Ammonium transporters in grasses: Molecular and functional characterization with special reference to the arbuscular mycorrhizal symbiosis

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Abbreviations

AM	arbuscular mycorrhiza
EM	ectomycorrhiza
AMT	ammonium transporter
Pht	phosphate transporter
C	carbon
N	nitrogen
P	phosphorus
S	sulfur
ERM	extraradical mycelium
IRM	intraradical mycelium
amiRNA	artificial micro RNA
LM	laser microdissection technology
Mbp	mega base pairs
Mya	million years ago
BAS	branched absorbing structures
CMN	common mycorrhizal network

Summary

Most herbaceous plants live in symbiosis with arbuscular mycorrhizal (AM) fungi. AM fungi colonize the roots of their host plant symbionts and provide them with mineral nutrients, especially phosphorus (P) and nitrogen (N) and receive, in exchange, photosynthetically fixed carbon.

In this work, we focused on the role of N in the AM symbiosis formed between *Glomus mosseae* or *Rhizophagus irregularis* and different plants belonging to the Poaceae: sorghum (*Sorghum bicolor*), maize (*Zea mays*), rice (*Oryza sativa*), foxtail millet (*Setaria italic*) and purple false brome (*Brachypodium distachyon*). It had been shown that AM fungi can take up N in form of nitrate, ammonium and amino acid and transfer it to the plant in form of ammonium. Thus, we hypothesized that some plant ammonium transporters (AMT) might be up-regulated at the interface between plant and fungus in the AM symbiosis.

As described in chapter 2, we established mycorrhized and non-mycorrhized sorghum plants and gave them different N treatments: no nitrogen, nitrate or ammonium. We found out that two AMTs, *AMT3;1* and *AMT4* were induced in mycorrhized plants (AM-inducible AMTs) independently of their N status. In sorghum, the pattern of expression of *AMT3;1* and *AMT4* was assessed with a split-root experiment combined with laser microdissection technology. Expression of both AMTs was not systemic in the roots of the plant. However, at a small scale, systemic expression around cells containing arbuscules could be observed. We conclude that expression of *AMT3;1* and *AMT4* could be part of the prepenetration response of the plant, preparing the cells to receive a new arbuscule. In addition, using immunolocalization, we localized the protein of AMT3;1 at the level of mature arbuscules.

As described in chapter 3, the up-regulation of *AMT3;1* and *AMT4* was conserved in all four Poaceae species studied. As the core Poaceae divided into two groups about 55 million years ago separating sorghum, foxtail millet and maize from rice and purple false brome, we assume that *AMT3;1* and *AMT4* were already induced by AM fungi in a common ancestor of all these plants.

In chapter 4, we looked at the fungal side and at the effect of the different N treatments on the expression of fungal transporters and enzymes of the N cycle. Our results show that the source of N has

an impact on the transcriptional regulation of enzymes from the fungal N cycle. Expression of the corresponding genes was modified in the fungal extraradical mycelium as well as in the intraradical mycelium.

In chapter 5, we studied the time needed by the AM fungus *Glomus mosseae* to transfer N from a ^{15}N-labeled source to sorghum plants. Labeled N was present in the plant leaves already after 48 hours revealing a very rapid transfer. This finding highlights the underestimated role of AM symbiosis in N-acquisition by the plant.

1. General introduction

1.1 The mycorrhizal symbiosis

The mycorrhizal symbiosis (from Greek, mykes – fungus and rhiza – root) is a mutualistic symbiosis formed between plants and fungi. The base of this mutualistic symbiosis is the bidirectional exchange of resources between both partners: the plant roots deliver products of the photosynthesis (carbon: C) to the fungi and in exchange receive mineral nutrients like nitrogen (N) or phosphorus (P). The fungi involved in the mycorrhizal symbiosis belong to different taxa (Zygomycota, Glomeromycota, Ascomycota and Basidiomycota) and colonize the roots of plant species throughout the plant kingdom (Anthocerophyta, Marchantiophyta, Bryophyta, Pteridophyta and Spermatophytes) (Smith & Read, 2008). Different types of mycorrhizae are formed depending on the identity of both symbiosis partners. Here, we will focus on the most common one, the arbuscular mycorrhiza.

1.2 The arbuscular mycorrhiza

The arbuscular mycorrhiza (AM) is the most common type of mycorrhizal symbiosis (Smith & Smith, 2011). It is formed between the roots of a majority of land plants and obligate symbiotic fungi belonging to the Glomeromycota (Schüssler *et al.*, 2001). AM fungi were probably involved in the colonization of land by plants. Indeed, AM structures and spores were found on fossils from the Ordovician (460 Mya) (Redecker *et al.*, 2000; Heckman *et al.*, 2001) and Brundrett (2002) suggests a co-evolution of roots and mycorrhiza since more than 400 Million years.

The name “arbuscular” comes from characteristic structures, the arbuscules (Fig.1.1) formed by some AM fungi in plant cortical cells. Arbuscules are the place of the nutrient exchange between plants and these fungi (Smith & Read, 2008). Outside the plant roots, AM fungi develop an extensive extraradical mycelium (ERM) interconnecting plants together and searching for mineral nutrients in the soil (Fig. 1.2). As propagules, they form large spores (up to 500 µm) containing storage lipid, carbohydrate and a large number of nuclei (Bécard & Pfeffer, 1993).

AM fungi are obligate symbionts and rely entirely on their plant partners for carbon (C) supply. In return, they deliver mineral nutrients to the plant and can improve drought and disease tolerance of the plant (Smith & Read, 2008).

1.3 Diversity, host specificity and ecological importance of AM fungi

It was first assumed that AM fungi were closely related to the phylum Zygomycota. However, in regard to the lack of Zygospores, the obligate symbiosis with host plants and rDNA phylogeny, Schüssler *et al.* (2001) revised this classification and placed the AM fungi in a newly defined phylum, the Glomeromycota. Members of the Glomeromycota appear to have haploid nuclei (Bianciotto *et al.*, 1995; Hijri & Sanders, 2004) and sexual structures are absent. This led to the conclusion that Glomeromycota are asexual and clonal (Smith & Read, 2008). However, closely related strains of a same species can exchange genetic material (De La Providencia *et al.*, 2005; Croll *et al.*, 2009). Indeed, hyphae form AM fungi were shown to form functional anastomoses that allow the exchange of nuclear material among growing mycelia (Croll *et al.*, 2009). Nonetheless, these gene exchanges are believed to be very rare (den Bakker *et al.*, 2010; Corradi & Lildhar, 2012).

Although asexual and clonal, the Glomeromycota harbor a high genetic diversity, even in single spores. Thus, different AFLP patterns or even variants of a given genes in one single spore were reported (Sanders *et al.*, 1995; Rosendahl & Taylor, 1997; Jansa *et al.*, 2002). It is still not totally clear, if the genetic diversity of a single spore is the consequence of genetically different nuclei present in one spore (heterocaryotism) (Kuhn *et al.*, 2001) or of identical nuclei, each containing the same sequence variants (homocaryotism) (Pawlowska & Taylor, 2004; Hijri & Sanders, 2005).

The actural taxonomic classification of the approximately 245 species of AM fungi described (http://schuessler.userweb.mwn.de/amphylo/belonging) is based on morphological and molecular criteria (Redecker *et al.*, 2003). However, difficulties are faced during molecular characterization due to the high genetic diversity in single spores mentioned before.

Lately, the AM fungal taxonomy was revised multiple times (Schüssler & Walker, 2010; Oehl *et al.*, 2011) causing confusion and difficulties among scientists working on AM fungi. Redecker *et al.* (2013) published recently a consensus containing the last taxonomic revisions. According to former

classifications, (Schüssler *et al.*, 2001; Walker & Schüssler, 2004; Msiska & Morton, 2009), they divided the Glomeromycota into four clades: the Paraglomerales, the Archaeosporales, the Glomerales and the Diversisporales.

The Paraglomerales contains one family, the Paraglomeraceae with one genus, Paraglomus.

The Archaeosporales contains three families with each one genus: the Ambisporaceae (genus Ambispora), the Geosiphonaceae (genus Geosipon) and the Archaeosporaceae (genus Archaeospora).

The Glomerales contains two families: the Glomeraceae (genus Glomus, Funneliformis, Septoglomus, Rhizophagus and Sclerocystis) and the Claroideoglomeraceae (genus Claroideoglomus).

The Diversisporales contains five families: the Gigasporaceae (genus Scutellospora, Gigaspora, Intraomatospora, Paradentiscutata, Dentiscutata, Cetraspora and Racocetra), the Pacisporaceae (genus pacispora), the Sacculosporaceae (genus Sacculospora), the Acaulosporaceae (genus Acaulospora) and the Diversisporaceae (genus Tricispora, Otospora, Diversispora, Corymbiglomus and Redeckera).

Nomenclature of the widespread model fungus MUCL43194 or DAOM197198 was also clarified: this fungus pereviously called *Glomus intraradices* is newly named *Rhizophagus irregularis* (Redecker *et al.*, 2013). *R. irregularis* is a fast growing fungus colonizing many plants including *M. truncatula*, poplar (*Populus trichocarpa*), maize (*Zea mays*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), *Setaria italica* and *Brachypodium distachyon*. It was shown to take up and transfer mineral nutrients like phosphorus (p), nitrogen (N) and sulfur (S) (Tian *et al.*, 2010; Smith & Smith, 2011) and was used to identify signaling pathways during root colonization processes, nutrient assimilation and transport. Interestingly, transcriptomic data of *R. irregularis* confirmed the fungal dependence to plant carbohydrate supply (Tisserant *et al.*, 2011).

In view of the wide range of plant host species (perhaps 80-90% of land plants) and the low number of described AM fungal species, very low host specificity is assumed to exist (Smith & Read, 2008). The same AM fungus was shown to colonize different plants at the same site and one plant can be colonized by different AM fungi (van Tuinen *et al.*, 1998; Fitter, 2005). However, Jansa *et al.* (2008) shown with real time PCR that the percentage of root colonization by coinoculated fungi varied depending on the host plant and on the fungal species. Additionally, Helgason *et al.* (2002) and Davison *et al.* (2011) observed non-random distribution of AM fungi among different host plants and suggested that some plant-fungus combinations might be more likely than others. Recently, Montesinos-Navarro *et al.* (2012) got similar results on plant-fungal networks. Moreover some AM fungi cannot be cultured in pot

cultures as described by Oehl *et al.* (2003) maybe due to the inappropriate host plant. These facts could indicate a degree of host specificity (Smith & Read, 2008). Among the described AM fungal species generalists (with low host preference) as well as specialists (with high host preference) might exist and different strategies of the host plant as well as of the AM fungi are very probable.

AM fungi are present in very diverse ecosystems like tropical rainforests or temperate grasslands, and also in highly disturbed, nutrient-rich or poor, arid or wet habitats (Smith & Read, 2008). They have an impact aboveground on plant biomass and diversity. The response of individual plant species to AM fungi can vary a lot. Thus, some plant species exhibit biomass decrease of up to 45% in the presence of AM fungi in contrast to others where biomass increase in the same range was observed (Klironomos, 2003). At the community level, presence of AM fungi can increase or in contrary decrease diversity and productivity (van der Heijden *et al.*, 1998; Hart *et al.*, 2003). The impact of AM fungi on plant diversity also seems to be linked to the identity of the plant dominating the community and its response to the association with AM fungi (van der Heijden & Horton, 2009). Interestingly, mycorrhizal growth response of a plant species in a community can be very different from the growth response of the same plant species grown individually (Facelli *et al.*, 2010). This fact could be explained by the formation of a so-called common mycorrhizal network (CMN) in plant communities. Indeed, due to the lack of specificity mentioned above, one AM fungus can colonize several plants, interconnecting them by a CMN. CMN promote plant interactions by allowing resource exchange directly from plant to plant (Simard & Durall, 2004) thus influencing the plant community. CMN can also help seedling establishment (Wilkinson, 1998). An interesting additional aspect of CMN is the carbon uptake from mycoheterotrophic plants through the CMN: these plants parasite the network. Mycoheterotrophic plants can have a reduced photosynthetic rate and become only part of their C from the CMN or be achlorophilous and rely entirely on their fungal partners to become C (Selosse *et al.*, 2006). However, the presence of mycoheterotrophic plants does not seem to reduce the fitness of the other plants linked to the CMN (van der Heijden & Sanders, 2003).

