 was greatly reduced, phenotypic changes of the nodule was not seen. This could be explained by the normally high expression of MtAPC1 in nodules, making the residual expression enough to carry its function, or that its closely-related *Medtr8g089360* gene, which is expressed in nodules, is functionally redundant. If the latter, MtAPC1 may be playing a function in a membrane system common to root and nodule cells, such as the plasma membrane, rather than localizing to a specific membrane system of infected cells (the symbiosome membrane).

Figure 4.8. MtAPC1 (*Medtr8g089360.1*) RNAi nodules are visually indistinct from non-transgenic nodules. Wild type and transformed RNAi nodules under fluorescent stereoscope. (A, left) Brightfield. (A, right) A constitutive DsRed marker was utilized to visualize transgenic root systems. The nodule sizes are representative of wild-type control and RNAi nodules. Scale bar: 500 μ m. (B) Real-time qPCR expression profile of MtAPC1 wild-type versus RNAi nodulating roots. Error bars represent standard error. Significant difference shown between control nodules and MtAPC1 RNAi using Tukey-Kramer $p < 0.05$.

MtAPC1 is expressed in infected cells within the nitrogen fixation zone

MtAPC1 is highly expressed in the nitrogen fixation and senescence zones (**Figure 4.9A**). Within the nitrogen fixation zone, infected cells show MtAPC1 expression (**Figure 4.9B**). This localization indicates MtAPC1 may be playing a role in nitrogen fixation.

Figure 4.9. *In situ* hybridization of mature nodule sections using *MtAPC1* probe. (A and B) Antisense probe (C and D) Sense probe. Scale bar: 200 μ m.

Subcellular localization of MtAPC1 shows localization to the plasma membrane

To investigate the subcellular localization of MtAPC1, localization was performed in epidermal peels of *Nicotiana benthamiana* leaves after infiltration of *Agrobacterium tumefaciens*. Red fluorescence at the periphery of epidermal cells indicated localization of the MtAPC1 to the plasma membrane of the epidermal cells (**Figure 4.10**).

Figure 4.10. Epidermal peel of *Nicotiana benthamiana* leaf three days after *Agrobacterium tumefaciens* infiltration with *MtAPC1-mRFP* fusion expressed under the CaMV35S constitutive promoter. In this heterologous transient system, MtAPC1 localized to the plasma membrane. Scale bar: 10 μ m.

MtAPC1 did not show amino acid import activity in yeast

Transformed 22 Δ 10AA *Saccharomyces cerevisiae* strain complemented with MtAPC1 was grown separately on 10 mM of each of the 16 amino acids strain 22 Δ 10AA cannot utilize as a sole nitrogen source. Growth was seen in empty vector and MtAPC1 lines at similar rates and suggests that MtAPC1 is not an importer. Growth in liquid cultures was also monitored with the 16 amino acids over a 24-h period (**Supplemental Figure S1**) and results corroborated the data on agar plates.

Figure 4.11. Amino acid import assay in yeast strain 22Δ10AA. Four ten-fold serial dilutions were made from 5 μ L liquid culture (initial $OD_{600}=1.0$). Plates contained YNB medium supplemented with 10 mM amino acid (or ammonium sulfate). Yeast was grown for three days at 28°C. The negative control medium lacks a nitrogen source. The positive control medium is supplemented with 10 mM ammonium sulfate. All except for tyrosine grew healthily at 10 mM. No differences in growth between empty vector (EV: pDR196-GW) and MtAPC1-complemented lines were noticed, denoting no amino acid import activity of MtAPC1 in this system.

Syntenic analysis revealed no synteny among *Medicago* CAT family members and other legume model organisms

Comparative analysis between the thirteen *Medicago truncatula* CAT genes and the *Arabidopsis thaliana*, *Lotus japonicus*, and the *Glycine max* genomes revealed no synteny. Microsynteny was not found within the *Medicago truncatula* genome regarding *Medtr8g089360* or any of the loci containing CAT genes. However, reiterated tandem duplications were found, forming a cluster formed by four CAT genes, of which three possess the typical 14 transmembrane domains and expected length of protein sequence (**Table 4.3**).

Table 4.3. Comparative Genomics analysis of APC tandem duplications using CoGe: Comparative Genomics web tool and SynMap (<https://genomeevolution.org/coge/>; Lyons and Freeling, 2008; Lyons et al., 2008).

APC Tandem Duplication Gene Set	<i>Medtr8g089360.1</i> <i>Medtr8g089320.1</i> <i>Medtr8g089360.2</i> <i>Medtr8g089340.1</i> <i>Medtr8g089342.1</i>
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Gene Network analysis shows correlation to Nodule-Specific Cysteine-Rich Secreted Proteins

A gene expression network was constructed with first-order Pearson's correlations centered on MtAPC1 (**Figure 4.12, Supplemental Table S1**). Interestingly, the three highest associations are with genes coding for Nodule-specific Cysteine-Rich (NCR) secreted proteins (*Medtr6g057520.1*, *Medtr3g015870.1*, *Medtr5g072420.1*). Additionally, the expression of two genes coding for peptide transporters (*Medtr7g098180.1* and *Medtr1g116930.1*) and another coding for a MATE transporter (*Medtr7g082810.1*) are also significantly correlated with MtAPC1.

Figure 4.12. Gene network centered on MtAPC1. Primary gene interactions were determined using Pearson's coefficient correlation. Correlation threshold was set at $p \geq 0.90$. Maximum correlation obtained for MtAPC1 was $p = 0.94$. Visualization was produced using Cytoscape (Shannon et al., 2003).

CONCLUSION

MtAPC1 is a nodule specific protein, with expression beginning at 4 days post inoculation. In the mature nodule, APC1 starts expressing during bacteroid differentiation in the interzone and shows highest expression in the nitrogen fixation and senescence zones. RNAi knockdown showed no visible phenotypic changes to the nodule size, form or color. It remains to be determined, however, whether the efficiency of symbiotic nitrogen fixation remained the same in both treatments. The heterologous transient system utilizing *Nicotiana benthamiana* showed protein localization to the plasma membrane indicating the MtAPC1 transporter may localize to the plasma membrane or a plasma membrane-derived membrane, such as the symbiosome membrane in *Medicago truncatula*. Altogether, our results support the hypothesis that MtAPC1 is as a non-essential transporter aiding in the flux of amino acids to sustain symbiotic nitrogen fixation.

The lack of syntenic regions of members of the CAT gene family between *Medicago truncatula* and *Arabidopsis thaliana* or even the other legume models indicates their function could be performed by other transporter families. This is a possibility given the broad substrate affinity of CAT transporters and that other families in the APC superfamily may have taken over the MtCAT1 function in nodules of other legumes.

Future studies should focus on knocking out both nodule-expressed CAT genes, *Medtr8g089360.1* and the closely related *Medtr8g089340.1* to assess the functional redundancy as well as essentiality of this function for symbiotic nitrogen fixation. Given the lack of synteny found for CAT genes within model legumes, a comparative analysis of the CAT gene family in legumes could elucidate whether nodule CAT transporters are specific to the Medicago clade to support symbiotic nitrogen fixation. The study of MtAPC1 subcellular localization in nodule cells will also help elucidate whether it localizes in the plasma membrane or in the symbiosome membrane, which has obvious implications to its physiological role in the nodule.

MATERIAL AND METHODS

Phylogenetic analysis and membrane topology prediction: The phylogenetic tree was produced using MEGA v.7 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2011; Tamura et al., 2012) by the maximum likelihood method with 1,000 bootstraps. Protein sequences were aligned using Muscle and imported into construct/test maximum parsimony tree with the following parameters: Ten initial trees, MP search level 1, and complete deletion of Gaps/Missing data. *Medicago truncatula* sequences (IMGAG annotation Mt3.5v4) were obtained from <http://bioinfo3.noble.org/getseq/>. TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>; Möller et al., 2001) and Protter (wlab.ethz.ch/protter) were used to predict transmembrane domains with protein sequences as input.

Gene expression analyses: Gene expression was assessed from the *Affymetrix* *Medicago truncatula* GeneChip microarray data deposited at the Samuel Roberts Noble Foundation *Medicago truncatula* Gene Expression Atlas (<http://mtgea.noble.org/v3/>; Benedito et al., 2008). Gene expression analyses of membrane transporters in rhizobial mutants was performed in R108 roots inoculated with *bacA*, *exoA*, *fixJ* or *hemA::LacZ* strains of *Sinorhizobium meliloti* in *Sm1021* background. A control group was not inoculated with rhizobia and was provided full nitrogen fertilization. Plant roots were harvested at 0, 3, 14, 21, and 28 dpi. Total RNA was extracted with mirVana miRNA Isolation kit (ThermoFisher Scientific) using the total RNA isolation protocol, followed by DNA removal with Turbo DNA-free kit (Applied Biosystems/ThermoFisher Scientific). First strand complementary DNA was synthesized by priming with oligo-dT using the SuperScript III First-Strand Synthesis Super mix (ThermoFisher Scientific) according to the manual. Primers used were: MtAPC1 forward: 5' TGC GTC GTT ATT ATT CAA CTG G, and MtAPC1 reverse: 5' CCA ACC ATC ACT CAT TGC TC. Three biological and three technical replicates were used for qPCR. SYBR green master mix (Applied Biosystems/ThermoFisher Scientific) and 5 μ L reactions were used on Applied Biosystems 7500 Real Time PCR System and 7500 software. All templates were amplified using the same protocol: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curves were produced following the 40 cycles by heating to 95°C for 15 seconds followed by 60°C for 15 seconds and 95°C for 15 seconds. 7500 Software (Applied Biosystems) gave C_T threshold cycle values. Excel was used to organize data and calculate geometric means of technical replications and calculate averages and standard errors. Technical outliers were eliminated from analysis.

The symbiotic marker genes used were: NCR2A (*Medtr2g042480.1* – marker of bacteroid differentiation; Moreau et al., 2011), LEG (*Medtr5g081000.1* – nitrogen fixation; Vinardell et al., 2003), (*Medtr7g104360.1* – senescence zone marker; Zhou et al., 2011), LYK3 (NCBI accession AY372406 – root hair curling; Smit et al., 2007), ENOD11 (AJ297721 – preinfection and infection; Svistoonoff et al., 2010), and HAP2-1 (EF488826 – meristem zone marker; Combier et al., 2006; Combier et al., 2008). The housekeeping genes chosen for this experiment were: EF1 α (*Medtr6g021800*), tubulin (*Medtr7g089120*), and MSC27 (X98618.1). NormFinder software (Andersen et al., 2004) was used to identify stably expressed housekeeping genes in the experiment and MSC27 was excluded from the calculations. Statistical analysis was performed using Tukey-Kramer at $p=0.05$.