In addition to their effect on plant biomass and productivity, AM fungi have positive effects on drought and pathogen resistance of the plant (Newsham *et al.*, 1995; Smith & Read, 2008) and influence the course of plant succession (Gange *et al.*, 1990)

Belowground, AM fungi influence the microbial diversity in the soil (Johnson *et al.*, 2004) and affect growth of some soil bacteria and fungi (Filion *et al.*, 1999). They also stabilize soil aggregates. Indeed,

AM fungi were shown to produce glomalin, a glycoprotein recalcitrant in the soil the concentration of which is correlated with aggregate stability (Wright & Upadhyaya, 1998).

1.3 The root colonization process

The establishment of the symbiosis is initiated by a mutual signal exchange between plants and fungi (Fig. 1). Plant roots were shown to stimulate germination of the spores (Graham, 1982), and the growth and branching of the fungal hyphae (Giovannetti *et al.*, 1993) by releasing root exudates. The most important signals in these exudates proved to be the strigolactones (reviewed in Parniske, 2008). Indeed, strigolactones induce hyphal branching and alterate fungal physiology and mitochondrial activity (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). The AM fungus, on its side, produces the so called "myc factors" (reviewed in Parniske, 2008). The presence of such "myc factors" was shown in experiments where plant symbiotic response was activated without direct contact with AM fungi (Kosuta *et al.*, 2003). "Myc factors" turned out to be lipochitooligosaccharides resembling the "Nod-factors" of rhizobia (Maillet *et al.*, 2011). Interestingly, as the rhizobium-legume symbiosis appeared about 340 million years after the AM symbiosis (Maillet *et al.*, 2011), "Nod-factors" could have been recruited and adapted from the "myc factors" explaining the similitude between both symbiosis pathways (Oldroyd & Downie, 2006; Parniske, 2008).

After germinating and when entering in contact with the roots of a host plant, hyphae adhere to the root surface and form after 2-3 days appressoria called hyphopodia. Simultaneously and before any plant cell wall penetration by the fungus occurs, plant cells produce a prepenetration apparatus (PPA) (Genre *et al.*, 2005) through which the hyphae can grow. Formation of the PPA is part of the processes involved in the reception of the fungus including among other things nuclear movements, alteration of the cytoskeletal activity and membrane proliferation (Genre *et al.*, 2005). Moreover, genes regulation is modified during AM colonization not only in colonized but also in non-colonized cells (Gaude *et al.*, 2011).

After penetrating the epidermis and the outer cortex, hyphae branch repeatedly inside the inner cortex cells to form arbuscules, tree like structures (*Arum* type) (Fig. 1.1) or coils (*Paris* type). Both arbuscules and coils are considerably increasing the surface of the fungal – plant interface and are the place of nutrient transfer. Arbuscules are usually short-lived (build and decay in about 7 days) (Smith & Read,

2008). The hyphae forming the arbuscules never penetrate the plasma membrane but always remain enveloped by it so that the fungus stays outside of the plant cell cytoplasm. The membrane enveloping the arbuscules is called periarbuscular membrane. It is derived from the plasma membrane but is functionally different: differential activities of specific enzymes, nutrient transporters and aquaporines have been reported (Gianinazzi-Pearson *et al.*, 2000; Rausch *et al.*, 2001; Harrison *et al.*, 2002b; Glassop *et al.*, 2005). The coils of the *Paris* type were less studied than the arbuscules of the *Arum* type. Here, hyphae are directly growing from one cell to the other without intercellular development. However, the rearrangement inside the cell (Cavagnaro *et al.*, 2001) and the capability to exchange nutrients seems to be similar in the coils and in the arbuscules (Dickson & Kolesik, 1999). Additionally, coils also remain enveloped by the plant plasma membrane (Smith & Read, 2008).

Vesicles are formed in the apoplast either intercellularly or intracellularly depending on the fungal species (members of the Gigasporaceae never form vesicles). They are thick-walled structures containing lipids and nuclei and are thought to be important storage organs (Fig. 1.1).

After the establishment of the intraradical mycelium, an extensive extraradical mycelium is formed. Finally, new spores are formed on the extracellular hyphal tips but also sometimes inside the roots as in *R. irregularis* (Smith & Read, 2008).

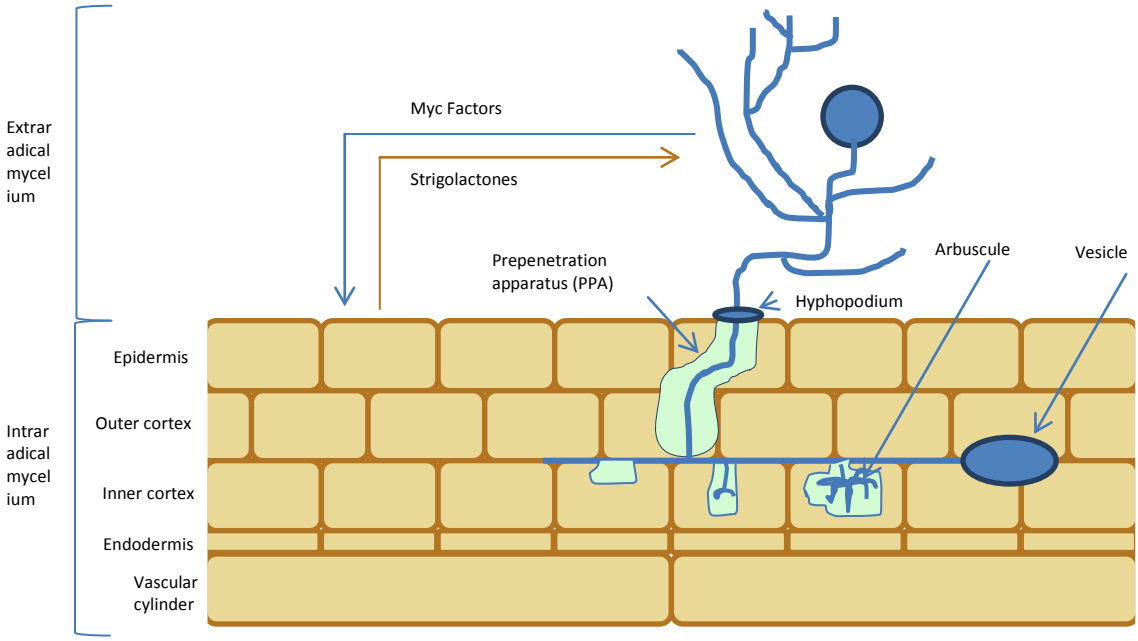

Figure 1.1: Root colonization: The strigolactones are exudates released from the plant root inducing spore germination and hyphal branching. Myc factors from the fungus induce symbiosis-related genes. In contact with plant roots, AM fungi form hyphopodia. In response, plant produces a prepenetration apparatus (PPA) that guides the entering fungal hyphae through the root outer cortex. In the inner cortex, PPA like structures are made by the plant to host tree like structures called arbuscules where nutrient transfer takes place. Sometimes vesicles, which are thought to be storage organs, are formed in the apoplast. Outside of the roots, fungal extraradical mycelium develops. Modified after Parniske (2008).

The extraradical mycelium (ERM) of the AM fungi (Figure 1.1 and 1.2) is very important for the nutrient uptake but also for the colonization of seedlings in perennial vegetation systems (Smith & Read, 2008). Two main types of ERM are present in the soil: (1) runner hyphae that are thick-walled and long-living and (2) branched absorbing structures (BAS) that are thin-walled and short-living hyphae. The runner hyphae search for nutrient patches and new host plants. They build a permanent base for the BAS hyphae that takes up nutrients and may associates with the roots of additional host plants. The diameter of the hyphae ranges from 10 to 15 µm for the thick-walled hyphae and from 1 to 7 µm for the thin-walled hyphae (Bago *et al.*, 2004) allowing the latter ones to explore very fine soil pores. Hyphal networks can extend over very long distances: molecular fingerprinting revealed that a single fungal clone could possibly connect the roots of *Hieraceum pilosella* plants over 10m distance in an undisturbed sand-dune (Rosendahl & Stukenbrock, 2004).

Figure 1.2: Pot culture of AM fungal spores and hyphae from the extraradical mycelium of *Glomus mosseae* ISCB13. Scale: 500 µm. Light microscopy picture done by myself.

1.4 Nutrient exchange in the AM symbiosis

The AM symbiosis is based on the reciprocal transfer of mineral nutrients and carbon (C) between AM fungi and plants. Plants forming AM symbiosis are mostly facultative symbionts and can grow in the absence of AM fungal colonization. In contrast, AM fungi are obligate symbionts and depend on the C from their host plant to complete their life cycle (Smith & Read, 2008).

1.4.1 Carbon transfer

In plants, sucrose (fructose β2↔1α glucose), the main form of sugar for long distance transport, is synthesized in the mesophyll and loaded into the phloem for transport before being released by sucrose transporters to a sink (Doidy *et al.*, 2012). When released, sucrose can be stored or cleaved by sucrose splitting enzymes to yield glucose and fructose. AM fungi absorb about 20% of plant fixed carbon (C) and thus are an important sugar sink (Jakobsen & Rosendahl, 1990). Plant react to the presence of AM fungi by increasing photosynthetic and invertase activity, as well as transcript levels of plant sucrose transporter. Additionally, accumulation of sucrose and monosaccharide in plant roots colonized by AM fungi could be shown (Casieri *et al.*, 2013). Sugar transfer between plant and fungi was demonstrated with ^{14}C CO_2 labeling and with nuclear magnetic resonance spectroscopy (Pfeffer *et al.*, 2001). The

transfer of sugar between plants and fungi occurs in the form of sucrose or monosaccharide. Indeed, plant sucrose transporters and plant monosaccharide transporters were found at the plant-fungus interface (Doidy *et al.*, 2012). However, AM fungi seem to have a higher capacity to absorb glucose and fructose than sucrose (Shachar-Hill *et al.*, 1995; Schüssler *et al.*, 2006). As a matter of fact, expression and activity of plant sucrose cleaving enzymes were shown to be regulated in the presence of AM fungi and to influence the sugar fluxes between both partners (Hohnjec *et al.*, 2003; Ravnskov *et al.*, 2003). Once absorbed by the IRM of the fungi, glucose and fructose are rapidly incorporated into trehalose and glycogen (Shachar-Hill *et al.*, 1995) to prevent accumulation in the cytoplasm. Additionally, lipids are synthesized in the IRM and transferred together with glycogen from the IRM to the ERM (Bago *et al.*, 2002). In the ERM, lipids are broken down to monosaccharide and trehalose or are stored in spores (Smith & Read, 2008).

1.4.2 Mineral nutrient transfer

BAS hyphae from the ERM of AM fungi (Bago *et al.*, 2004) proliferate on nutrient rich patches or on organic matter (Joner & Jakobsen, 1995; Hodge *et al.*, 2001; Cavagnaro *et al.*, 2005) but relay on saprotrophic microorganisms to decompose organic matter (Hawkins *et al.*, 2000; Whiteside *et al.*, 2009). From the soil, BAS hyphae take up mineral nutrients including phosphorus (P), nitrogen (N) or sulfur (S). This uptake is followed by the transport of nutrients from the ERM to the IRM. In the IRM, transfer occurs at the symbiotic interface between plants and fungi. Symbiotic interfaces are intracellular (interface with arbuscules or coils) or intercellular (interface with intercellular hyphae). However, intracellular interfaces are believed to be the main place of nutrient exchange. The interfaces are built as followed: the plasma membrane of the fungus is separated from the plant plasma membrane by an apoplastic interfacial compartment. For this reason, nutrient exchange requires the efflux from one partner and the uptake by the other (Smith & Read, 2008). The plant membrane at the symbiotic interface is called periarbuscular membrane (Pumplin & Harrison, 2009; Kobae & Hata, 2010). Interestingly, AM-mediated nutrient acquisition can be increased in plants by simultaneously harboring several fungal symbionts (Jansa *et al.*, 2008) hypothesizing a degree of functional complementarity of different species in their nutrient acquisition.

Phosphorus transfer

Phosphorus (P) is an important macro nutrient for plant growth. It is mainly absorbed by the plant in form of inorganic P (P_i), specifically H_2PO_4. However, it is often present only at very low concentrations in the soil (between 5 and 10 µM, Schachtman *et al.*, 1998) and is nearly immobile (Hinsinger, 2001). For this reason a narrow so-called "depletion zone" is formed around plants roots: after rapid absorption of the available P_i, massflow of soil solution is unable to bring sufficient amount of P_i to plant roots (Smith & Read, 2008). Plants mycorrhized by AM fungi can absorb P_i through two different pathways; directly *via* the root epidermis including root hairs, or *via* the mycorrhizal pathway starting at the hyphae of the ERM (Smith & Read, 2008). Although both pathways absorb the same form of P_i, plants dramatically increase their P supply in association with AM fungi (Marschner & Dell, 1994) as fungal BAS hyphae forage the soil for nutrients beyond the depletion zone of the roots. They have a much smaller diameter (1-7 µm) than plant roots (>300 µm) and root hairs (5-17 µm) and get access to nutrients not accessible to the roots, which increase the explored soil volume of the plant (Drew *et al.*, 2003; Schnepf *et al.*, 2011).

In the ERM, P_i is taken up by fungal phosphate transporters (Pht) closely related to plant Phts. AM fungal Phts were cloned and appear to be high affinity transporters able to take up P_i in low concentration in the soil (Harrison & van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001; Benedetto *et al.*, 2005). The absorbed P_i is then transformed to polyphosphate in the vacuoles to be translocated in vacuolar compartments from the fungal ERM to the IRM (Ezawa *et al.*, 2004). In the IRM, polyphosphate is hydrolyzed to be transferred as P_i from the fungi to the plant. This whole transfer process is much faster than diffusion of P_i through the soil (Smith & Read, 2008). Consequently, hyphal transfer can overcome the limited P availability for plants resulting from the fast development of a depletion zone around the roots.

Plant Phts are classified in three families: Pht1, Pht2 and Pht3 (Smith *et al.*, 2003). The Pht1 family is further divided in four subfamilies (I-IV). Phts from the Pht1 family are responsible for a substantial part of total P uptake (Shin *et al.*, 2004; Ai *et al.*, 2009). A small group of Pht1 is induced by AM fungi. Most of these AM-inducible Phts are clustering together into the distinctive subfamily I, which contains Phts of monocots and dicots (Yang & Paszkowski, 2011; Casieri *et al.*, 2013). These Phts are only expressed in AM plants as *MtPT4* in *Medicago truncatula* or *OsPT11* in rice (Harrison *et al.*, 2002b; Paszkowski *et al.*,

2002). Other AM-inducible Phts cluster in the evolutionary younger subfamily III containing only dicotyledonous species (Bucher, 2007). They have a basal expression also in non-mycorrhized plants (Rausch *et al.*, 2001; Güimil *et al.*, 2005; Maeda *et al.*, 2006).

At the symbiotic interface, AM-inducible Phts are believed to take up P_i delivered by the fungi. Indeed, AM-inducible Phts were localized mainly in cells harboring arbuscules (Rausch *et al.*, 2001; Harrison *et al.*, 2002b) and protein of an AM-inducible Pht, MtPT4, was found on the fine branches of developing mature arbuscules (Pumplin & Harrison, 2009). Moreover, *M. truncatula* mutants with a down-regulated *MtPT4* expression showed a premature arbuscule death and a decreased colonization level (Javot *et al.*, 2007). The mutants had also significantly lower shoot P content than wild types in the presence of AM fungi. Similarly, *L. japonicus* mutants of LjPT3 had a reduced symbiotic P uptake and a reduced arbuscule development (Maeda *et al.*, 2006).

Plants can have more than one AM-inducible Pht raising the question of the redundancy of AM-inducible Phts. In *L. esculentum,* mutants of the AM-inducible Pht *LePT4* had a similar symbiotic P uptake and arbuscule development than wild type plants, indicating a possible redundancy with another AM-inducible Pht1 (Nagy *et al.*, 2005). However, in rice plants, Yang *et al.,* (2012) found that only one of the two AM-inducible Pht1 was necessary for a functional symbiosis rejecting the hypothesis of a redundant function of both transporters in this plant.

Nitrogen uptake and transfer

In the soil, Nitrogen (N) is present in organic (N_o) and inorganic (N_i) form but N_o is the dominating form. Plants and the ERM of AM fungi can absorb N_i as nitrate or ammonium and some soluble forms of N_o like amino acids (Smith & Read, 2008). In contrast, the role of AM fungi in the mineralization of organic forms of N is still not totally clear. Hodge *et al.* (2001) observed that AM fungi enhance N_o decomposition as well as plant N capture but other work does not confirm these findings (Frey & Schüepp, 1993; Hawkins *et al.*, 2000). Indeed, Ames *et al.* (1983) and Hawkins *et al.* (2000) both observed the transfer of ^{15}N from an organic source but at a very slow and small rate, insufficient to influence the N status of the plants.

Nitrate and ammonium are relatively mobile in the soil (nitrate more than ammonium) and can be transported to plant roots by mass flow, limiting the depletion zone around the roots. For this reason,

the question of the relevance of AM fungi in plant N nutrition was raised (Smith & Smith, 2011). Nevertheless, drought can restrict the mobility of nitrate and ammonium (Tinker & Nye, 2000) which could increase the relevance of AM fungi in N transfer (Tobar *et al.*, 1994; Smith & Read, 2008). Additionally, N transfer by AM fungi could play an important role for plants growth on marginal soils, where nutrients are in short supply in soil. In the soil, plant roots also compete with microorganisms for N. The capability of microorganisms to absorb N was shown to be higher than the one of plants (Kuzyakov & Xu, 2013). Here, AM symbiosis enhances the competitive capacities of the plant to obtain the N they need. As a matter of fact, Mader et al. (2000) estimated the amount of N present in the plant and coming from the AM pathway to up to 42%. In monoxenic cultures of carrot roots, this amount was of about 30% (Govindarajulu *et al.*, 2005). Tanaka & Yano (2005) found that even 74% of maize shoot N was derived from the AM fungi.

More precisely, AM fungal ERM take up N_i as ammonium or nitrate. Thus, Govindarajulu *et al*. (2005) supplied $^{15}NO_3^-$ and $^{15}NH_4^+$ to *in vitro* AM cultures of carrot (*Daucus carota*) colonized by *Rhizophagus irregularis* and observed the labeling of free amino acids in the ERM. In addition, in the AM fungus *R. irregularis,* ammonium transporters (AMT) were described (Lopez-Pedrosa *et al.*, 2006; Pérez-Tienda *et al.*, 2011).

N_o is also taken up by the fungal ERM. Indeed, Whiteside *et al.* (2012) reported the uptake of N_o in the form of chitosan and glycine. Additionally, uptake of N_o from a labeled N_o patch was reported by Leigh *et al.* (2009). Like other fungi, AM fungi might have a preference for ammonium in comparison to nitrate because of the extra energy required to reduce nitrate to ammonium before its N can be incorporated into organic compounds (Marzluf, 1997).

Although AM fungi are able to take up different forms of N, not all forms of N are transferred equally to the plant. Indeed, Ngwene *et al.* (2012) found out that more ^{15}N was transferred to cowpea plants when the AM fungus *R. irregularis* had access to labeled nitrate compared to ammonium. In fact, the ^{15}N shoot/root ratio of the plants was clearly higher in the $^{15}NO_3^-$ treatments. Ammonium was preferentially kept in the fungal mycelium or in plant roots indicating a much lower rate of transfer in the case of ammonium. However, contradictory results were obtained by Tanaka and Yano (2005): they observed that the AM fungus *Glomus aggregatum* can rapidly deliver ammonium-N to maize plants but not nitrate-N. These contrasting results might be due to the different AM fungi and host plant used in these experiments and could indicate a degree of functional complementarity of AM fungi.

Once internalized by the ERM, N has been showed to be assimilated and translocated to the IRM in form of amino acids, mainly in the form of arginine (Govindarajulu *et al.*, 2005; Jin *et al.*, 2005). More precisely, the N_i absorbed from the soil is converted to glutamine via glutamine synthase (GS) and then to arginine via Carbamoyl phosphate synthetase (CPS), Arginosuccinate synthase (ASS) and Arginosuccinate lyase (AL) in the urea cycle. The arginine obtained is transferred to the IRM but not to the plant as shown by labeling experiments using ^{14}C (Govindarajulu *et al.*, 2005). Indeed, arginine seems to be retransformed to glutamate via Ornithine oxoacid transaminase (OAT) and then to ammonium in the IRM to be transferred to the plant (Govindarajulu *et al.*, 2005). This hypothesis has also been confirmed by studying the enzymatic activities in the ERM and in the IRM: Tian *et al.* (2010) shown that GS, CPS, ASS and AL were more expressed in the ERM and OAT more in the IRM.

At the symbiotic interface, the transfer of ammonium from the IRM to the plant is expected to proceed by way of secretion of ammonium through unknown transporters present in the fungal plasma membrane into the periarbuscular space, followed by uptake through plant AMTs in the periarbuscular membrane (Fig.1.4). Here, a higher number of symbiotic interfaces could be important as Ames *et al.* (1983) reported that amount of N transferred to the plant was correlated with the percentage of root length colonized and with the hyphal length density.

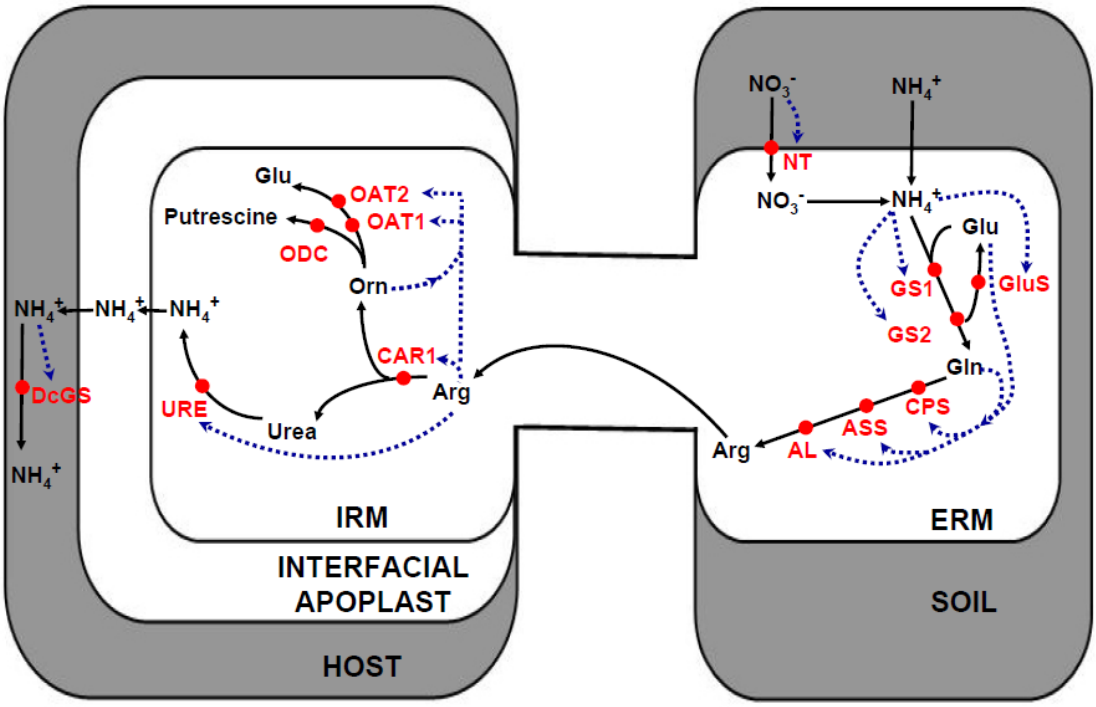

Fig.1.4: Working model of nitrogen transport and metabolism in the symbiosis between plant roots and AM fungi. N moves (black arrows) from the soil into the fungal ERM, through a series of metabolic conversion reactions into Arginine, which is transported into the intraradical mycelium within the root (Host) and is there broken down; nitrogen is transferred to and assimilated by the host as ammonium (Tian *et al.*, 2010).