Cloning of coding regions: cDNA of the gene of interest was amplified using KOD Hot Start DNA polymerase (EMD Millipore) and cloned into the pENTR Gateway entry vector using the Directional pENTR-D/TOPO Cloning Kit (Life Technologies). The transcribed region was obtained from the IMGAG gene model (<http://bioinfo3.noble.org/getseq/>) and primers were designed using Primer3 (<http://primer3.ut.ee>) using the following parameters: primer size between 18-25 bp, melting temperature of primers (T_m) between 58-62°C, GC content between 40-60%, max self-complementarity=3 bases, max 3' end complementarity=1 base. Primers used for cloning the coding regions were: forward MtAPC1: 5' CAC CAT GGA AGA CGC AAT TTC TTC, reverse MtAPC1 with stop codon: 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TAG AAT AAC AAT AGC ATT ATT TAT C, reverse MtAPC1 with no stop codon: 5' ATT TTT ACA TTC CTC TTC AAC ATT G. PCR cycling conditions used were: initiation at 95°C for 2 minutes, followed by 35 to 39 cycles at 95°C for 20 seconds, 60°C for 10 seconds, and 70°C for 1 minute; termination at 70°C for 5 minutes, followed by constant 4°C. Applied Biosystems GeneAmp PCR System 9700 or Eppendorf Mastercycler EP thermocyclers were used.

cDNA amplicons were cloned into pUBIcGFP-DR destination vector for complementation and subcellular localization by LR Gateway recombination (Life Technologies) before being moved into *Agrobacterium rhizogenes* strain *ARqual* for *ex vitro* root transformation purposes.

Yeast analyses: Transcript coding regions with stop codons of gene of interest were transferred from the entry vector to the destination vector pDR196-GW via LR recombination, followed by yeast transformation with the LiAc/SS-DNA/PEG transformation procedure (Gietz and Woods, 2002). Yeast transport mutant strains for substrates of interest were selected.

For influx transport, the wild-type non-transformed yeast strain and the empty vector yeast strains were the positive controls for auxotrophic yeast strains with the plasmid containing the transcripts' coding region (Kraidlova et al., 2011). Yeast cells were grown on selective media (YNB) supplemented with ammonium sulfate or amino acids. Tenfold serial dilutions of yeast cell suspensions were spotted and incubated for 3 days at 28°C.

Amino acid import assays: MtAPC1 was cloned into pDR196-GW. Empty pDR196-GW was the control. Both were transformed into 22Δ10AA (MAT α gap1-1 put4-1 uga4-1 can1::HisG lyp1-alp1::HisG hip1::HisG dip5::HisG gnp1Δ agp1Δ ura3-1) received from Dr. Guillaume Pilot of Virginia Tech. This line can for the 16 amino acids (Gly, Ala, Val, Met, Ile, Leu, Phe, Trp, Pro, Ser, Thr, Tyr, Asp, Asn, Glu, or Gln) whereas it cannot test for Lys, His, or Cys (Besnard et al., 2016). 22Δ10AA grows normally on Arg at 10 mM and 10 mM ammonium sulfate and therefore cannot test Arg import. MtAPC1 were inserted into pDR196-GW vector and transformed into 22Δ10AA cells and selected on medium without uracil. The line complemented with MtAPC1 was grown separately on 10mM of each of the 16 amino acids the original strain 22Δ10AA cannot utilize as a sole nitrogen source.

The BioTek Gen5 software with the BioTek Epoch 2 microplate reader was used for yeast import assays. A 24-hour read period was recorded with readings taken every 20 minutes. Plates were shaken in a linear fashion for 8 minutes prior to each reading. Five biological replications were used for each treatment.

RNAi knockdown of *MtAPC1* in *Medicago truncatula* cv. ‘R108’ roots: Since no *Medicago* mutant *Tnt1* line were identified for *MtAPC1* genetic analysis, RNAi was employed. The 481-bp segment was received from Dr. Carroll Vance’s lab (University of Minnesota) in the pK7GWIWG2 RNAi vector (Limpens et al., 2005) and transformed into *Agrobacterium rhizogenes* strain ArQual1 for *ex-vitro* root transformation. Root systems were subsequently inoculated with *SmABS7-LacZ* rhizobial line and nodules harvested at 28dpi. Control and RNAi transformed nodulating roots were harvested, RNA isolation was performed, followed by cDNA synthesis and qPCR. Olympus MVX10 MacroView microscope with the RFP filter was used. Camera was Hamamatsu ORCA_Flash4.0 v2 CMOS (C11440-22CU) for fluorescence imaging at the WVU Imaging Core Facility.

Leaf infiltration of *Nicotiana benthamiana*: *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* according to the protocol described by Li (2011). *MtAPC1* (primers: forward 5’ CACCATGGAAGACGCAATTTCTTC, reverse 5’ CACCCTAATTTTACATTCCTCTTCAACATTG) in pDR196-GW was transformed into *Agrobacterium tumefaciens*. Leaves were infiltrated by a needle-less syringe and imaged on a Leica M125 stereoscope with a red filter and Leica MC170HD camera three days later.

***In situ* hybridization:** Mature 28-dpi nodules (*Medicago truncatula* ‘R108’ inoculated with rhizobial strain ABS7) were harvested, fixed with 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1M sodium phosphate buffer under benchtop vacuum for one hour at room temperature and stored overnight at room temperature. Nodules were dehydrated by a graded ethanol series (50%, 75%, 80%, 95% and 100% twice) for 45 mins each, followed by xylene for 15 min and moved to a 50:50 mixture of xylene and paraplast for two hours at 60°C and then embedded in paraplast and sectioned into 10-mm slices with a microtome. Sections were dried overnight at 37°C and incubated at 60°C for one hour, emerged twice in xylene (15 min each), and rehydrated according to BioChain protocol. The probe was produced by cloning a 150-bp fragment of the *MtAPC1* gene (primers: forward 5’ CACCAGCAGCCACCAAATGAAGAA, reverse 5’ CGGAGACAGCATAACGACAAA) into the pGEM-T Easy vector. The cloned vector was opened with restriction enzymes *SpeI* and *SacII* and transcribed *in vitro* with T7 RNA polymerase (antisense probe) and Sp6 RNA polymerase (sense probe). *In situ* hybridization was performed according to BioChain manual and instructions (catalog number K2191020) and Sigma Aldrich DIG RNA Labeling (SP6/T7) protocol.

Syntenic analysis: Comparison of output from CoGe: Comparative Genomics web tool and SynMap software using coding sequence (was used to compare the thirteen CAT family members of *Medicago truncatula* (JCVI unmasked v4, id22582), to *Lotus japonicus* (unmasked v3.0, id37292), *Glycine max* (unmasked, id37627), and *Arabidopsis thaliana* (unmasked v1, id18626) to find syntenic regions of genomes. Syntenic blocks in microsynteny within a genome or synteny between genomes was considered at a minimum of 5 gene pairs aligning within a span of 20 genes. Default settings were utilized. Tandem duplication gene sets were also examined using the comparative genomics tool (<https://genomevolution.org/coge/>; Lyons and Freeling, 2008; Lyons et al., 2008).

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APPENDICES

Supplemental Table S1. RNA-seq data normalized and reanalyzed. Organ data comes from Young et al., 2011 and nodule zone data comes from Roux et al., 2014).

APC	Annotation	Correlation	P-Value	Blade	Bud	Flower	Root	Nodule	Fl	Flld	FlIp	IZ	Zlll
Medtr8g089360.1	cationic amino acid transporter-like protein	1	0	0	0	0	0	20	0	0	0	0	4
Medtr5g026850.1	cyclops protein, putative	0.94	0	0	0	0	21	52	1	0	1	2	5
Medtr6g057520.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.94	0	0	0	0	3	864	0	1	157	419	369
Medtr7g011790.1	DUF1336 family protein	0.94	0	0	0	2	0	10	0	0	0	1	1
Medtr1g061050.1	fructose-bisphosphate aldolase	0.93	0	4	13	11	127	195	2	1	4	10	25
Medtr4g007090.1	phospholipid N-methyltransferase	0.93	0	0	0	0	1	53	1	6	8	12	11
Medtr7g079430.1	UDP-glucose:sterol glucosyltransferase	0.93	0	0	1	1	1	14	0	1	0	1	2
Medtr7g098180.1	peptide transporter	0.93	0	0	0	0	8	40	0	0	1	1	3
Medtr8g087710.1	major intrinsic protein (MIP) family transporter	0.93	0	7	8	1	3	395	1	2	12	23	110
Medtr1g116930.1	peptide/nitrate transporter	0.92	0	0	0	0	0	30	0	1	1	2	3
Medtr4g094360.3	nodulin MtN21/EamA-like transporter family protein	0.92	0	0	0	0	0	26	0	0	4	7	17
Medtr8g018290.1	phospholipid-transporting ATPase-like protein	0.92	0	1	3	4	2	12	0	0	0	0	1
Medtr2g076010.1	pathogenesis-like protein	0.91	0	1	4	58	0	88	0	0	1	2	2
Medtr2g094620.1	pleckstrin-like (PH) and lipid-binding START domain protein	0.91	0	1	2	0	2	11	0	0	0	1	1
Medtr6g071070.1	glutamine synthetase domain protein	0.91	0	61	363	208	355	1435	4	3	14	39	94
Medtr6g086170.1	sulfate/bicarbonate/oxalate exchanger and transporter sat-1	0.91	0	0	0	1	6	193	0	1	5	10	31
Medtr1g079730.1	carboxyl-terminal peptidase	0.9	0	0	2	1	1	19	0	0	1	1	2
Medtr2g098420.1	hypothetical protein	0.9	0	2	2	3	2	11	0	0	0	1	1
Medtr2g100710.1	6-phosphofructokinase	0.9	0	5	5	42	15	49	0	0	1	2	4
Medtr3g015870.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	1	577	0	1	70	186	285
Medtr4g103495.1	subtilisin-like serine protease	0.9	0	0	0	0	0	1	0	0	0	0	0
Medtr4g108170.1	ABC transporter A family protein	0.9	0	0	0	0	0	21	0	0	1	1	1
Medtr5g011750.1	hypothetical protein	0.9	0	0	3	2	0	19	2	2	2	4	9
Medtr5g072420.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	1	33	0	0	2	3	3
Medtr7g078060.1	O-acyltransferase WSD1-like protein	0.9	0	0	0	0	0	14	0	0	1	1	1
Medtr7g082810.1	MATE efflux family protein	0.9	0	0	0	0	1	38	0	0	2	4	8
Medtr7g090950.1	eukaryotic-type carbonic anhydrase	0.9	0	0	0	9	0	16	0	0	2	5	14
Medtr7g096870.1	high affinity inorganic phosphate transporter	0.9	0	0	0	0	0	27	0	0	1	2	5
Medtr8g076190.1	ATP-binding microtubule motor family protein	0.9	0	0	0	2	0	19	0	0	1	1	3

Supplemental Figure S1. Liquid cultures for amino acid import assays. Negative control lacked a nitrogen source. Ammonium sulfate was positive control. All concentrations of amino acids as well as ammonium sulfate were 10 mM. Blue line represents untransformed yeast strain 22Δ10AA, orange line represents empty vector, and gray line represents yeast complemented with MtAPC1. Bars represent standard errors of 5 replicates. Growth curves were collected over 24 hours with readings every 20 minutes.

Chapter 5

Functional characterization of the *Medicago truncatula* nodule-specific copper transporter *MtCOPT1*

ABSTRACT

Copper (Cu^{2+}) is an essential micronutrient for plants, playing important roles in photosynthesis, mitochondrial respiration, detoxification of reactive oxygen species, and oxidative stress protection. A fine equilibrium must be maintained to move Cu^{2+} to the enzymes that require it as a cofactor, but not move so much as to cause toxicity through Fenton-like chemistry, cellular damage or displacement of other essential ions. This equilibrium is supported by specific membrane transporters. Cu^{2+} import into the cell by channels of the Copper transporter (Ctr) family (TCDB #1.A.56) is conserved across eukaryotes. The three-transmembrane domain topology of this family is highly conserved. The signature domain motif, $\text{MX}_3\text{MX}_{12}\text{GX}_3\text{G}$ (where X represents any amino acid residue), spans from the end of the second through the third transmembrane domain. In legumes, the exact function of Cu^{2+} during symbiotic nitrogen fixation remains undetermined. We identified eight *CTR* genes in the genome of the model legume, *Medicago truncatula*. One in particular is expressed solely in the nodule (*Medtr4g019870*, MtCOPT1), leading us to hypothesize that it plays an essential role during symbiotic nitrogen fixation. MtCOPT1 is highly and specifically expressed in the interzone of infected nodule cells. Analysis of gene architecture, transmembrane topology, and gene expression data was performed and showed gene architecture and transmembrane domains comparable to COPT family members. Import assay in yeast showed MtCOPT1 has affinity for Cu^{2+} , revealing that its expression in nodules may be essential for Cu^{2+} homeostasis during symbiotic nitrogen fixation.

INTRODUCTION

The roles of copper in plant physiology

Copper (Cu^{2+}) is an essential micronutrient utilized as a cofactor of enzymes involved in many biochemical plant processes including photosynthesis, cellular respiration, and oxidative stress protection through detoxification of reactive oxygen species (ROS) (Brunori et al., 2005; Andrés-Colás et al., 2010; Perea-García et al., 2013). Oxidative stress tolerance is regarded as especially important in legume nodules during symbiotic nitrogen fixation, as the reduction process is highly energetic (16 ATP/ N_2 fixed; $\Delta G^\circ = -33$ KJ/mol NH_3) (van Heeswijk et al., 2013), utilizes aerobic respiration and generates reactive oxygen species. ROS are formed during aerobic metabolism and include hydrogen peroxide (H_2O_2), singlet oxygen molecules ($^1\text{O}_2$), superoxide anions (O_2^-), and hydroxyl radicals ($\cdot\text{OH}$). The high reactivity of these ROS leads to degradation of DNA, lipids, carbohydrates and proteins and, subsequently cell death (Overmyer et al., 2003; Moller et al., 2007). Although much is known about Cu^{2+} as an essential cofactor in plants, very little remains known about its precise role in symbiotic nitrogen fixation in legume nodules.

Copper is a transition metal found in the plant under two different redox states: Cu^+ [Cu(I), the reduced state] and Cu^{2+} [Cu(II), the oxidized state]. This metal binds to different substrates depending on its redox state. In its reduced form, Cu^+ will preferentially bind to sulfur-containing compounds with a thiol or thioester group, while in the oxidized state, Cu^{2+} will preferentially bind to imidazole nitrogen groups or oxygen (Cohu and Pilon, 2010; Printz et al., 2016). Plant compounds to which Cu^{2+} bind include ascorbate oxidase, polyphenol oxidase, Cu-Zn superoxidase dismutase, plastocyanin, cytochrome c oxidase, and copper chaperones (O'Halloran and Culotta, 2000; Hansch and Mendel, 2009). Given the dual redox nature of Cu in physiological conditions, it can be used a wide range of interactions within the cell from stabilizing structures to driving reactions. However, excess Cu^{2+} is highly toxic and can lead to cell damage and death. This indicates a fine equilibrium must be maintained in cells to stave off damage but allow necessary physiological processes to occur with Cu^{2+} as a cofactor (Hansch and Mandel, 2009; Festa and Thiele, 2011).

Copper toxicity is due to the excess production of hydroxyl radicals when present in excess as free Cu ions, which can participate in Fenton-like redox reactions with organic molecules (Pham

et al., 2013), thus damaging nucleic acids, proteins, and lipids in the cell. Additionally, it can also displace other essential cofactor metals, thus hampering enzymatic reactions (Burkhead et al., 2009). Organisms have developed mechanisms to maintain metal homeostasis to avoid Cu^{2+} deficiency as well as its toxicity within their cells.

Cu^{2+} is found in many plant cells spaces and organelles, including the cytosol, apoplast, thylakoid lumen, inner mitochondrial membrane and the endoplasmic reticulum. As a cofactor, Cu^{2+} is involved in photosynthesis by participating in the electron transfer process with plastocyanin, in addition to roles with $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase (SOD) to detoxify ROS in chloroplasts, and as a cofactor in ethylene receptors, apoplastic oxidases, and mitochondrial cytochrome c oxidase in the electron transport chain (Tainer et al., 1983; Redinbo et al., 1994; Brunori et al., 1998). Additionally, superoxide dismutase (SOD) utilizes Cu^{2+} to activate the two-step dismutation of superoxide radicals to hydrogen peroxide and oxygen during aerobic respiration (Tainer et al., 1983; Culotta et al., 2006).

Plant phenotypes associated with Cu toxicity are quite similar to those of Fe deficiency, including increased oxidative stress and leaf chlorosis (Pätsikkä et al., 2002). Cu^{2+} -deficient plants show chlorosis that first develops in young leaf tips before the formation of necrotic lesions. These symptoms are consistent with a decreased function of plastocyanin and evidenced by an impaired photosynthetic transport chain and a reduction in non-photochemical quenching (Abdel-Ghany and Pilon, 2008).

Copper is taken up from the soil as Cu^{2+} by specific transporters and reduced by a ferric reductase oxidase at the plasma membrane (Mukherjee et al., 2006; Bernal et al., 2012; Zhou et al., 2016; Printz et al., 2016). In plants, only a few transporter families have been specifically associated with Cu transport: channels of the Copper Transporter family (Ctr, TCDB 1.A.56) and secondary transporters of Plant Copper Uptake Porter family (PI-Cu-UP, 2.A.1.82) (Andrés-Colás et al. 2010; Klaumann et al., 2011; Martins et al., 2012; Li et al., 2013). Additional families reported to be involved with transport of divalent metals, including copper, are the Oligopeptide Transporter (OPT) Family (2.A.67) (Waters et al., 2006) and the Zinc (Zn^{2+})-Iron (Fe^{2+}) Permease (ZIP) Family (2.A.5) (Wintz et al., 2003; Puig et al., 2007; Del Pozo et al., 2010). Below, we further explore the Ctr channels in plants to contextualize the physiology roles members of this family play in Cu homeostasis.

The Copper transporter family in plants

The transporter classification database (TCDB: www.tcdb.org) contains the family 1.A.56 known as the copper transporter (Ctr) family of channels.

Figure 5.1. Phylogenetic analysis of all members of the Ctr/COPT channel family in select plant species. Some of the CTR protein in *Chlamydomonas reinhardtii* are more distantly related to members in higher plant species, including *Arabidopsis thaliana*, rice (*Oryza sativa*), and the legumes soybean (*Glycine max*), *Medicago truncatula*, and common bean (*Phaseolus vulgaris*). Branch length represents average residue substitution per site. Species are color coded with *Medicago truncatula* represented with black dots.

Within the Ctr family, proteins are denoted CTR in yeast, fungi and animals, whereas they have been named COPT in plants. Different quaternary structures can be utilized to create a functional Cu^{2+} transporter. CTR/COPT proteins can be functional as monomers, homodimers, homotrimers (Lee et al., 2002), or as heterocomplexes with other CTR/COPT proteins or non-CTR/COPT proteins to transport Cu^{2+} (Beaudoin et al., 2006; Yuan et al., 2010).

The founding member of this family is the *Saccharomyces cerevisiae* CTR1 transporter, which encodes a high affinity Cu^{2+} uptake protein localized to the plasma membrane. CTR1 loss of function leads to altered cellular responses to extracellular Cu^{2+} , therefore demonstrating a physiologic role of Cu^{2+} transport in the cytosol (Dancis et al., 1994a; Dancis et al., 1994b). Unlike other members of the Ctr family, which can form heterogeneous complexes, the functional CTR1

channel can only be formed as homotrimers (Dancis et al., 1994a; Beaudoin et al., 2006; Yuan et al., 2010).