Plant ammonium transporters

The first ammonium transporters isolated were MEP1 (Marini *et al.*, 1994) from yeast and AtAMT1;1 (Ninnemann *et al.*, 1994) from *Arabidopsis thaliana*. Both were shown to be high affinity NH_4^+ transporters (K_m <0.5 µM) and were characterized in a yeast mutant defective in ammonium transport. Related proteins were found in bacteria (Siewe *et al.*, 1996; Montesinos *et al.*, 1998; van Dommelen *et al.*, 1998), yeast (Marini *et al.*, 1997) and animals (*Caenorhabditis elegans*), as well as in plants including *Arabidopsis thaliana* (AtAMT1;2 and AtAMT1;3 ; Gazzarrini *et al.* 1999), *Brassica napus* (BnAMT1;2; Pearson *et al.,* 2002), *Lotus japonicus* (LjAMt1;1, LjAMT1;2 and LjAMT1;3: ; Salvemini *et al.,* 2001; D'Apuzzo *et al.,* 2004), *Lycopersicon esculentum* (LeAMT1;1, LeAMT1;2 and LeAMT1;3; Lauter *et al.* 1996; von Wiren *et al.,* 2000; Becker *et al.,* 2002; Ludewig *et al.,* 2002) and *Oryza sativa* (OsAMT1;1, OsAMT1;2 and OsAMT1;3 ; Sonoda *et al.,* 2003). All these plant transporters had homology in the amino

acid sequence over 70% and were classified in the AMT1 family (Fig.5) but not all of them were characterized as high affinity AMTs: AtAMT1;2 and AtAMT1;3 for example encoded low-affinity transporters with K_m value of 25 to 40 µM (Gazzarrini *et al.*, 1999).

Three years after the discovery of AtAMT1;1, an entirely different AMT was discovered in *A. thaliana*: AtAMT2;1. It has only 25% homology with the other described *A. thaliana* AMTs and was shown to be more closely related to yeast MEP transporters than to the AMT1 subfamily (Marini *et al.*, 1997). It was classified in a distinct family (Howitt & Udvardi, 2000; Loque & von Wiren, 2004), the AMT2 family. Members of the AMT2 family have introns in their DNA sequence in contrary to members of the AMT1 family (except for *LjAMT1;1*; Salvemini *et al.,* 2001). According to Suenaga et al. (2003), the AMT2 family can be further divided in three clades: AMT2, AMT3 and AMT4. Well-characterized members of the AMT2 family come from *Glycine max* (GmAMT4;1; Kobae *et al.,* 2010), *L. japonicus* (LjAMT2;2; Guether *et al.,* 2009b), and *O. sativa* (OsAMT2;1; Suenaga *et al.* 2003) (Fig.1.5).

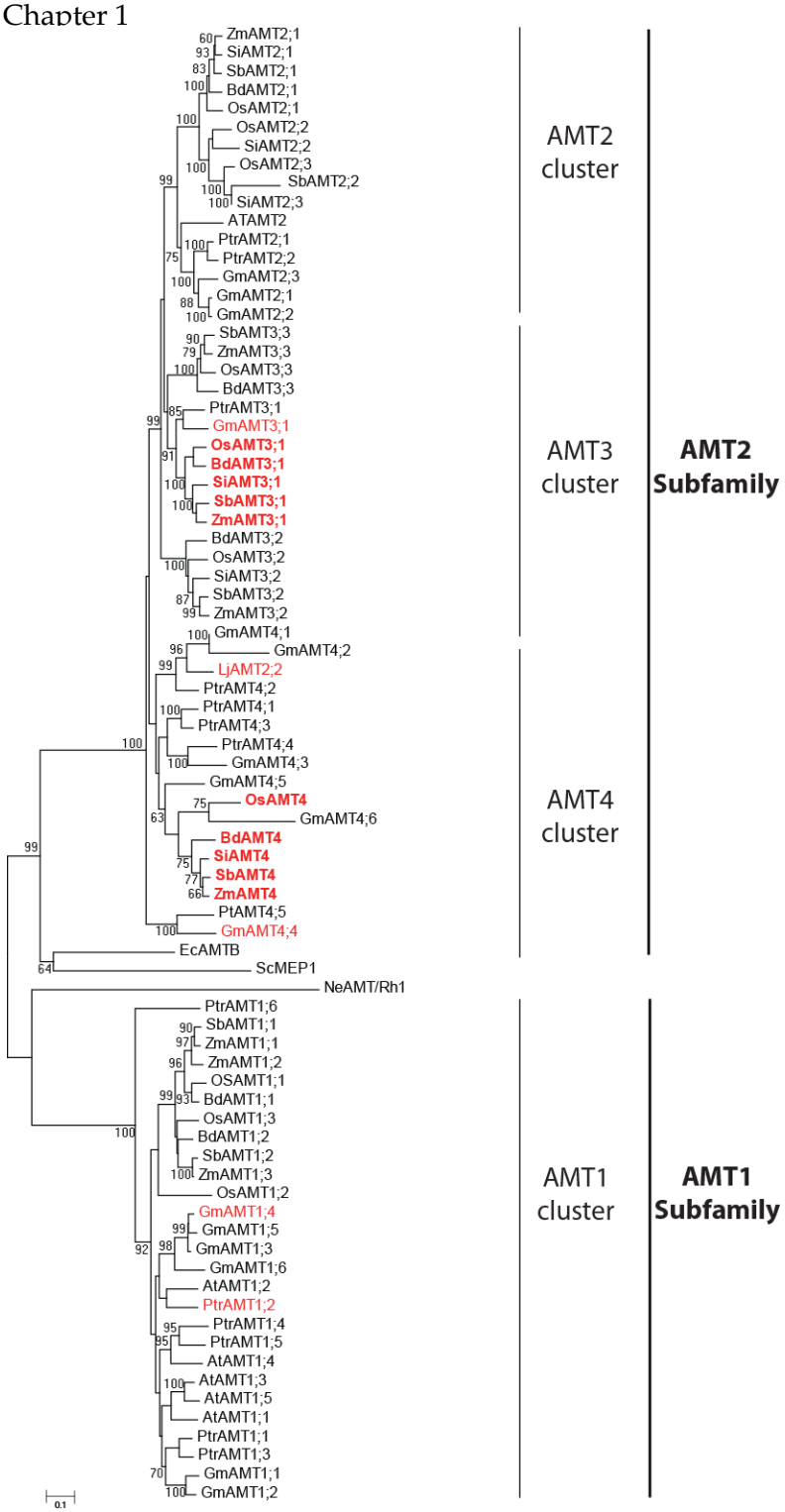

Figure 1.5. Neighbor joining tree of the ammonium transporter (AMT) family, based on the full open reading frames. Bootstrap values are from 1000 replications. Sequence names consist of species code (first letter of genus and first letter of species name) and the AMT number. The scale indicates a distance equivalent to 0.1 amino acid substitutions per site. Species codes: Ec, *Escherichia coli,* Ne, *Nitrosomonas europaea,* Sc, *Saccharomyces cerevisiae* (chosen as outgroups); At, *Arabidopsis thaliana,* Gm, *Glycine max,* Lj, *Lotus japonicus,* Os, *Oryza sativa,* Ptr, *Populus trichocarpa,* Sb, *Sorghum bicolor,* Zm, *Zea mays,* Bd, *Brachypodium distachyon,* Si, *Setaria italica.* AM inducible AMTs are in red, AM-inducible AMTs from the Poaceae are in red and bold.

Plant AMTs have eleven transmembrane domains (Simon-Rosin *et al.*, 2003; Sonoda *et al.*, 2003; Couturier *et al.*, 2007) similarly to AmtB from the bacteria *E. coli* (Zheng *et al.*, 2004), GintAMT1 from the AM fungus *G. intraradices* (Lopez-Pedrosa *et al.*, 2006) and AMT1 from the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Javelle *et al.*, 2003). Transport of ammonium through the pore also appears to be similar between plants, fungi and bacteria (Khademi *et al.*, 2004) (Fig.1.6): NH_4^+ is binding with high affinity to the pore, then transfers a proton to a pore-lining histidine residue. This proton is transported through the pore simultaneously with NH_3 and goes back to the NH_3 molecule shortly before it leaves the pore (Wang, S *et al.*, 2012). In contrast, Human Rh-type AMTs were shown to have a different transport mechanism than plant AMTs (Mayer *et al.*, 2006).

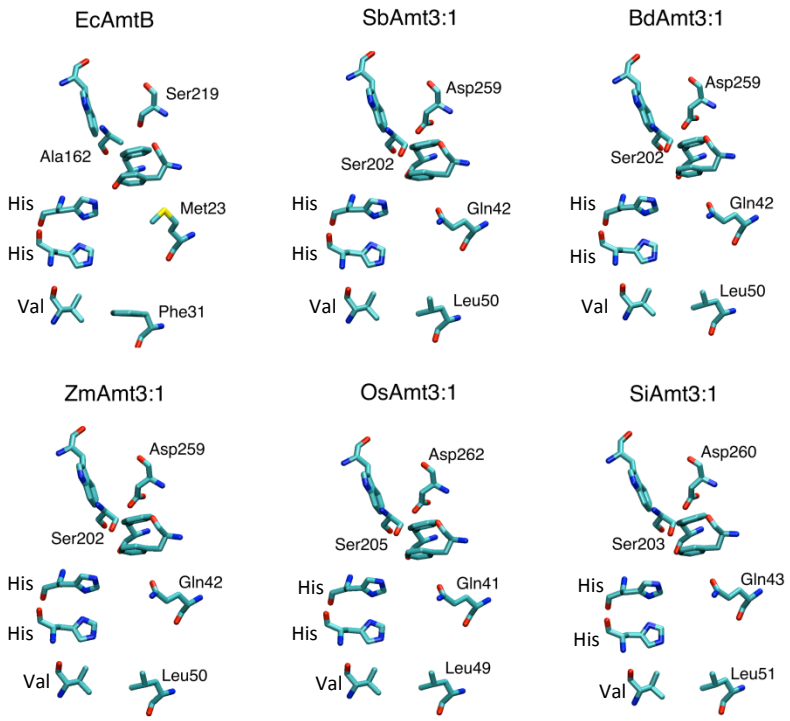

Figure 1.6. Homology models of the *E. Coli* EcAmtB, *S. bicolor* SbAMT3;1, *O. sativa* OsAMT3;1, *Z. mays* ZmAMT3;1, *B. distachyon* BdAMT3;1, and *S. italica* SiMT3;1 transporters built using swiss-model web server.

Interestingly, some AMTs are up-regulated during mycorrhizal symbiosis in different plants, such as poplar (*Populus trichocarpa*: *PtAMT1;2*; Couturier *et al.,* 2007), birdfoot trefoil (*Lotus japonicus: LjAMT2;2*; Guether *et al.,* 2009b), and soybean (*G. max: GmAMT3;1*, *GmAMT4;4*, *GmAMT4;1* and *GmAMT1;4*; Kobae *et al.,* 2010). AM inducible AMTs are present in the AMT1, AMT2, AMT3 and AMT4 cluster (Fig.1.5). Some AM-inducible AMTs seem to be conserved among plant families like AMT3;1 and could have evolved from a common ancestor. Others seem to have evolved independently (like *GmAMT1;4* or *PtAMT1;2*). Interestingly, both PtAMT1;2 and LjAMT2;2 were characterized as high affinity AMTs.

In *L. japonicus*, transcript of *LjAMT2;2* was localized in arbusculated cells (Guether *et al.*, 2009) using microdissection technology. Additionally, the highest uptake rate of this transporter at acidic pH 4.5 is fitting with the hypothesized localization at the periarbuscular membrane. Similarly, already described AM-inducible Phts were localized at the periarbuscular membrane (Harrison *et al.*, 2002b; Balestrini *et al.*, 2007). Nevertheless, transcript of *LjAMT2;2* was also observed in non-colonized cortical cell near arbusculated cells raising the hypothesis that expression of AM-inducible AMTs is part of the prepenetration response of the plant.

1.4.3 Reciprocity of nutrient exchange

The question of the reciprocity of the exchange was addressed as the evolutionary success of the AM symbiosis supposed a regulation of nutrient exchange between both partners (Kiers & Denison, 2008). Reward mechanisms were studied first on the exchange of P and C between fungi and plants. Interestingly plant appeared to be able to reward with more C the fungi transferring more P (Kiers *et al.*, 2011). In a split-root experiment where both sides of the root system were mycorrhized with different AM fungi, a reward of the most beneficial fungus was also observed (Bever *et al.*, 2009). In the opposite direction, AM fungi can reward an increased C transfer from the plant by delivering more P (Kiers *et al.,* 2011). However, different fungal responses to an increase in C supply were also observed: *R. irregularis* provided more P to the plant in direct exchange with more C but *Glomus aggregatum* did not (Kiers *et al.,* 2011). Thus, the exchange of nutrients between plant and AM fungi is hardly one to one relation. Indeed, Walder *et al.* (2012) shown that different plants sharing a common mycorrhizal network do not invest and do not get the same amount of resources. In this study, sorghum (*Sorghum bicolor*) was

interconnected to flax (*Linum usitatissimum*) by *G. mosseae* or *R. irregularis*. The authors found out that sorghum invested much higher amounts of C in the common mycorrhizal network than flax when both plants were grown in mixed-cultures. However, flax obtained up to 94% of the N and P taken up by the fungi. Interestingly, *G. mosseae* and *R. irregularis* differed in their nutrient repartition between flax and sorghum revealing different strategies of these AM fungi. Interestingly, the biomass of sorghum was nearly not affected in mixed-culture even if it invested most C in the common mycorrhizal network. This finding supports the hypothesis that photosynthesis is not a limiting factor for plant growth (Körner, 2003).