Pertaining to the structure of Ctr transporters, in members across all kingdoms of life there is a well conserved MX_3M motif (where X is any amino acid residue) within transmembrane domain (TMD) 2 and a conserved GX_3G domain in TMD 3 (van den Berghe et al., 2007) – the so called $\text{MX}_3\text{MX}_{12}\text{GX}_3\text{G}$ motif. which interacts with the extracellular matrix. The MX_3M motif is important to Cu^{2+} uptake, but not to protein-protein interactions (Puig et al., 2002). In turn, the GX_3G motif is essential to the trimeric assembly of Ctr proteins in yeast (Puig et al., 2002; Aller et al., 2004). Both motifs are thought to play a role in the Cu^{2+} uptake and assembly of the functional heterotrimeric Ctr4/Ctr5 in yeast (Puig et al., 2002; Aller et al., 2004; Ioannoni et al., 2010; Beaudoin et al., 2011; Plante et al., 2014). With the exception of ScCTR3, all Ctr family members are rich in methionine residues within their hydrophilic N-terminal tails (Puig et al., 2002).

Saccharomyces cerevisiae imports Cu^{2+} into the cytosol via three high-affinity transporters: ScCTR1, ScCTR2, and ScCTR3. ScCTR1 and ScCTR3 are located in the plasma membrane and attain Cu^{2+} from the environment (Dancis et al., 1994a; Marjorette et al., 2000). Meanwhile ScCTR2 is found in the tonoplast and imports Cu^{2+} stored in the vacuole of the cell into the cytoplasm (Portnoy et al., 2001; Puig and Thiele, 2002). ScCTR proteins are highly specific for reduced Cu^+ . Copper exists extensively as Cu^{2+} , and it must first be reduced by a ferric/cupric reductase in order to be transported (Hassett and Kosman, 1995).

The human CTR1 (hCTR1) forms homotrimer complexes that localize to the plasma membrane (Lee et al., 2002; Aller and Unger, 2006; Gupta and Lutsenko, 2009; Maryon et al., 2013). Certain intracellular elements of the protein, such as the carboxyl tail are not essential for the human hCTR1 transporter permeation, but they are essential for regulation of Cu^{2+} entry (Aller and Unger, 2006; Mayron et al., 2013). CTRs have metal-binding motifs in the N terminus as well as the second TMD (Puig et al., 2002; Guo et al., 2004; Eisses and Kaplan, 2005; Beaudoin et al., 2006). The two methionine residues in the N- terminus as well as two near in the extracellular end of the second TMD of hCTR1 are necessary for Cu^{2+} import (Puig et al., 2002; Eisses et al., 2005; Mayron et al., 2013). The TMD hydrophobicity pattern of CTR/COPT proteins indicates a conserved presence of three transmembrane domains, an external N-terminus, and a cytosolic C-terminus across species (Yuan et al., 2011; Jung et al., 2012; Bellemare et al., 2002; Eisses and

Kaplan, 2002).

There are six COPT genes in the plant model, *Arabidopsis thaliana*. AtCOPT1 is a high-affinity Cu^{2+} transporter expressed in primary and secondary root tips (Perea-García et al., 2013) and localized to the plasma membrane (Andrés-Colás et al., 2010). AtCOPT6 also localizes to the plasma membrane, it is highly expressed in leaves and can interact with itself or form complex with AtCOPT1 *in vitro* (Jung et al., 2012). AtCOPT2 is a high-affinity Cu^{2+} transporter that is induced under Cu^{2+} and iron deficiencies and expressed in subapical root regions where it localizes to the plasma membrane (Perea-García et al., 2013). In *Arabidopsis*, the tonoplast-localized COPT5 is mostly expressed in siliques and root vascular tissues and mobilizes Cu^{2+} under severe deficiency (Sancenón et al., 2004; Garcia-Molina et al., 2011; Klaumann et al., 2011; Perea-García et al., 2013). There are seven COPT genes in the monocot model, rice (Yuan et al., 2011). OsCOPT3 localizes to the plasma membrane and possibly cooperates with OsCOPT2, OsCOPT4 and OsCOPT6 to mediate Cu^{2+} transport (Yuan et al., 2011).

Following, we explore the genomic context of the model legume *Medicago truncatula* in search of key COPT transporters potentially related with Cu homeostasis in root nodules during symbiotic nitrogen fixation.

RESULTS AND DISCUSSION

Phylogenetic analyses of the CTR/COPT transporters

A phylogenetic analysis was performed with all eight Ctr family members in the *Medicago truncatula* genome and all members listed in the TCDB curated database (**Figure 5.2**). The closest paralog to MtCOPT1 in the *Medicago* genome is *Medtr7g066070.1* which shares 56% identity with MtCOPT1. *Arabidopsis thaliana* AtCOPT1 and AtCOPT6 are belong to the same clade as MtCOPT1. *Arabidopsis thaliana* COPT1 and COPT6 localize to the plasma membrane (Andrés-Colás et al., 2010, Jung et al., 2012). *Arabidopsis* COPT6 has been shown to interact with itself at the plasma membrane and also form heterologous interactions with COPT1 (Jung et al., 2012). The closely related *Oryza sativa* COPT3 also localizes to the plasma membrane and possibly cooperates *in vivo* with COPT2, COPT4 and COPT6 to mediate Cu^{2+} transport (Yuan et al., 2011). Whereas Ctr transporters from animals and fungi, as well as green algae, cluster in a clade, those

from higher plants are grouped in their own clade, with the exception of *Medtr6g061710.1*, which locates as an outgroup member.

Figure 5.2. Molecular phylogenetic tree of the complete Ctr protein family in *Medicago truncatula* and the CTR family members found in the Transporter Classification Database (www.TCDB.org). At, *Arabidopsis thaliana*; Ca, *Candida albicans*; Cg, *Colletotrichum gloeosporioides* (fungus); Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Gs, *Galdieria sulphuraria* (red alga); Hs, *Homo sapiens*; Os, *Oryza sativa*; Pf, *Plasmodium falciparum* (malarial parasite, Alveolata, protist); Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Vv, *Vitis vinifera*. MtCOPT1 is represented by a black dot, green dots represent plants, blue represents fungi, and all others are red. Vv, CBI27797.3; Pf, XP_001348543.1; Gs, XP_005705236.1.

***Medicago truncatula* nodule specific putative copper transporter**

To analyze the *Medicago* Ctr family members, a phylogenetic analysis with the eight *Medicago truncatula* COPT (**Figure 5.3**) and a heatmap was constructed with gene expression of the six major organs of the mature plant. Only *Medtr4g019870.1* is expressed nodule specifically, indicating a potential function related to symbiotic nitrogen fixation.

Figure 5.3. Phylogenetic tree of the eight *Medicago truncatula* COPT family members. The tree was constructed using Mega 7 using the Maximum Likelihood method with 1000 bootstraps. Heatmap was constructed using MeV. White is low expression with highest expression shown by red. MtCOPT1 (*Medtr7g019870.1*) is the only family member to be nodule specific and show high expression in the nodule. Scale is shown below the heatmap. Gene architecture is shown for the 8 *Medicago truncatula* family members of the Ctr family of transporters (TCDB 1.A.56). Exons are shown in light blue, UTR regions are shown as darker blue and introns are shown as black lines linking exons or sections of UTR.

Of the eight *Medicago* Ctr family members, only four showed expression in organs of the mature plant growing under optimal conditions, in which plants were fertilized with 1/2X B&D solution (Broughton and Dilworth, 1971), which contains 0.2 μM Cu^{2+} (**Figure 5.3**). Under these conditions, just one Ctr gene was nodule specific, indicating it may play an essential function of transporting Cu^{2+} to participate in the symbiotic nitrogen fixation process. It is possible, however, that under lower Cu^{2+} conditions, significant changes in transcription may occur. The phylogenetic analysis based on protein sequence corroborates the distinctions found in gene architecture. All but one of the *Ctr* genes in *Medicago* have no introns. Indeed, *Medtr6g061710* has a distinct predicted exon-intron structure, including both UTRs split into two exons.

Based on their gene expression, *Medtr3g105330.1* and *Medtr4g065660.1* may encompass Cu uptake and xylem loading systems in the root under normal Cu^{2+} supply levels. The nodule-specific expression of *Medtr7g019870.1* suggests it to be a delivery system into the nodules, perhaps into infected cells carrying out active symbiotic nitrogen fixation.

Gene architecture of COPT genes and predicted transmembrane domains are representative of the Ctr family

Congruent with the other functionally characterized members of this family (Sancenon et al., 2003; Sancenon et al., 2004; Jung et al., 2012), MtCOPT1 contains three TMDs with a fully consistent $\text{MX}_3\text{MX}_{12}\text{GX}_3\text{G}$ motif (**Figure 5.4B**), as predicted by the topology analysis programs TMHMM (**Figure 5.5A**) (Sonnhammer et al., 1998; Krogh et al., 2001) and Protter (Omasits et al., 2014) (**Figure 5.5B**). (Both programs also predicted an extracellular N-terminus and an intracellular C-terminus. As expected, the MtCOPT1 hydrophilic N-terminal tail also contains the conserved methionine residues present in members of this family (**Figure 5.5B**).

A

B

MKMIHMMKEDMKMHMNLWYWGKDAIILFPGWPNQSLGMYILALS
 FVFFLALVVEFLPNKSTIKQGTNHIKGGGLIQAIYFFRISFLYLMLAVMS
FNIGIFIAAVVGHTIGFFLVKSHDIFAANKEQNRESAMTIDDI

Figure 5.4. Predicted gene architecture and protein sequence of the nodule-specific *MtCOPT1* transporter gene *Medtr4g019870*. (A) *MtCOPT1* is found on the p arm of chromosome 4 of *Medicago truncatula*. This gene has one predicted exon (gray bar) and no introns. Untranslated regions (UTRs) are shown in black on the 5' and 3' ends of the exon. Chromosomal coordinates of start and stop codons and limits of each UTR (IMGAG annotation v.4.1) are indicated. (B) Protein sequence of *Medtr4g019870.1* is shown with the three transmembrane domains underlined and conserved amino acids of the $\text{MX}_3\text{MX}_{12}\text{GX}_3\text{G}$ motif shown in red.

Figure 5.5. Membrane topology of MtCOPT1 (*Medtr4g019870.1*). (A) The three transmembrane α -helices characteristic of the Ctr family were predicted using TMHMM v.2 (Sonnhammer et al., 1998; Krogh et al., 2001). (B) Predicted transmembrane domains of MtCOPT1 protein using the Protter program (Omasits et al., 2014). The expected positions of the terminal regions of the protein, extracellular N-terminus and intracellular C-terminus were confirmed by both topology programs. The conserved methionine residues in the N-terminal tail is highlighted in blue. (C) The conserved MX₃MX₁₂GX₃G (Pfam PF04145) identified in the MtCOPT1 protein. The conserved methionine residues of the MX₃M motif spanning from the second to third transmembrane domains is shown as yellow circles and the conserved glycine residues of the GX₃G in the third transmembrane domain is shown as green circles.

Protein modification of MtCOPT1 was prospected using the Medicago Protein Compendium (Marx et al., 2016). Two amino acid residues were identified as phosphorylated in the C-terminus from protein sequencing results in the database. S130 can be phosphorylated alone or in company with T133 (**Figure 5.6**). The implications of these protein modifications and the kinases involved remain to be determined.

Figure 5.6. Protein sequencing analysis of MtCOPT1 in the Medicago Protein Compendium (<http://compendium.medicago.wisc.edu>). (A) Protein features related to the three transmembrane domains (underlined), the Ctr conserved domain (MX₃MX₁₂GX₃G, in red), the phosphorylated serine and threonine residues (in blue). The protein sequencing data currently offers 54% coverage for MtCOPT1. (B, C) Expression profiles for the two identified MtCOPT1 phosphorylation isoforms relative to apical meristem. TMT: Tandem Mass Tag.

MtCOPT1 expression is highest in the nodule interzone

Expression of *MtCOPT1* was first examined using information from the *Medicago truncatula* Gene Expression Atlas (Benedito et al., 2008; He et al., 2009). Expression of *MtCOPT1* is nodule specific (**Figure 5.3**). Within zones of the mature nodule, *MtCOPT1* expression is highest when the nodule cells have been infected and the bacteria have begun to differentiate into fully functional bacteroids within the nodule and afterwards (**Figure 5.7A**). The interzone has peak expression followed by the fixation zone (**Figure 5.7B**). As nodules develop, expression is first seen at 10 days post inoculation (dpi) and becomes steady through 28 dpi (**Figure 5.7A and 5.8B**). The addition of nitrate, an inhibitor of symbiotic nitrogen fixation, led to *MtCOPT1* downregulation (**Figure 5.7B**). *MtCOPT1* expression is only seen after 10 dpi and required bacteroid differentiation (**Figure 5.8A**), suggesting a symbiotic function in mature nodules.

Figure 5.7. Transcriptional profile of the *MtCOPT1* gene (*Medtr4g019870.1*). (A) During nodule development, *MtCOPT1* expression starts at ten days post inoculation. Data from Medicago Gene Atlas (<https://mtgea.noble.org/v3/>; Benedito et al., 2008, He et al., 2009). (B) RNA-seq laser-capture microdissection (LCM) data (Roux et al., 2014) shows highest expression in the inter and nitrogen fixation zones.

Figure 5.8. RT-qPCR of *MtCOPT1* in root systems of *Medicago truncatula* ecotype ‘R108’ inoculated with *Sm1021* rhizobia. (A) *MtCOPT1* expression at 28 dpi nodulating roots inoculated with rhizobial strains *exoA*, *bacA*, *fixJ*, or a functional strain containing *LacZ* relative to uninoculated root. (B) Time-course of *MtCOPT1* expression in root systems inoculated with the functional rhizobial strain.

Yeast import assays show substrate affinity for copper

Copper import assays were performed with the *Saccharomyces cerevisiae* mutant strain DTY272 (*ctr1Δ ctr3Δ. ctr1Δ ctr3Δ*), which shows Cu^{2+} uptake impairment and allows testing of import by genetic complementation.

To test whether *MtCOPT1* is a Cu^{2+} importer, we expressed this gene in the mutant yeast strain on agar media containing increasing concentrations of CuSO_4 . Very little growth was observed without any Cu^{2+} supplementation (**Figure 5.9**). Growth on 0.1 mM CuSO_4 and was steady through 8 mM (**Figure 5.9**). Toxicity levels started at 16 mM CuSO_4 growth decreases and no growth was observed at 32 mM CuSO_4 . Deficiency is overcome at 0.1 mM. *MtCOPT1* induced better growth than either the DTY272 yeast strain alone, or the DTY272 transformed with the empty vector at both deficiency levels and at toxicity levels. At deficiency levels (0.01 mM), better growth was seen with pDR196-GW-*MtCOPT1* compared to both the original mutant yeast strain or the line complemented with the empty vector, indicating import of Cu^{2+} across the plasma membrane. It was expected that the import of Cu^{2+} increased survival of cells at deficiency levels, but it was not expected it to confer an advantage at toxicity levels.

Figure 5.9. Copper import assays. YPEG media + CuSO₄ (0, 0.01, 0.1, 1, 2, 4, 8, 16, 32 mM CuSO₄). Yeast was grown to OD₆₀₀=1.0. Cells were washed three times in sterile water and readjusted to OD₆₀₀=1.0. 10-fold dilutions of 5 uL drops were pipetted onto YPEG + CuSO₄. Plates were grown at 28°C and photographed at 3 days. Top row is DTY272, second row is DTY272 empty vector (pDR196-GW), third row is DTY272 COPT in pDR196-GW. *S. cerevisiae* DTY272 *ctr1Δctr3Δ* mutant was a gift from Dr. Dennis Thiele from Duke University.

Gene Network shows correlation with nodule specific cysteine rich peptides

A gene network based on gene expression of the Medicago transcriptome and centered on *MtCOPT1* was generated. Fifty-one genes correlated with a stringent threshold ($\rho \geq 0.90$). Among them, nineteen Nodule-specific Cysteine-Rich (NCR) secreted proteins showed first-order associations with *MtCOPT1*. Three genes coding for leghemoglobin, a protein essential for the nitrogen fixation process, were also found strongly associated with *MtCOPT1* (**Supplemental Table S1**). These results point out to a symbiotic role of *MtCOPT1* during nitrogen fixation. The identification of common transcription factors as well as *cis*-elements in promoters of correlated genes in this network may reveal how symbiotic genes coordinate gene expression to allow symbiotic nitrogen fixation to occur in infected cells of the nodule after bacteroid differentiation.

Figure 5.10. Gene network centered on *MtCOPT1* gene (yellow node). First-order gene associations were determined using Pearson Coefficient Correlation ($\rho \geq 0.90$). Visualization was generated in Cytoscape (Shannon et al., 2003). The highest correlation found was $\rho = 0.94$. Supplemental Table S1 in the appendix shows the expression matrix and individual correlation values for the 51 genes in this network.

CONCLUSION

Despite the ubiquitous importance of Cu^{2+} in plant physiology, and especially in ROS detoxification in cells with high intensity of aerobic respiration activity, such as in nodule cells, little attention had been placed in identifying Cu^{2+} import systems in legume nodules. We identified a nodule-specific Cu^{2+} transporter with import activity that is expressed after bacteroid differentiation and continues its expression during active nitrogen fixation.

NOTE: Recently, Senovilla et al. (2018) found *MtCOPT1* to localize to the plasma membrane of cells in the differentiation, interzone and early fixation zones and hypothesized *MtCOPT1* imports Cu^{2+} from the apoplast to the cytosol to feed the metabolic processes of symbiotic nitrogen fixation. Their results confirm our findings and precluded the continuation of this work due to the lack of novelty for publication in a peer-reviewed journal.

MATERIALS AND METHODS

Phylogenetic analyses and membrane topology prediction: Phylogenetic trees were produced using MEGA v.7 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2011; Tamura et al., 2012). Sequences were aligned by Muscle (Edgar, 2004) and the Maximum Likelihood method used the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood is shown. The analysis involved 29 amino acid sequences. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted in MEGA7 (Felsenstein 1985; Kumar et al., 2015). *Medicago truncatula* sequences were obtained from <http://bioinfo3.noble.org/getseq/>. TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>; Möller et al., 2001) and Protter (wlab.ethz.ch/protter) were used to predict transmembrane domains with protein sequences as input.

Gene expression analyses: Gene expression analysis was assessed from the Affymetrix *Medicago truncatula* GeneChip microarray data deposited at the Samuel Roberts Noble Foundation *Medicago truncatula* Gene Expression Atlas (<http://mtgea.noble.org/v3/>; Benedito et al., 2008). Gene expression analyses of membrane transporters in rhizobial mutants was performed on R108 root systems inoculated with *fixJ*, *bacA*, *exoA*, or *hemA::LacZ* strains of *Sinorhizium meliloti* in *Sm1021* background. A control group was not inoculated with rhizobia and was provided full nitrogen fertilization. Plant roots were harvested at 0, 3, 14, 21, and 28 dpi. RNA was extracted with mirVana miRNA Isolation kit (Life Technologies) followed by DNA removal with Turbo DNA-free kit (Applied Biosystems). First strand complementary DNA was synthesized by priming with oligo-dT using the SuperScript III First-Strand Synthesis Super mix (Life Technologies) following the manual instructions. Primers used were: MtCOPT1 forward: 5' CAT TGC TGC TGT TGG TC, MtCOPT1 reverse: 5' CAA TAG TCA TGG CGG ACT CG. Three biological and three technical replicates were run. SYBR green master mix and 5 μ L reactions were used on Applied Biosystems 7500 Real Time PCR System and 7500 software. All templates were amplified using the same protocol: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curves were produced following the 40 cycles by heating to 95°C for 15 seconds followed by 60°C for 15 seconds and 95°C for 15 seconds. 7500 Software (Applied Biosystems) gave C_T threshold cycle values. Excel was used to organize data and calculate geometric means of technical replications and calculate averages and standard errors. Technical outliers were eliminated from analysis.

The housekeeping genes chosen for this experiment were: EF1 α (*Medtr6g021800*), tubulin (*Medtr7g089120*), and MSC27 (X98618.1). NormFinder software (Andersen et al., 2004) was used to identify stably expressed housekeeping genes in the experiment and MSC27 was excluded from the calculations.