Interestingly, a recent study by Fellbaum *et al.* (2012) shown that fungal N transfer might also be linked to C transfer from the plant. By manipulating the C supply to the fungi, the author shown that an increased C supply triggered the uptake and transport of N. They also found an influence of the C supply on the expression of fungal genes coding for enzymes of the N cycle. Expression of the enzymes argininosuccinate synthase, argininosuccinate lyase, carbamoyl-phosphate synthase, glutamate synthase, and glutamine synthetase 1 and 2 were up-regulated in the ERM of *R. irregularis* under conditions of increased C availability.

All these results together underline the complex cross-talk between the symbiosis partners and show that both partners are controlling together the nutrient exchange during AM symbiosis.

1.5 Host plants

AM fungi have the capability to form a symbiosis with the roots of most land plants. Among the angiosperms, there are only a few exceptions: Colonization is unlikely to occur in families like Chenopodiaceae, Brassicaceae, Caryophyllaceae, Polygonaceae, Juncaceae and Proteaceae. *Arabidopsis thaliana*, the model organism used in plant biology and genetics belonging to the Brassicaceae family is a good example of a non-mycorrhizal plant: this is a severe disadvantage for studying molecular processes in the AM symbiosis. Nevertheless, also in non-mycorrhizal plant families, colonization can sometimes be observed (Veiga *et al.*, 2013). As a matter of fact, environmental factors play an important role for AM colonization and changes could affect the mycorrhizal status of the plants (Smith & Read, 2008). Indeed, some plant species from the family of the Chenopodiaceae were found to form AM

symbiosis in saline environments (Sengupta & Chaudhuri, 1990; van Duin *et al.*, 1990). The genome of all plants might carry evolutionary footprints of present or former AM status and important genes for the AM symbiosis might be conserved between plant families.

In temperate ecosystems, AM fungi are mostly colonizing herbs but also some trees as *Populus, Acer*, *Malus* or *Salix.* For this reason, AM fungi can be of crucial importance for reforestation programs and as inocula basis for annual plants. Interestingly, *Populus* and *Salix* are not only forming arbuscular mycorrhiza but can also form ectomycorrhiza (Walker & McNabb, 1984; Dhillion, 1994), another type of mycorrhizal symbiosis where the fungi stay outside of the roots and forms a Hartig net around root tips. In the case of poplar, the form of the symbiosis depends on the age of the plant, on soil depth, and on environmental factors like soil moisture (Lodge, 1989; Neville *et al.*, 2002; Gehring *et al.*, 2006). Remarkably, it was shown that both symbiosis forms have a different impact on plant gene expression. Actually, poplar gene expression of phosphate transporters can vary when plants are mycorrhized with ectomycorrhizal or with AM fungi (Loth-Pereda *et al.*, 2011): PtPT10 was only induced in plants mycorrhized with *G.mosseae* or *R. irregularis*. The transporter was not expressed in the non-mycorrhized roots or in the roots mycorrhized with *Paxillus involutus* or *Laccaria bicolor*. Also PtPT3, PtPT4/PtPT7 and PtPT5 shown a higher expression in the roots mycorrhized with *G.mosseae* or *R. irregularis* compared to non-mycorrhized roots or roots mycorrhized with *P. involutus* or *L. bicolor*. However, little is known about functional complementarity of both symbiosis forms when colonizing simultaneously the plant.

Nevertheless, as mentioned before, in temperate ecosystems herbs are mostly forming AM symbiosis. Some of these herbs are of crucial importance in agriculture systems as for example cereals in the family of the Poaceae including maize, rice or sorghum. In my thesis, I focused on this plant family.

1.5.1 The Poaceae

The grass family Poaceae includes approx. 1000 extant species (Glémin & Bataillon, 2009). Maize, rice and sorghum are part of the plants domesticated by the human societies. Domestication of plants played an important role in the shift from hunting and gathering to agriculture. Through the domestication process, plant characteristics, like high yield, are selected and lead to a fast evolution of the species. Most of these plant alterations would be deleterious in the wild (Zohary, 2004).

The Poaceae originated in the late Cretaceous (-80 million years ago (Mya)) (Prasad *et al.*, 2005). In its evolution, the core Poaceae split into two major clades the BEP and PACCMAD clades (BEP: Bambusoideae, Ehrhartoideae, Pooideae and PACCMAD: Panicoideae, Arundinoideae, Centothecoideae, Chloridoideae, Aristidoideae, Danthonioideae) (Bouchenak-Khelladi *et al.*, 2008), which diverged around or even before -55 Mya (Prasad *et al.*, 2005) (Fig. 1.7). The subdomesticated and cultivated species found in the Poaceae belong to these two clades (BEP and PACCMAD) and are distributed in four subfamilies, two in each clade: the Ehrartoideae (rices) and the Pooideae (wheat, barley, rye and oat) in the BEP clade; and the Panicoideae (maize, sorghum, foxtail millet and sugar cane) and the Chloridoideae (finger millet and tef) in the PACCMAD clade. The Pooideae can be classified further in numerous tribes. Among them, the tribe Aveneae contains oat and the tribe Triticeae contains species such as wheat, barley and rye which emerged ca. 12 Mya (Gaut, 2002; Huang *et al.*, 2002). Within the Panicoideae, a tribe of closely related species, the Andropogoneae, includes maize sorghum and sugar cane and emerged between 9 and 16 Mya (Gaut, 2002).

Probably, domestication started with the Pooideae about 12000 years ago in the region of the Fertile Crescent (today Israel, Jordan , Syria, Irak, Libanon and parts of the Turkey) (Frankel *et al.*, 1995). The Pooideae include only C3 species and were domesticated in boreal regions as well as in temperate regions. The Panicoideae and Chloridoideae were domesticated probably a little later in the tropics and subtropics and include also C4 species (Glémin & Bataillon, 2009). Interestingly, in America, maize was nearly the only domesticated cereal grass and other crops like potato, bean and pumpkin were playing an important role for the establishment of agriculture.

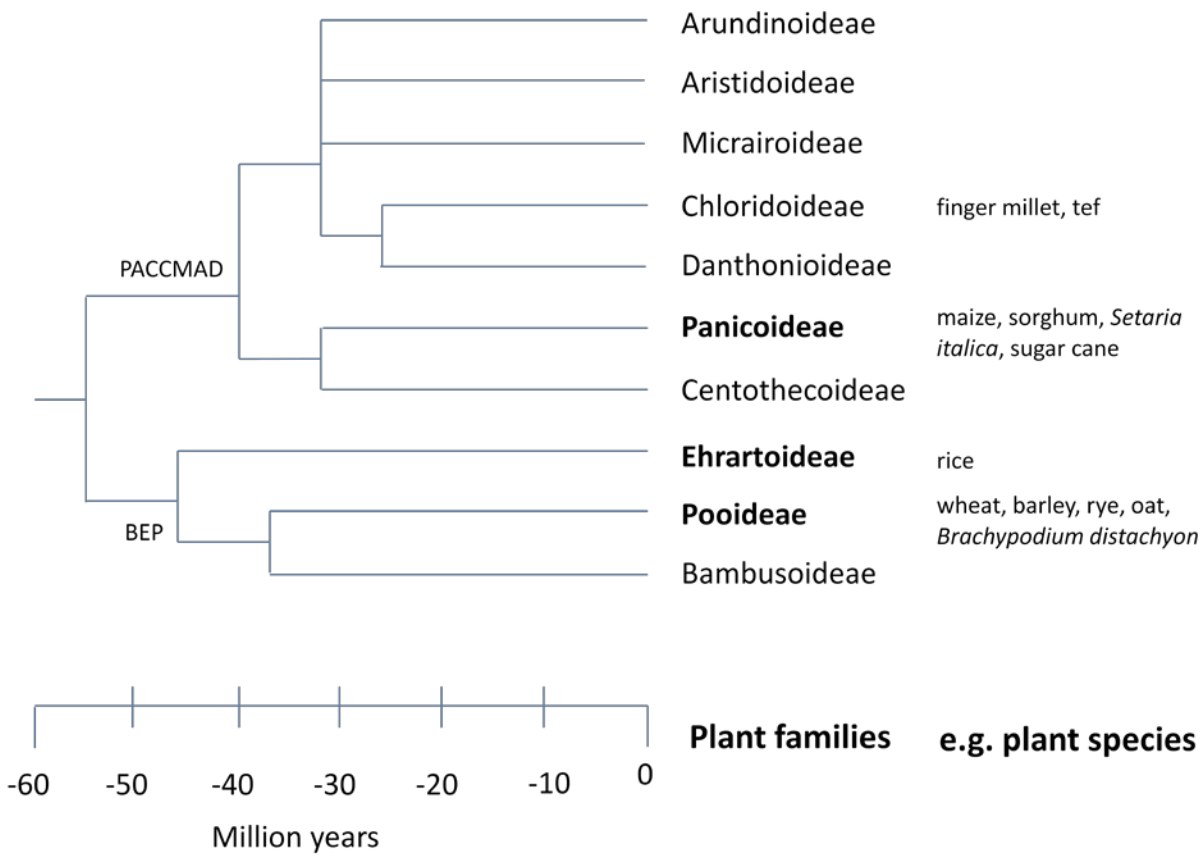

Fig. 1.7: Evolution of the Poaceae (modified after Glémin and Bataillon 2009)

Domestication is usually associated with a loss of diversity in the genome due to a rather intense selection on a subset of wild genotypes with desirable characteristics. Indeed, it was shown that genomic diversity present in wild ancestors is higher than in the domesticated species (Glémin & Bataillon, 2009). However, the ability to form AM symbiosis did not get lost in domesticated grasses even under conditions of intensive agriculture that are common today.

In the following paragraphs, the grasses used as models in my thesis are briefly presented.

Sorghum

Sorghum (*Sorghum bicolor*) is an herbaceous plant belonging to the Panicoideae (Fig. 1.7). It is a C4 plant and can produce good yield under more arid conditions than most other cereal crops. Thus, it is an existential source of food, feed, or fiber for farmers living in the semi-arid tropics of Africa, Asia and South America (Paterson *et al.*, 2009). The semi-arid tropics include most of India, South-East Asia, Sub-Saharan Africa, most of southern Africa and part of Latin America. Typically, the semi-arid tropics have limited, erratic rainfall. Often, sorghum is grown in drought prone areas that are not suitable for maize cultivation. In the southern plains of the USA, sorghum is mainly grown for the production of biofuel. The total production of sorghum around the world in 2010 was of over 55 million tones and the total cultivated area over 40 million hectares (http://faostat3.fao.org/home/index.html). In 2010 the USA was the country with highest production of sorghum (8.7 million tones). Sorghum is the fifth most cultivated crop plant just after maize, rice, wheat and barley.

The sorghum genome was sequenced and annotated (Paterson *et al.*, 2009), revealing a relatively small genome (about 730 Mb). Its ability to form AM symbiosis makes sorghum to a good model plant to study AM-plant interactions at molecular level.

Maize

Maize (*Zea mays*) is the domesticated variant of the grass teosinte and originates from Central America (Doebley *et al.*, 2006). As sorghum, it a C4 plant belonging to the Panicoideae (tribe Andropogoneae). Maize is a monoecious plant and develops unisexual male and female flowers in separated parts of the plant. Maize is used mainly for food, feed and biofuel production. It is the worldwide most cultivated crop plant with a production of 840 million tons per year, thereof 316 million tons in the USA in 2010 (http://faostat3.fao.org/home/index.html). Additionally, maize is an important model organism for fundamental research into the inheritance and functions of genes, epigenetic silencing or transposition (Schnable *et al.*, 2009). In contrast to sorghum, maize has undergone several genome duplications and its genome size increased to 2300 Mb over the last 3 million years (Schnable *et al.*, 2009).

Rice

Rice (*Oryza sativa* or *Oryza glaberrima*) belongs to the Ehrartoideae. It is a C3 plant which can be grown in relatively wet environment where no other crop are capable to grow. Rice is the second most cultivated crop after maize. It is of crucial importance especially in Asia and is the staple food for over half of the world's population (www.rice2004.org). The worldwide rice production reached 696 million tons in 2010 thereof 197 million tons in China, the country producing most rice over the world (http://faostat3.fao.org/home/index.html).

The genome of rice has a size of about 389 Mb, one of the smallest cereal genome sizes. Moreover, rice is relatively easily genetically transformed (stable transformation with *Agrobacterium* for example; Toki, 1997), making rice an ideal model organism for the cereal grasses (Sequencing ProjectInternational Rice, 2005).

Brachypodium distachyon

Brachypodium distachyon (Purple false brome) is a wild annual grass endemic to the Mediterranean and Middle East (Draper *et al.*, 2001). It belongs to the Pooideae, like wheat, barley and most cool season cereals. Actually, it was the first member of the Pooideae the genome of which was fully sequenced (Initiative, 2010) (the genome is available at http://www.brachypodium.org/). Purple false brome has a relatively small genome size compared to the other Poaceae (about 272 Mb) and has a short generation time, making it a good model for plant molecular geneticists (Draper *et al.*, 2001).

Setaria italica

Setaria italica (Foxtail millet) belongs to the Panicoideae like sorghum and maize. It is grown mainly in Asia. *Setaria italica*'s genome was sequenced and annotated recently (Zhang *et al.*, 2012). Its genome size is similar to rice with about 490 Mb. The agricultural importance of foxtail millet is limited, due to its low productivity. However, foxtail millet is closely related to switchgrass (*Panicum virgatum*) and napier grass (*Pennisteum purpureum*) used for the production of biofuel. Additionally, high-throughput platforms for its transformation exist.

1.6 Aims and structure of this thesis

In order to understand the factors regulating the transfer of mineral N from different AM fungi to an important crop plant, *Sorghum bicolor*, the following objectives were defined for this thesis:

(I) identify and characterize of the AMTs of *S. bicolor* by genome analysis and qRT-PCR analysis to measure transcript accumulated in various nitrogen treatments and in the presence or absence of AM fungi

(II) study the functional evolution of AM-inducible AMTs: did they evolve independently in *S. bicolor* and in other cereals like maize, rice, foxtail millet and purple false brome or were they present in a common ancestor of these plants?

(III) find out the effect of different N sources on the N cycle of the AM fungi *R. irregularis* by studying the expression of gene coding for enzymes from the N cycle as well as ammonium and nitrate transporters under various nitrogen statuses.

(IV) determinate the time course of N transfer between *G. mosseae* and sorghum

(V) assess the importance of functional AM-inducible AMTs for a working AM symbiosis by producing transgenic rice plants in which these transporters are silenced

Chapter 1 is an introduction on mycorrhizal fungi.

In **chapter 2**, I present my results on the characterization of *S. bicolor* AMTs. These data provide information on the expanded family of AMTs present in the genome of *S. bicolor*, on their expression in different plant tissues and under different N status and mycorrhizal conditions. The AM-inducible AMTs were characterized in yeast to confirm their functionality. We also localized the transcript and the protein of one AM-inducible AMT in mycorrhized *S. bicolor* roots and elucidated the pattern of expression of this transporter using a combination of various and complementary methods: laser microdissection technology, western blots and immunolocalization. This chapter has been published already in “New Phytologist”.

The question of the evolution of AM-inducible AMTs is raised in **chapter 3**. The aim of this study was to understand the stability of AM-inducible AMTs in an evolutionary point of view and to study the

polymorphism of these genes among different cereal plants. We studied the expression of two AM-inducible AMTs (AMT3;1 and AMT4) in different plant species belonging to the family of the Poaceae. For all the plant studied, AMT3;1 was additionally characterized in yeast and it's three dimensional structure was simulated revealing a very interesting conserved evolution. A publication on this study is in preparation.

Chapter 4 highlights the role of N source in the transfer of N from the AM fungi to the plant. As shown by Tian *et al.* (2010), AM fungi can take up N in different forms and transform it into arginine to transfer it from their ERM to their IRM. Finally, the arginine is released in glutamate and ammonium to be transferred from the AM fungi to the plant. For this reason, different enzymes are expressed in the ERM and in the IRM of AM fungi depending on the N source. Here we studied the effect of different N sources on the expression of genes coding for enzymes described by Tian *et al.* (2010). Additionally, to complete the picture, we looked at the expression level of additional enzymes and of fungal ammonium and nitrate transporters. The plant side was also analyzed: expression level of the plant glutamine synthase under the different N sources was quantified. The publication on this study is in preparation.

A short communication submitted to "Plant signaling and behavior" is presented in **chapter 5**. In contrast to P, N is relatively mobile in the soil compensating the depletion zone around roots. For this reason the question of the role of AM fungi for plant N nutrition has been raised. Here, we demonstrate the efficiency and rapidity by which the AM fungus *G. mosseae* to takes up N and transfers it to its plant partner. These data highlight the role of AM fungi for plant N acquisition.

A paper by Florian Walder *et al.* of which I am a co-author is shown in the **appendix I**. Florian Walder made a PhD thesis on common mycorrhizal network at our institute. I contributed to this paper collaborating with Florian Walder and particularly by sampling, extracting RNA and synthesizing cDNA of the field *S.bicolor* plants as well as by designing quantitative PCR primers for the reference gene ubiquitin.

In **appendix II**, two special techniques are explained :(I) lasermicrodissection and (II) amiRNA. In (I) a poster from Arnould *et al.* about laser microdissection technology is also shown. This poster was presented at the third "Journée Francophones Mycorhizes" in Nancy. It highlights the methods we

developed in collaboration with the group of Prof. Daniel Wipf (INRA, Dijon, France) to obtain high quantity and quality RNA and proteins using laser microdissection technology.

Appendix III concerns the functional analysis of transgenic rice plants with silenced OsAMT3;1 or OsAMT4 transporters. After the results of chapter 2 and 3, it appears that AMT3;1 and AMT4 are up-regulated in sorghum as well as in maize, rice, foxtail millet and purple false brome. Rice can be more easily transformed compared to sorghum. So, we produced transgenic rice plants which AMT3;1 and AMT4 genes were silenced, using the amiRNA technology. Our goal was to understand the importance of AM-inducible AMTs for a functioning symbiosis by silencing the AMT3;1 and AMT4. This work was made in collaboration with CIRAD Montpellier (France). Rice cali were transformed with artificial micro RNA constructs designed to silence OsAMT3;1 or OsAMT4. Regenerated plants containing only one copy of the construct were selected. Seeds of these selected plants were then planted in presence or absence of AM fungi to check functionality of the construct and preliminary results are shown in this chapter. We plan to publish these data together with additional experiments on the capability of the mutants to transport N in the presence of AM fungi.

2. The Family of Ammonium Transporters (AMT) in *Sorghum bicolor*: Two mycorrhizal-induced AMTs are expressed locally, but not systemically in colonized roots

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Research

The family of ammonium transporters (AMT) in *Sorghum bicolor*: two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi

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Summary

- Arbuscular mycorrhizal (AM) fungi contribute to plant nitrogen (N) acquisition. Recent studies demonstrated the transport of N in the form of ammonium during AM symbiosis. Here, we hypothesize that induction of specific ammonium transporter (AMT) genes in *Sorghum bicolor* during AM colonization might play a key role in the functionality of the symbiosis.
- For the first time, combining a split-root experiment and microdissection technology, we were able to assess the precise expression pattern of two AM-inducible AMTs, *SbAMT3;1* and *SbAMT4*. Immunolocalization was used to localize the protein of *SbAMT3;1*.
- The expression of *SbAMT3;1* and *SbAMT4* was greatly induced locally in root cells containing arbuscules and in adjacent cells. However, a split-root experiment revealed that this induction was not systemic. By contrast, a strictly AM-induced phosphate transporter (*SbPt11*) was expressed systemically in the split-root experiment. However, a gradient of expression was apparent. Immunolocalization analyses demonstrated that SbAMT3;1 was present only in cells containing developing arbuscules.
- Our results show that the *SbAMT3;1* and *SbAMT4* genes are expressed in root cortical cells, which makes them ready to accommodate arbuscules, a process of considerable importance in view of the short life span of arbuscules. Additionally, SbAMT3;1 might play an important role in N transfer during AM symbiosis.

Introduction

Arbuscular mycorrhizal (AM) fungi play a key role in the nutrition of many herbaceous land plants, including crops such as *Sorghum bicolor*. They take up nutrients from the soil via their extraradical mycelium and translocate them to the plant partner, receiving carbohydrates in return (Smith & Read, 2008). Nutrients, such as phosphorus (P) and nitrogen (N), are then transferred from the fungus to the host plant at the level of a specialized structure, the arbuscule, and the plant takes up these nutrients through a special membrane derived from the plant plasma membrane, the so-called 'periarbuscular membrane' (Harrison *et al.*, 2002; Kobae & Hata, 2010).

It is well known that the AM symbiosis modifies the expression of plant transporter genes. For example, some plant P transporters have been shown to be induced or expressed only in arbuscule-containing cells of *Lotus japonicus*, *Medicago truncatula* or *Oryza sativa* (Karandashov *et al.*, 2004; Glassop *et al.*, 2007; Javot *et al.*, 2007; Guether *et al.*, 2009a). In *M. truncatula*, the P transporter *MtPht1;4*, which is only expressed during AM symbiosis, has been shown to be localized in the periarbuscular membrane (Harrison *et al.*, 2002).

The role of AM fungi in plant N nutrition has been studied. In the soil, N is present in organic and inorganic forms, but the former is the dominant form. Plants and the extraradical mycelium (ERM) of AM fungi can absorb inorganic N as nitrate or ammonium (Govindarajulu *et al.*, 2005) and some soluble forms of organic N, such as amino acids (Smith & Read, 2008; Leigh *et al.*, 2009). Nitrate and ammonium are relatively mobile in the soil and can be transported to plant roots by mass flow, limiting the depletion zone around the roots. For this reason, the question of the relevance of AM fungi in plant N nutrition was raised (Johnson, 2010; Smith & Smith, 2011). Evidence for inorganic N uptake by AM fungi in the form of ammonium was obtained through the characterization of two ammonium transporters (AMTs) in the AM fungus *Glomus intraradices* (Lopez-Pedrosa *et al.*, 2006; Pérez-Tienda *et al.*, 2011). Furthermore, Mäder *et al.* (2000) have shown that the amount of N present in the plant

and coming from the AM pathway was up to 42%. In monoxenic cultures of mycorrhizal carrot roots, this amount was *c.* 30% (Govindarajulu *et al.*, 2005). By contrast, the role of AM fungi in the mineralization of organic forms of N is unclear. Hodge *et al.* (2001) reported that AM fungi enhance organic N decomposition as well as plant N capture, but other studies have not confirmed these findings (Frey & Schüepp, 1993; Hawkins *et al.*, 2000). Once internalized, N is assimilated and translocated to the intraradical hyphae in the form of amino acids, mainly arginine (Govindarajulu *et al.*, 2005), and is finally transferred to the plant as ammonium (Tian *et al.*, 2010). In a root cell with an arbuscule, this transfer is expected to proceed by the secretion of ammonium into the periarbuscular space through unknown transporters present in the fungal plasma membrane, followed by its uptake through plant AMTs from the periarbuscular membrane.

Plant AMTs have been studied extensively, and phylogenetic analysis has revealed two distinct subfamilies: the AMT1 subfamily and the AMT2 subfamily (Loque & von Wiren, 2004; Supporting Information, Table S1). Here, we focus on the role of AMTs in the mycorrhizal symbiosis of sorghum (*S. bicolor*), an important crop plant whose genome has been fully sequenced (Paterson *et al.*, 2009). Sorghum, a herbaceous plant belonging to the *Poaceae*, is the world's fifth biggest crop (after maize, rice, wheat and barley). It can grow under relative arid conditions and is an important source of food, feed and fibers in many developing countries. Bioinformatic analyses of the *S. bicolor* genome revealed eight genes coding for AMTs. The transcript abundance of these transporters was measured in roots of *S. bicolor* in the presence or absence of AM fungi under different regimes of N nutrition. Laser microdissection was used to isolate individual root cells with or without mycorrhizal structures (Balestrini *et al.*, 2007; Gomez *et al.*, 2009) to measure cell-specific gene expression. Additionally, a split-root experiment was performed to determine whether the induction process was systemic. Our goal was to identify AM-inducible AMTs in sorghum and to define their pattern of expression during AM symbiosis. This could lead to new insights into the importance of AM-inducible AMTs for AM symbiosis.