Copper import assays: Transcript coding regions with stop codons of gene of interest were transferred from the entry vector to the destination vector pDR196-GW via LR recombination, followed by yeast transformation with the LiAc/SS-DNA/PEG transformation procedure (Gietz and Woods, 2002). Yeast transport mutant strains for substrates of interest were selected. For

influx transport, the wild-type non-transformed yeast strain and the empty vector yeast strains were the positive controls for auxotrophic yeast strains with the plasmid containing the transcripts' coding region (Kraidlova et al., 2011). DTY272 (*ctr1* Δ *ctr3* Δ mutant strain of *S. cerevisiae*) was used for import assays. DTY272 yeast strain (control), DTY272 with empty pDR196-GW (control), and DTY272 with COPT in pDR196-GW were grown on YPEG + CuSO₄ (0mM, 0.01 mM, 0.1 mM, 1 mM, 2 mM, 4 mM, 8 mM, 16 mM, and 32 mM CuSO₄). Five microliter drops of ten-fold dilutions of exponentially growing cells were normalized to A₆₀₀=1.0 and were spotted on plates and grown at 28°C for 3 days.

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APPENDIX

Supplemental Table S1. First-order correlations ($0.9 \geq p \geq 1.0$) of gene network centered on *MtCOPT1*, shown in Figure 10. Raw RNA-Seq data (SRA files) from mature organs of the plant (Young et al., 2011) and zones of the mature nodule (Roux et al., 2014) were reanalyzed and normalized to transcripts per million (TPM).

Gene	Annotation	Correlation	P-Value	Blade	Bud	Bud	Flower	Root	Nodule	Fl	FlId	FlIp	IZ	ZIII
Medtr4g019870.1	Ctr family copper transporter	1	0	0	0	0	0	1	201	0	0	46	112	61
Medtr1g071880.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.94	0	0	0	0	0	0	171	0	0	16	41	31
Medtr5g084260.1	Nodule-specific Glycine Rich Peptide	0.94	0	0	1	0	0	9	1983	0	0	46	370	104
Medtr1g079730.1	carboxyl-terminal peptidase	0.93	0	0	2	3	1	1	19	0	0	1	1	2
Medtr1g090820.3	leghemoglobin Lb120-1	0.93	0	0	0	0	0	34	3359	1	8	198	1003	377
Medtr3g015870.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	1	577	0	1	70	186	285
Medtr4g013270.1	CASP ARALYDRAFT-like protein	0.93	0	0	0	0	0	0	205	0	3	8	19	13
Medtr6g006220.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	0	67	0	0	13	38	19
Medtr6g445080.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	12	8000	1	2	137	623	501
Medtr6g090485.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	0	316	0	0	116	250	130
Medtr7g031915.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	0	295	0	0	33	97	65
Medtr7g055763.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	0	56	0	0	20	73	28
Medtr7g057560.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.93	0	0	0	0	0	0	56	0	0	20	73	28
Medtr1g088150.1	transmembrane protein, putative	0.92	0	0	0	0	0	0	17	0	0	1	3	3
Medtr2g073170.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.92	0	0	0	0	0	0	541	0	1	280	809	645
Medtr2g103303.1	embryo-specific protein	0.92	0	0	0	0	0	1	632	1	11	13	21	24
Medtr4g108170.1	ABC transporter A family protein	0.92	0	0	0	1	0	0	21	0	0	1	1	1
Medtr7g071690.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.92	0	0	0	0	0	4	714	0	1	286	848	98
Medtr8g074890.1	prolyl 4-hydroxylase alpha-like protein	0.92	0	0	0	0	0	0	17	0	0	2	4	2
Medtr1g042940.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.91	0	0	0	0	0	3	1058	2	10	804	1439	1231
Medtr2g016230.1	RmlC-type cupin	0.91	0	1	5	2	4	2	40	0	0	3	6	5
Medtr3g055440.5	nodulin-25 protein	0.91	0	0	0	0	0	0	0	0	0	0	0	0
Medtr4g007090.1	phospholipid N-methyltransferase	0.91	0	0	0	0	0	1	53	1	6	8	12	11

Medtr4g103495.1	subtilisin-like serine protease	0.91	0	0	0	0	0	0	1	0	0	0	0	0
Medtr5g008040.1	12-oxophytodienoate reductase-like protein	0.91	0	0	0	0	0	0	54	0	0	7	10	1
Medtr5g022390.1	rhodanese-related sulfurtransferase	0.91	0	0	0	0	1	136	401	0	0	24	66	39
Medtr6g452900.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.91	0	0	0	0	0	0	229	0	1	152	445	262
Medtr7g008130.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.91	0	0	0	0	0	0	40	0	0	10	25	20
Medtr7g028550.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.91	0	0	0	0	0	0	40	0	0	15	27	17
Medtr7g056460.1	leguminosin group567 LEED...PEED secreted peptide	0.91	0	0	0	0	0	0	10	0	0	3	9	7
Medtr7g058880.1	late nodulin	0.91	0	0	0	0	0	0	304	1	12	29	82	66
Medtr1g008280.2	signal peptide peptidase-like protein	0.9	0	10	21	26	11	29	127	1	4	14	21	9
Medtr1g064350.1	GRF zinc finger protein	0.9	0	0	0	0	0	0	219	0	3	15	66	26
Medtr1g116930.1	peptide/nitrate transporter	0.9	0	0	0	0	0	0	30	0	1	1	2	3
Medtr2g032710.1	polygalacturonase plant-like protein	0.9	0	3	6	6	5	1	13	0	0	1	1	0
Medtr2g103307.1	embryo-specific protein	0.9	0	0	0	0	0	0	547	0	0	9	19	14
Medtr3g012380.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	0	20	1529	0	2	340	1038	652
Medtr3g068070.1	NAD(P)-binding rossmann-fold protein	0.9	0	0	0	0	0	0	26	0	0	3	5	1
Medtr3g084820.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	1	0	0	0	19	3461	0	1	273	1026	566
Medtr4g057600.1	serine carboxypeptidase-like protein	0.9	0	0	0	0	0	0	16	0	1	1	2	2
Medtr4g063735.1	wuschel-related homeobox protein	0.9	0	0	0	0	0	0	23	0	0	2	8	5
Medtr4g079830.1	agenet domain protein	0.9	0	0	0	0	0	2	35	0	0	5	17	8
Medtr5g063600.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	0	2	560	0	1	103	216	114
Medtr5g080440.1	leghemoglobin Lb120-1	0.9	0	0	0	0	0	3	3209	0	4	309	1105	364
Medtr5g081030.1	leghemoglobin Lb120-1	0.9	0	1	1	0	0	16	9753	2	34	245	1113	471
Medtr6g464450.1	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	0	0	272	0	0	45	114	69
Medtr6g066040.1	plant/F12B17-70 protein	0.9	0	0	0	0	0	0	26	0	0	7	12	2
Medtr7g071720.2	Nodule Cysteine-Rich (NCR) secreted peptide	0.9	0	0	0	0	0	0	280	0	0	59	159	86
Medtr7g082810.1	MATE efflux family protein	0.9	0	0	0	0	0	1	38	0	0	2	4	8
Medtr8g087720.1	major intrinsic protein (MIP) family transporter	0.9	0	0	3	3	0	5	137	0	4	7	8	15
Medtr8g103233.1	peptide/nitrate transporter plant	0.9	0	0	1	1	0	3	27	1	5	2	3	4

Chapter 6

Introducing additional nodule specific membrane transporters as potential essential participants in symbiotic nitrogen fixation in *Medicago truncatula*

Abstract

Successful symbiotic nitrogen fixation relies heavily on the movement of essential substrates to allow reactions to occur within the infected cells of nodules. Membrane transporters play a key role in moving molecules between symbionts to assist in protection during high energy production within the cell and to provide essential cofactors to enable enzyme activity. Two potential membrane transporter families were investigated. The Oligopeptide Transporter (OPT) family is classified in the Transporter Classification Database as 2.A.67. Members of the family have been shown to transport peptides from 3-8 amino acids in length and are capable of transporting glutathione. Family members typically have 12-15 transmembrane domains (TMDs) and are 600-900 amino acids in length. *Medicago truncatula* has 11 OPT family members identified in the genome. One, *Medtr7g092230.1* (*MtOPT1*) is expressed exclusively in the nodule. Expression begins early in the developing nodule and can be seen at 4 days post inoculation with expression seen in all zones except the meristem, indicating importance throughout the developing nodule process. Rhizobial mutant analysis revealed only infection by rhizobia is essential for gene expression and not bacterial differentiation or nitrogen fixation. The Vacuolar Iron Transporter (VIT) family belongs to the Transporter Classification Database 2.A.89 family. Family members have been identified in plants and yeast. Members of the vacuolar iron transporter family have been shown to transport Fe^{2+} and Mn^{2+} . The *Medicago truncatula* genome codes for six VIT transporters. One of those members, *Medtr4g094335.1*, herein called *MtSEN1*, is highly and exclusively expressed in the nodule. RT-qPCR analysis with mutant rhizobia found *MtSEN1* requires rhizobial infection for expression and upregulated expression is seen in nodules 3 days post inoculation, where most likely, in preparation for nitrogen fixation, iron is being transported for use as a cofactor in nitrogenase and leghemoglobin. Iron, glutathione, and homoglutathione are found in nodules and transporters belonging to families known for transport of those molecules

are probable transporters. Therefore, nodule specific expression of these two putative membrane transporters could potentially play an essential role in supporting symbiotic nitrogen fixation.

INTRODUCTION

The Oligopeptide transporter (OPT) and Vacuolar Iron transporter (VIT) families

In plants, glutathione (GSH, γ -L-glutamyl-L-cysteinylglycine) is the primary form of mobilized organic sulfur (Lubkowitz, 2011). As a scavenger of reactive oxygen species (ROS), glutathione and homoglutathione are thought to provide defense against stresses especially during the high consumption of ATP during cell differentiation and nitrogen fixation, when electrons can be lost and damage the cell (Pasternak et al., 2014). Additionally, glutathione is the most abundant non-protein thiol in almost all prokaryotic and eukaryotic cells (Bourbouloux et al., 2000). AtOPT6 from *Arabidopsis thaliana* was found to transport glutathione derivatives (Cagnac et al., 2004) and Opt1p from the fungus *Candida albicans* is a high-affinity glutathione transporter (Koh et al., 2002).

The OPT family of transporters (TCDB 2.A.67) is also known to have affinity to peptides of three to five residues in prokaryotes, yeast, and plants, potentially useful to help protect from ROS, created during the energy intensive process of symbiotic nitrogen fixation (Becana et al., 2010). (Homo)glutathione transported to infected cells of nodules could protect against damage. (Homo)glutathione, unique to the Leguminosae family, is composed of three amino acid residues and has been recognized as an important modulator of the efficiency of the nitrogen fixation process in legumes (Macnicol, 1987; El Msehli et al., 2011; Colville et al., 2015). OPT transporters are found exclusively in prokaryotes, plants, and fungi. The OPT family is divided into two distant clades, the OligoPeptide Transporters and the Yellow Stripe-Like proteins with affinity for secondary amino acids bound to metals (Lubkowitz, 2011). Given the number of organisms for whom genomes have already been sequenced, it is unlikely OPT genes will be found in eukaryotes other than the aforementioned domains of life (Lubkowitz, 2006). This well characterized family includes members that transport oligopeptides of 3-8 amino acid residues in length (Gomolplitinant and Saier Jr., 2011). Among the 9 OPT genes in *Arabidopsis thaliana*, OPT1, 4, 5, 6, and 7 were demonstrated to transport the pentapeptide KLLLG, while OPT4 also transports

the tetrapeptides KLGL and KLLG (Koh et al., 2002). The OPT family is one of three families responsible for peptide transport in plants. The other two are the Peptide Transporter (PTR) and the ATP-Binding Cassette (ABC) transporter superfamilies (Stacey et al., 2002). Some OPT members have also been shown to transport metal ions. *Thlaspi caerulescens* OPT3 transports Fe^{2+} , Zn^{2+} , Cd^{2+} and Cu^{2+} (Hu et al., 2012).

In *Arabidopsis thaliana*, VIT1 has been shown to transport Fe^{2+} into the vacuole of seeds (Kim et al., 2006; Roschzttardtz et al., 2009). In *Lotus japonicas*, the VIT1 homolog SEN1 is required for symbiotic nitrogen fixation and bacteroid differentiation, but its subcellular localization was not determined (Hakoyama et al., 2012). The *Saccharomyces cerevisiae* homolog CCC1 is a transporter of iron and manganese and localizes to the tonoplast and golgi (Lapinskas et al., 1996). Wheat vacuolar transporter TaVIT2 has also been shown to transport iron and manganese and localizes to the vacuolar membrane (Connorton et al., 2017). The VIT family of transporters (TCDB 2.A.89) have been shown to transport Fe^{2+} in plants.

Iron is an essential micronutrient for humans and more than a billion people suffer from anemia due to iron deficiency (WHO, 2008). Pulses and seeds are great sources of iron, and biofortification can be accomplished through transgenic crops or traditional breeding practices. Iron is the fourth most abundant element in the earth's crust, but it is the third most limiting nutrient of plant growth with over a third of the world's soils considered to be iron deficient (Grotz and Guerinot, 2006). Iron demand is increased during symbiotic nitrogen fixation as it is required in the synthesis of iron containing proteins, including leghemoglobin in the plant and nitrogenase and cytochromes of the electron transport chain within bacteroids (Seefeldt, 1994; Seefeldt et al., 2009).

RESULTS AND DISCUSSION

The transporter prediction program TransportTP was utilized to determine the number of family members in the OPT family (Li et al., 2009). In the Medicago genome, there are 11 identified OPT genes. Expression of Medicago OPT family members were analyzed to determine organ expression. The *Medicago truncatula* MtOPT1, *Medtr7g092230.1*, was found to have high nodule specific expression (**Figure 6.1C**), unlike its two closely related genes on chromosome 7. *Medtr7g092240.1* shows expression in the flower and *Medtr7g093350.1* shows expression in the

nodule but at lower levels than MtOPT1 (**Figure 6.1C**). The three closely related genes have varying gene architecture (**Figure 6.1B**). MtOPT1 has 4 predicted exons and three predicted introns and lacks predicted UTRs (**Figure 6.1D**).

Figure 6.1. MtOPT1 and closely related genes' chromosomal location, gene architecture, and organ expression. (A) Map of chromosome 7 with expanded view of adjoining OPT genes, followed by predicted gene architecture of OPT. (B) Gene architecture of adjoining *Medicago* genes. All three have one large exon and are in the same orientation. (C) RNA-Seq heatmap of mature organs of same three genes (Young et al., 2011) using MultiExperiment Viewer (MeV.tn4.org; Howe et al., 2011). (D) MtOPT1 (*Medtr7g092230.1*) has four predicted exons (red boxes) and three introns (red lines). The chromosome 7 coordinates for start and stop codons are shown. Remarkably, the MtOPT1 gene model has no predicted UTRs.

Medicago truncatula MtOPT1 is closely related to AtOPT9 from *Arabidopsis*, which has not been fully characterized. Also related are the *Candida albicans* Opt1p, as well as *Schizosaccharomyces pombe* Pgt1, which are both glutathione transporters (Thakur et al., 2008). A homolog of *Medtr7g092230.1* in *Saccharomyces cerevisiae* was characterized as a peptide

uptake system (Hauser et al., 2000). Notice the clustering of YSL, or yellow stripe 1, proteins on a separate clade (**Figure 6.2**).

Figure 6.2. Molecular Phylogenetic analysis by Maximum Likelihood method of *Medtr7g092230.1* and all eukaryotic OPT homologs of the Transporter Classification Database (www.TCDB.org; Kumar et al., 2016).

The most closely related proteins to MtOPT1 (*Medtr7g092230.1*) in the Medicago genome are *Medtr7g092240.1* and *Medtr7g092250.1* (**Figure 6.3**). *Medtr7g092230.1* and *Medtr7g092240.1* share 91% identity at the protein level. However, *Medtr7g092240.1* shows high expression in flowers and a basal constitutive activity, including in roots and nodules, while the other closely related protein, *Medtr7g092250.1*, shows high expression in nodules and basal expression elsewhere in the plant. It is tempting to suggest that this positional situation was created by asymmetrical recombinations at some point in the evolution of legumes and that one of the duplicates evolved through neofunctionalization towards nodule development and the nitrogen symbiosis.

However, **Figure 6.3** shows that the branch that holds MtOPT1 encompasses 6 legume transporters (including the three closely related proteins aforementioned) and neighbors an Arabidopsis branch holding two Arabidopsis orthologs, AtOPT1 and AtOPT5. AtOPT1 is highly expressed in flower with less expression in leaf and stem, while AtOPT5 is expressed primarily in flowers (Koh et al., 2002). AtOPT9 is expressed in pollen and microspores (Bock et al., 2006). *Medtr7g092230.1* also shows high expression in flowers with basal expression elsewhere.

Figure 6.3. Molecular Phylogenetic analysis by Maximum Likelihood method of the complete OPT gene family in *Medicago truncatula* and *Arabidopsis thaliana* genomes constructed in MEGA7 (Felsenstein 1985; Kumar et al., 2015). Notice that the two closest *Medicago* homologs to *Medtr7g092230.1* are localized next to it on the tree.

A protein alignment of the three OPT clustered on chromosome 7 (*Medtr7g092230.1*, *Medtr7g092240.1*, and *Medtr7g092250.1*) was constructed to examine the thirteen transmembrane domains of the transporter (Figure 6.4). Two OPT domains highly conserved in the OPT family are present in all three members (Figure 6.4).

Figure 6.4. Protein alignment of *Medtr7g092230.1*, *Medtr7g092240.1*, and *Medtr7g092250.1* with thirteen transmembrane domains shown below the sequences (red lines). Blue boxes show NPG and KIPPR motifs (Pfam domain: PF02669), respectively, which are conserved domains of the OPT family.

TMHMM software (<http://www.cbs.dtu.dk/services/TMHMM/>) revealed thirteen putative transmembrane domains for family member *Medtr7g092240.1*. Indeed, OPT family proteins typically have 12 to 15 transmembrane α -helical spanners (TMS). MtOPT1 analysis reveals 13 predicted transmembrane domains with a C-terminus facing outside of the cell. Transmembrane domains were predicted using two prediction programs, both TMHMM and Protter predicted 13 transmembrane domains with intracellular N-terminal domains and extracellular C-terminal domains (Sonnhammer et al., 1998; Krogh et al., 2001; Omasits et al., 2014) (Figure 6.5A and 6.5B).

A

B

Figure 6.5. Membrane topology of MtOPT1 (*Medtr7g092230.1*). (A) Thirteen predicted transmembrane α -helices were found using TMHMM v.2 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>; Sonnhammer et al., 1998; Krogh et al., 2001). (B) Thirteen TMDs was also predicted by Protter. Model was created using the Protter website to predict the protein's secondary structure within the membrane (<http://wlab.ethz.ch/protter>; Omasits et al., 2014).

Real time analysis shows rhizobial infection is essential for MtOPT1 expression

RT-qPCR analysis was conducted on *Medicago truncatula* ecotype 'R108' inoculated with *Sm1021* rhizobia and rhizobial mutants that do not proceed past certain developmental stages. *exoA*

rhizobia cannot infect plants, *bacA* rhizobia are unable to differentiate, and *fixJ* rhizobia are unable to fix nitrogen (Mitra and Long, 2004; Griffiths and Long, 2008). MtOPT1 requires infection for expression, but does not require bacteroid differentiation or nitrogen fixation (**Figure 6.6A**). Over a time course with LacZ rhizobia, expression is not seen at 0 dpi or 3 dpi, but is seen from 14 dpi through mature, nitrogen fixing nodules at 28 dpi indicating expression is important only after the initial infection and invasion of rhizobia which would include bacteroid differentiation and nitrogen fixation (**Figure 6.6B**).

Figure 6.6. RT-qPCR analysis of *MtOPT1* in root systems of *Medicago truncatula* ecotype ‘R108’ inoculated with *Sm1021* rhizobia. (A) *MtOPT1* expression at 28 dpi nodulating roots inoculated with rhizobial strains *exoA*, *bacA*, *fixJ*, or a functional strain containing *LacZ*. (B) Time-course of *MtOPT1* expression in root systems inoculated with the functional rhizobial strain.

The MtOPT1 transporter may be involved in the transport of antioxidants essential to protect from Reactive Oxygen Species (ROS) during early development of nodules and infection and differentiation of bacteroids during symbiotic nitrogen fixation.