Materials and Methods

Plant growth conditions for tissue analysis

Experiments were performed with sorghum (*Sorghum bicolor* (L.) Moench), cv Pant-5. This cultivar is closely related to BTx623, the sorghum cultivar used for genome sequencing (Paterson *et al.*, 2009). Seeds of cv Pant-5, kindly provided by sorghum breeders of I.G.F.R.I. (CCS Agriculture University of Hissar, Haryana, India) and G. B. Pant University of Agriculture and Technology (Pantanagar, Uttaranchal, India), were surface-sterilized (10 min in 2.5% KClO) and then rinsed with sterile deionized water several times for 1 d and soaked in sterile deionized water overnight. Seeds were pregerminated on autoclaved Terra Green (Oil Dri US-special, American aluminiumoxide, type III/R; Lobbe Umwelttechnik, Iserlohn, Germany) at 25°C for 24 h and then grown in the dark at room temperature for 72 h. The fungal strains *G. intraradices* BEG-75 and *G. mosseae* ISCB13 (Botanical Institute, Basel, Switzerland) were propagated by trap cultures as previously described (Oehl *et al.*, 2004). To establish AM symbiosis, pregerminated seeds were individually inoculated in pots containing a mixture of acid-washed Terragreen, sand and loess soil (5 : 4 : 1 w/w/w). About 100 spores were added to the mixture. For the controls (nonmycorrhizal plants), the same amount of autoclaved inoculum was added to the mixture. To correct for possible differences in microbial communities, each pot received 1 ml of filtered washing of AM fungal inoculum (van der Heijden *et al.*, 1998). Plants were grown in a glasshouse with day : night temperatures of *c.* 28 : 15°C.

Plants were watered twice a week during experiments. From the first week on, 8 ml of modified Hoagland solution was applied weekly. Five different Hoagland solutions, modified after Gamborg & Wetter (1975), were prepared to obtain different N sources or N concentrations (Table S2): '−N', '1 × NO_3^-', '0.3 × NO_3^-', '0.1 × NO_3' and '1 × NH_4^+'.

Experimental setup

For all experiments, seeds were surface-sterilized and grown in 500 ml pots containing the soil mixture as already described.

Time-course experiment Three different AM fungal treatments were applied (+AMF: *G. intraradices* or *G. mosseae*, −AMF). Five different Hoagland solutions were applied to the *G. mosseae* and −AMF treatments, namely '−N', '1 × NO_3^-', '0.3 × NO_3^-', '0.1 × NO_3' and '1 × NH_4^+ and three different Hoagland solutions were applied to the *G. intraradices* treatment, namely '−N', '1 × NO_3^-', and '1 × NH_4^+'. All treatments were independently repeated four times. A total of 156 pots were prepared. Shoots and roots of one-third of the plants (52) were harvested separately 5, 8 and 13 wk after inoculation.

Split root experiment After 2 wk of growth, plantlets were dug out gently and roots were distributed equally between two 350 ml pots fixed together with tape and containing the same mixture as described before. Three different AM fungal treatments were applied: AM fungi on both sides of the split-root system (+AMF: *G. mosseae*/+AMF: *G. mosseae*); AM fungi on one side and no AM fungi on the other side of the split-root system (+AMF: *G. mosseae*/−AMF); and no AM fungi on both sides of the split-root system (−AMF/−AMF). Two different Hoagland solutions were applied, namely '−N' and '1 × NO_3^-'. All treatments were independently repeated four times. A total of 40 split-roots systems were prepared. Shoots and roots were harvested separately 13 wk after inoculation.

Laser microdissection experiment Two different Hoagland solutions were applied, namely '−N' and '1 × NO_3^-'. Two different AM fungal treatments were applied (+AMF: *G. mosseae*, −AMF). All treatments were independently repeated four times. A total of 16 pots were prepared. Shoots and roots were harvested separately 13 wk after inoculation.

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Staining of AM fungi in plant roots and quantification of root colonization

From each analyzed plant, one subsample of 100 mg of fresh roots was used to determine the degree of AM fungal colonization, as follows. Root subsamples were stained with trypan blue (0.005% w/v in lactic acid, glycerol, water, 1 : 1 : 1, w/w/w) at 60°C for 10 min in 15 ml tubes in a water bath and destained for 24 h in glycerol : 1% HCl (w/w). Root colonization was quantified according to the grid intersection method as described by Brundrett *et al.* (1984). Total colonization comprised intersections containing hyphae, vesicles, spores or arbuscules. Differences between means of variables were analysed by ANOVA ($P \leq 0.05$), using SPSS 18.0 (IBM, Chicago, IL, USA).

DNA isolation from field samples and fungal diversity analyses

The diversity of AM fungal species associated with sorghum roots harvested in a field site (northeastern France; 47°62′N, 7°52′E; September 2011) was assessed. For each of the three plants, two subsamples (*c.* 100 mg) of fresh roots were snap-frozen and stored at −80°C. DNA was extracted using the NucleoSpin tissue KS kit (Macherey-Nagel, Düren, Germany). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA was amplified on a T3 thermocycler (Biometra, Goettingen, Germany) and the amplified fragments were then subcloned using the TOPO-TA cloning kit (Invitrogen). The full procedure is described in Courty *et al.* (2011). Sequences were manually corrected using Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA). To identify fungal species, BLASTN searches were carried out against the sequence databases at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

Identification and characterization of sorghum genes encoding AMT transporters

Sequencing, assembly, and annotation of the *S. bicolor* genome was described by Paterson *et al.* (2009). All *S. bicolor* sequences are available at the Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/Sorbi1/Sorbi1.home.html) and have been deposited at GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan. Using BLAST search and the INTERPRO domains (IPR018047 and IPR001905) at the JGI website, we identified gene models coding for putative AMTs (AMTs) in the draft genome. Gene prediction at the JGI was performed using gene predictors (FGENESH, and GENEWISE), and gene models were selected by the JGI annotation pipeline (Paterson *et al.*, 2009). Selection of the AMT models was based on expressed sequence tag (EST) support, completeness, and homology to a curated set of proteins. The putative homologs that were detected were characterized based on conserved domains, identities, and *E*-values in comparison with the use of a range of AMT sequences available from plants at the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and UNIPROT (http://expasy.org/) (Table S3). All full-length cDNAs were sequenced by cDNA walking (Methods S1). Sequences of the cDNAs described here are available at the NCBI database under accession numbers JX294852 to JX294859.

Signal peptides were predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and subcellular location with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/). Conserved protein domains were analyzed using prosite (http://us.expasy.org/prosite) and InterProScan (http://www.ebi.ac.uk/InterProScan).

For phylogenetic analysis, the AMT amino acid sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbour joining trees were constructed using Poisson correction model for distance computation in MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was carried out with 1000 replicates. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Gene accession numbers of amino acids sequences are given in the Methods S1.

Samples, RNA isolation and quantitative reverse transcription-PCR

RNA extraction and cDNA synthesis were performed as described by Courty *et al.* (2009), using the conditions specified in Methods S1. Primers used as controls or for analysis had an efficiency ranging between 90 and 110%. In the split-root and laser microdissection experiments, the transcript abundance of the AM-specific phosphate transporter from *S. bicolor* SbPt11 was also measured (F. Walder *et al.*, unpublished). In the split-root experiment, the *G. mosseae* elongation initiation factor (EIF) gene was used as an additional control to confirm the presence/absence of AM fungi. All primers used are listed in Table S4.

From each of the three *S. bicolor* plants harvested in the field site described, three subsamples (*c.* 100 mg) of roots, shoots, stem, pistils and stamens were snap-frozen and stored at −80°C for further gene expression analysis.

Concerning the time course, the split root and the laser microdissection experiments, plant roots were carefully washed under tap water to remove all soil adhering to the roots. Three subsamples of 100 mg of fresh roots were snap-frozen and stored at −80°C for further gene expression analysis.

C and N analysis

The remainder of the root samples and the shoot material were dried at 80°C for 72 h and weighed. These samples were ground in 1.5 ml Eppendorf® tubes using 1.1-mm-diameter tungsten carbide balls (Biospec Products Inc., Bartlesville, OK, USA) in a Retch MM301 vortexer (Retch GmbH & Co., Haan, Germany). Total N and C were measured using an online continuous flow CN analyzer coupled with an isotope ratio mass spectrometer (ANCA-SL MS 20-20 system; Sercon Ltd, Crewe, UK).

Tissue analysis by laser microdissection

Roots were washed with running water to remove the substrate. Pieces of 10–15 mm were cut with a razor blade from differentiated regions of the mycorrhizal and nonmycorrhizal roots. The root segments were embedded in OCT (EMS, Delta Microscopies Aygues-Vives, France) and then frozen at −23°C. Some 40 μm thin sections were cut with a Cryocut (Cryocut 1800 Leica), and the cuts were placed on Fisher Probe-On slides (Fisher Scientific, Ilkirch, France). The sections were washed and fixed as follows: 3 min 70% EtOH, 30 min DEPC H O, 2 min 100% EtOH. The slides were then dried for 20 min at 37°C on a warming plate and kept at −80°C before use.

An Arcturus XT microdissection system (Applied Biosystems, Foster City, CA, USA) was used to collect the cells from the mycorrhizal and nonmycorrhizal root sections. The slides with the dissected cells were thawed at 4°C for 15 min and then dried in a dessicator at room temperature for 15 min. Eight replicates of three different cell types were collected: arbuscule-containing cells (ARBs), noncolonized cortical cells from mycorrhizal roots (MNMs) and cortical cells from nonmycorrhizal roots (Cs). A total of 5000–15 000 cells were cut for each sample. RNA from the collected cells was extracted using the Arcturus Pico Pure RNA isolation Kit (Excilone, Applied Biosystems, Foster City, CA, USA), with a DNAse treatment in the column kit following manufacturer's instructions. Quantity and quality of the extracted RNAs were verified using a bioanalyzer with RNA pico chips (Agilent, Santa Clara, CA, USA). Synthesis of cDNA and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was done as previously described using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), starting with 100 pg RNA.

Heterologous complementation of a yeast mutant defective in ammonium uptake

The full-length *SbAMT3;1* and *SbAMT4* (Table S2) cDNA was cloned in pDR196 using Gateway technology (Invitrogen), as described earlier (Wipf *et al.*, 2003). The resulting plasmids were called pDR196-*SbAMT3;1* and pDR196-*SbAMT4*. The yeast strain 31019b (*MATa ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) (Marini *et al.*, 1997) was transformed with pDR196-*SbAMT3;1* or pDR196-*SbAMT4* according to Dohmen *et al.* (1991). As a control, we also cloned and transformed similarly the low-affinity transporter AtAMT1;3 from *Arabidopsis thaliana* described by Gazzarrini *et al.* (1999).

Western blot analysis

Soluble and cell-wall-adhering proteins were extracted as described in Methods S1. The proteins were separated on SDS/12% polyacrylamide gels and transferred to nitrocellulose membranes in Towbin buffer. Proteins were stained with Ponceau red, indicating equal amounts of protein in each lane. Membranes were washed twice with MilliQ water and then blocked with MTBS (3% milk powder, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) at 4°C overnight, and then washed twice with MTBS for 5 min each. The blots were incubated at 4°C overnight with a 1 : 1000 dilution of SbAMT3;1 antibodies in MTBS. The membranes were washed three times with MTBS and then incubated with secondary antibody (alkaline phosphatase-conjugated mouse anti-rabbit antibody, 1 : 30 000 dilution; Sigma-Aldrich) at room temperature for 1 h. After two washes with MTBS and one wash with alkaline phosphatase buffer (1 M NaCl, 1 M Tris-HCl pH 9.5), the membranes were incubated in chemiluminescence substrate (CDP-star; Roche).

Immunolocalization

Antibodies specific for the SbAMT3;1-derived peptides 469-[5′-H2N- WYSDNDTQHNKAPSG-CONH2-3′]-483 and 117-[5′-H2N-QHYYHDSDVVETFEIT-CONH2-3′]-132, corresponding to a nonconserved region in the protein, were produced in rabbits. The antisera and the peptides were obtained from the custom peptide antibody production program (Eurogentec, Seraing, Belgium). Immunolocalization was performed as described by Blancaflor *et al.* (2001) and Harrison *et al.* (2002) with some modifications (Methods S1) on nonmycorrhizal and mycorrhizal sorghum roots colonized by *G. mosseae*. Control images in which the mycorrhizal root sections were treated by replacing primary anti-PtPT10 antibodies with preimmune immunoglobulin at the same concentration showed no specific fluorescence.