Iron and the Vacuolar Iron Transporter Family

The transporter prediction program TransportTP was utilized to determine the number of family members in the VIT family (Li et al., 2009). In the *Medicago* genome, there are 6 identified VIT genes. Expression of *Medicago* VIT family members were analyzed to determine organ expression. One of those members, *Medtr4g094335.1*, herein called *MtSEN1*, is highly and exclusively expressed in the nodule. MtSEN1 has a nodule specific expression fashion (**Figure**

6.7A). MtSEN1 expression is high during early nodule development starting at 4 days post inoculation (**Figure 6.7B**). Expression occurs in all zones of the mature nodule, with higher expression in the distal part of the interzone, just below the nodule meristem, but it's expression remains high throughout nodule development, including in the nitrogen fixation zone (**Figure 6.7C**). Early expression in the meristematic zone of the nodule suggests involvement in symbiosome/bacteroid differentiation and nitrogen fixation.

Figure 6.7. Expression profile of MtSEN1 gene. (A) Expression in mature plant organs, (B) during nodule development and (C) in zones of mature nodule. Data from the Medicago Gene Atlas (<http://bioinfo.noble.org/gene-atlas>; Benedito et al., 2008; He et al., 2009) and Roux et al. (2014).

MtSEN1 is on chromosome 4. The gene is predicted to have a single exon and no introns. Chromosomal coordinates of untranslated regions (UTRs) are shown in relation to start and stop codons (**Figure 6.8**).

Figure 6.8. Gene structure of MtSEN1. The MtSEN1 gene has one exon shown in gray and no introns. 5' and 3' untranslated regions (UTRs) can be seen as black boxes. MtSEN1 is located on chromosome 4 of the Medicago genome.

The VIT family of transporters commonly have 5 TMDs as is seen in members from the fungus *Ustilago maydis* to *Arabidopsis thaliana* VIT1 (Kim et al., 2006). Although the TMHMM

software predicts four TMDs, it can be seen in the prediction model that there is potentially a fifth transmembrane domain that did not quite reach the cutoff value to be counted as a transmembrane domain (Möller et al., 2001). However, the Protter program predicts that region as a fifth transmembrane domain (TMD) (**Figure 6.9B**) (Omasits et al., 2014).

Figure 6.9. Membrane topology of MtSEN1. (A) Four predicted transmembrane helices are predicted by TMHMM. Both N- and C- terminus tails are predicted to intracellular. Figure generated using TMHMM v.2 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). (B) Although TMHMM only predicts 4 transmembrane domains, Protter predicts five transmembrane domains from the protein sequence. The N-terminus tail is predicted to be extracellular and the C-terminus tail is predicted to be intracellular. Model was created using the Protter website to predict the protein's secondary structure within the membrane (<http://wlab.ethz.ch/protter>; Omasits et al., 2014).

MtSEN1 (*Medtr4g094335.1*) is closely related to *Medtr2g008110.1*, *AtVITH2.1*, *AtVITH1*, *AtVITH2*, *AtVITH3*, and *AtVITH4* (**Figure 6.10**).

Figure 6.10. Molecular Phylogenetic analysis by Maximum Likelihood method of *MtSEN1*, *Medicago truncatula* family members and *Arabidopsis* and homologs constructed in MEGA7 (Felsenstein 1985; Kumar et al., 2016).

Mutant *S. meliloti* rhizobial strains were used to determine which nodule development events were necessary for gene expression. *exoA* is a rhizobial mutant that cannot infect the plant, *bacA* cannot differentiate bacteroids, and *fixJ* cannot fix nitrogen (Mitra and Long, 2004; Griffitts and Long, 2008). Infection by *S. meliloti* rhizobia is necessary for expression of *MtSEN1*, but bacterial differentiation and nitrogen fixation are not essential to expression (**Figure 6.11A**). Expression of *MtSEN1* is seen after the 3-day post inoculation mark and expression is high from 10 dpi to 28 dpi (**Figure 6.11B**) indicating involvement in differentiating bacteria and nitrogen fixation. Overall, *MtSEN1* is highly expressed early in nodule development with peak expression

during rhizobial infection of cells suggesting function related to processes prior to supporting fully functional, nitrogen fixing bacteroids.

Figure 6.11. RT-qPCR of MtSEN1 in root systems of *Medicago truncatula* ecotype ‘R108’ inoculated with *Sm1021* rhizobia. (A) MtSEN1 expression at 28 dpi nodulating roots inoculated with rhizobial strains *exoA*, *bacA*, *fixJ*, or a functional strain containing *LacZ*. (B) Time-course of *MtSEN1* expression in root systems inoculated with the functional rhizobial strain.

CONCLUSION

Together, MtOPT1 and MtSEN1 are putative nodule specific transporters predicted to have involvement in symbiotic nitrogen fixation through the transport of small oligopeptides ranging from three to eight amino acids in length, or glutathione or homoglutathione via MtOPT1 or iron via MtSEN1. Future studies to functionally characterize MtOPT1 and MtSEN1 would include transport affinity substrate assays, subcellular localization, and CRISPR experiments to determine knockout phenotype and potentially could reveal key players in symbiotic nitrogen fixation.

MATERIALS AND METHODS

Phylogenetic analysis and membrane topology predictions: The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>; Möller et al., 2001) and Protter

(wlab.ethz.ch/protter) were used to predict transmembrane domains with protein sequences as input.

Gene expression analyses: Gene expression analysis was assessed from the *Affymetrix* Medicago GeneChip microarray data deposited at the Samuel Roberts Noble Foundation *Medicago truncatula* Gene Expression Atlas (<http://mtgea.noble.org/v3/>; Benedito et al., 2008). Gene expression analyses of membrane transporters in rhizobial mutants was performed on R108 root systems inoculated with *fixJ*, *bacA*, *exoA*, or *hemA::LacZ* strains of *Sinorhizorium meliloti* in *Sm1021* background. A control group was not inoculated with rhizobia and was provided full nitrogen fertilization. Plant roots were harvested at 0, 3, 14, 21, and 28 dpi. RNA was extracted with mirVana miRNA Isolation kit (Life Technologies) followed by DNA removal with Turbo DNA-free kit (Applied Biosystems). First strand complementary DNA was synthesized by priming with oligo-dT using the SuperScript III First-Strand Synthesis Super mix (Life Technologies) following the manual instructions. Primers used were: MtOPT1 forward: 5'ACCTGTTCCAGTGTGGCTTC, MtOPT1 reverse: 5'ACCTGCGGCGATAATAGG, MtSEN1 forward: 5' TTAGGAGCAGCATTGGTAAGG, MtSEN1 reverse: 5' CCAACCACCACTCTAACACAAGA. Three biological and three technical replicates were run. SYBR green master mix and 5µL reactions were used on Applied Biosystems 7500 Real Time PCR System and 7500 software. All templates were amplified using the same protocol: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curves were produced following the 40 cycles by heating to 95°C for 15 seconds followed by 60°C for 15 seconds and 95°C for 15 seconds. 7500 Software (Applied Biosystems) gave C_T threshold cycle values. Excel was used to organize data and calculate geometric means of technical replications and calculate averages and standard errors. Technical outliers were eliminated from analysis.

The housekeeping genes chosen for this experiment were: EF1α (*Medtr6g021800*), tubulin (*Medtr7g089120*), and MSC27 (X98618.1). NormFinder software (Andersen et al., 2004) was used to identify stably expressed housekeeping genes in the experiment and MSC27 was excluded from the calculations.

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Chapter 7

GENERAL CONCLUSIONS

Membrane transporters are essential to move substrates between organelles within cells and from cell to cell within plants. As reviewed in Chapter 2, membrane transporters support symbiotic nitrogen fixation during the relationship between plants and rhizobia which introduces another level of nutritional flux not seen in plants unable to form a symbiosis. Although there are more than 2,000 putative membrane transporters in the *Medicago truncatula* genome, relatively few have been characterized, and the genetic identity of transporters central to the nitrogen fixation process have not fully been elucidated. An analysis to identify putative functional transporters of the APC superfamily of transporters in *Medicago truncatula* playing roles in the symbiotic nitrogen fixation process is presented (Chapter 3). This research also aimed to functionally characterize transporters involved in amino acid and copper transport during symbiotic nitrogen fixation (Chapters 4 and 5). We examined nodule specific transporters to define substrates, examine knockdown expression, and delineate patterns of expression.

In Chapter 3, I classified APC superfamily members through manual curation of confidence levels for expected transporter functionality by comparing to functionally characterized family members within the Transporter Classification Database, Swiss-Prot annotation, Gene Ontology, and expected Pfam domains. Additionally, for APC superfamily members, I compiled information relating to gene expression in plant organs as well as in nodule zones leading to the identification of candidate gene coding for amino acid transporters within nodules supporting symbiotic nitrogen fixation.

In Chapter 4, I set out to functionally characterize MtAPC1 (*Medtr8g089360.1*). In silico analysis expression patterns were analyzed to begin analysis of the gene. In situ analysis showed expression in infected cells in the nitrogen fixation zone. Yeast import assays were performed to test affinity to 16 amino acids and it was concluded that Mt APC1 was not an importer as previously thought. Real time analysis of RNAi showed reduced expression of MtAPC1 expression with no visible phenotype, potentially due to gene redundancy.

In Chapter 5, MtCOPT1 (*Medtr4g019870.1*) was functionally characterized through *in silico* analysis to determine expression patterns of the gene. RT-qPCR analysis in our lab confirmed expression patterns with highest expression at 10 days post inoculation consistent with

involvement in nitrogen fixation. Protein modifications, by phosphorylation of serine and threonine residues in the C-terminus were identified, but the potential functional effects of such modifications remain to be determined. Yeast import assays of MtCOPT1 showed affinity to copper.

In Chapter 6, I preliminarily analyzed two nodule specific transporters, MtOPT1 (*Medtr7g092230.1*) and MtSEN1 (*Medtr4g094335.1*), to evaluate potential function in relation to symbiotic nitrogen fixation. *In silico* analysis revealed early expression at 4 days post inoculation with expression seen in the invasion zone and sustained through the nitrogen fixation zone for MtOPT1 and expression in the infection zone and also sustained through the nitrogen fixation zone in MtSEN1 (*Medtr4g094335.1*) suggesting roles during symbiotic nitrogen fixation. These results will foster future research to identify the exact roles they play in nodule development and the symbiosis.

In summary, this dissertation contributes to the identification and classification of putative APC Superfamily membrane transporters. It provides insights and gives direction for membrane transporters that would be of interest to further study for functional characterization. In addition, this dissertation contributed to functional characterization of nodule specific membrane transporters of amino acids and copper, with the identification of nodule-specific putative transporters with potential affinities to iron and oligopeptides.