Statistical analyses

An ANOVA was performed on the total biomass, on the C and N content, and on the total and arbuscular colonization for each treatment separately, where the two latter parameters were arcsine-transformed to fit the assumption of normal distribution. The ANOVA was based on N treatments and AMF treatments. Pairwise comparisons between the treatments were done with planned contrast analysis. Independent paired *t*-tests were performed. A probability of $P \leq 0.05$ was considered to be significant.

Results

Identification of AMT-encoding genes in *S. bicolor*

Eight genes coding for putative AMTs were identified in the predicted gene catalog resulting from the automated annotation of the *S. bicolor* genome assembly (v1.0, http://genome.jgi-psf.org/sorghum/sorghum.home.htm). The whole genome assembly was used. We performed a phylogenetic analysis, based on the alignment of the corresponding sequences, in comparison to other plant species (Fig. 1). Two of the AMTs identified in the *S. bicolor* genome (SbAMT1;1 and SbAMT1;2) are members of the AMT1 subfamily, a well-defined group in both monocots and dicots. According to current knowledge, genes encoding AMT1 proteins contain no intron, with the exception of LjAMT1;1 from *L. japonicus* (Salvemini *et al.*, 2001). All the other AMTs of *S. bicolor* (SbAMT2;1, SbAMT2;2, SbAMT3;1, SbAMT3;2,

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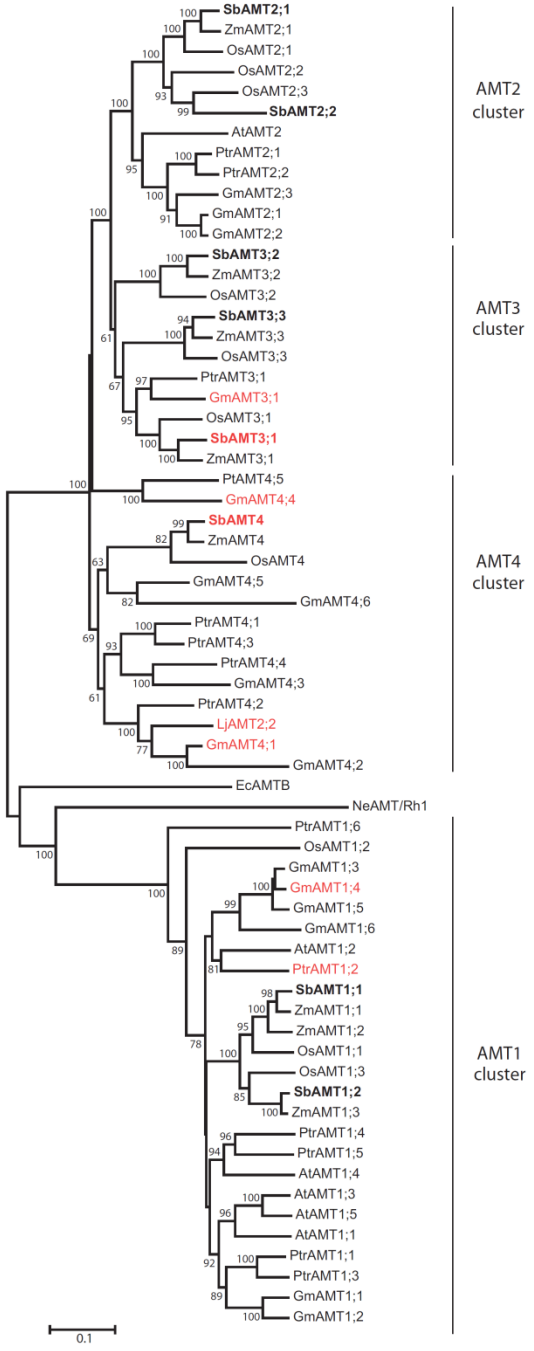
SbAMT3;3 and SbAMT4) belong to a separate clade comprising three clusters, each of which seems to be conserved between monocots and dicots (Fig. 1), with introns roughly conserved in each subclade with regard to size and splicing location (data not shown). The transmembrane prediction programme TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) indicates that each of the eight AMTs has 11 transmembrane domains with an extracellular N-terminus and a cytosolic C-terminus, like other plant AMT members (Marini & Andre, 2000; Thomas *et al.*, 2000). Characteristics of the *S. bicolor* AMT gene family are summarized in Table S3. Similarity and identity between the AMT amino acid sequences of *S. bicolor* are summarized in Table S5.



Effect of N source and of mycorrhization

Root colonization rates were *c.* 20–50% and 25–75% at 5 and 9 wk postinoculation, respectively. The percentage of roots colonized increased up to 80% after 13 wk (Fig. S1). The colonization rate was higher for *G. intraradices* than for *G. mosseae* in all the treatments at the second and third harvest (Table 1). N content per plant (Fig. S2) and shoot and root DW (Fig. S3) increased over time and were highest after 13 wk when NO_3^- 1× and NH_4^+ 1× were applied. After 13 wk, plant N nutrition had no significant effect on the mycorrhizal colonization with *G. mosseae* ($P = 0.4$) and *G. intraradices* ($P = 0.8$) and the plant N content was significantly lower in the '−N' treatment than in the '+NO_3^-' and '+NH_4^+' treatments for *G. intraradices* ($P = 0.008$) and *G. mosseae* ($P = 0.01$), respectively.

In the absence of mycorrhizal fungi, there were only minor effects of N nutrition on AMT gene expression in the roots (Table S6). By contrast, *SbAMT3;1* and *SbAMT4* were highly induced in mycorrhizal compared with nonmycorrhizal plants, independently of the N supply (Fig. 2a,b). *SbAMT3;1* and *SbAMT4* were similarly up-regulated after 5, 9 and 13 wk although plants were less colonized after 5 wk (Table S7).

Heterologous complementation of a yeast mutant defective in ammonium uptake

A yeast mutant complementation test was used to demonstrate the NH_4^+ transport function and to biochemically characterize *SbAMT3;1* and *SbAMT4*. *AtAMT1;3* was used as control. All three transporters were expressed through the yeast expression vector pDR196 (Wipf *et al.*, 2003) in a mutant yeast strain, 31019b, which lacked the three endogenous NH_4^+ transporter genes (MEP1, MEP2, MEP3) and was unable to grow on a medium containing < 3 mM NH_4^+ as the sole N source (Marini

Fig. 1 Neighbour joining tree of the ammonium transporter (AMT) family, based on the full open reading frames. Bootstrap values are from 1000 replications. Sequence names consist of species code (first letter of genus and first letter of species name) and the AMT number. The scale indicates a distance equivalent to 0.1 amino acid substitutions per site. Species codes: Ec, *Eschericha coli*; Ne, *Nitrosomonas europaea* (chosen as outgroups); At, *Arabidopsis thaliana*; Gm, *Glycine max*; Lj, *Lotus japonicas*; Os, *Oryza sativa*; Ptr, *Populus trichocarpa*; Sb, *Sorghum bicolor*; Zm, *Zea mays*. *S. bicolor* AMTs are in bold font. AM-inducible AMTs are in red.

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Table 1 One-way ANOVA comparing the percentage of colonization and of arbuscules in roots colonized by *Glomus intraradices* with roots colonized by *Glomus mosseae* for each of the three sampling dates (5, 8 and 13 wk postinoculation, respectively)

	Harvest 1 (5 wk)			Harvest 2 (8 wk)			Harvest 3 (13 wk)		
Treatment	−N	NH_4^+	NO_3^-	−N	NH_4^+	NO_3^-	−N	NH_4^+	NO_3^-
Colonization	0.76	**0.024**	0.803	**0.036**	**0.010**	**0.000**	**0.000**	**0.001**	**0.002**
Arbuscules	**0.037**	0.068	0.940	**0.004**	0.943	0.325	**0.003**	0.458	**0.031**

Percentages of root colonization were recorded on four plants for each treatment. *P*-values are given in each cell. Bold values indicate that roots were significantly ($P < 0.05$) more highlyi colonized by *G. intraradices* than by *G. mosseae*.

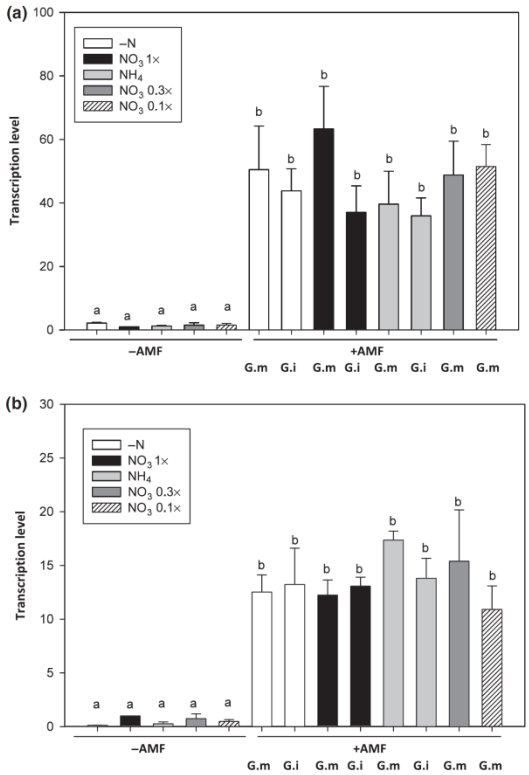

Fig. 2 Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *SbAMT3;1* (a) and *SbAMT4* (b) in *Sorghum bicolor* roots either noncolonized or colonized by arbuscular mycorrhizal fungi (AMF: G.i, *Glomus intraradices*; G.m, *Glomus mosseae*) 9 wk postinoculation (second harvest) in the different nitrogen (N) treatments (−N, $1\times NO_3^+$, $1\times NO_3^-$, $0.3\times NO_3^-$ and $0.1\times NO_3^-$). The values are the means of four replicates, and error bars represent SD. Ubiquitin was used as the reference transcript. Gene expression was normalized to the '−AMF, $1\times NO_3^-$' treatment. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's *F*-test). Letters indicate a *P*-value < 0.05.

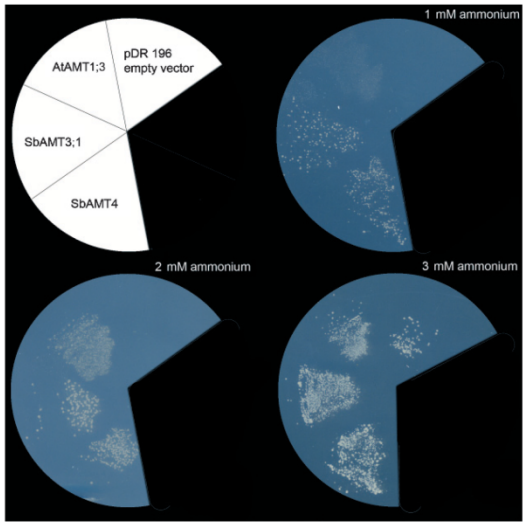

Fig. 3 Complementation of a yeast mutant defective in ammonium uptake by SbAMT3;1 and SbAMT4. Growth of the yeast strain 31019b, transformed with various constructs, on minimal medium supplemented with various NH_4^+ concentrations (1, 2 or 3 mM) as a sole nitrogen source. All strains were incubated for 5 d at 29°C. AtAMT2 from *Arabidopsis thaliana* was used as a control (Sohlenkamp *et al.*, 2000). pDR196 empty vector (control), *mep1Δmep2Δmep3Δ* + pDR196; AtAMT1;3, *mep1Δmep2Δmep3Δ* + pDR196-AtAMT1;3; SbAMT3;1, *mep1Δmep2Δmep3Δ* + pDR196-SbAMT3;1; SbAMT4, *mep1Δmep2Δmep3Δ* + pDR196-SbAMT4.

et al., 1997). SbAMT3;1cDNA or SbAMT4cDNA functionally complemented the yeast mutant efficiently, when 1 and 2 mM NH_4^+ were supplied to the agar medium (Fig. 3). As expected, Arabidopsis *AtAMT1;3* complemented as well, but the cells expressing one of the sorghum transporters grew more vigorously.

Tissue-specific expression of the different AMTs

In *S. bicolor* field samples, *SbAMT1;1*, *SbAMT1;2*, *SbAMT2;1* and *SbAMT3;3* showed a similar expression pattern; they all were expressed at a similar level in the five different tissues (root, shoot, stem, pistils and stamens) (Fig. 4). Additionally, *SbAMT3;3* had the highest expression level overall.

SbAMT2;2 and *SbAMT3;2* were not expressed in the stamens. *SbAMT3;2* was additionally highly expressed in the pistils.

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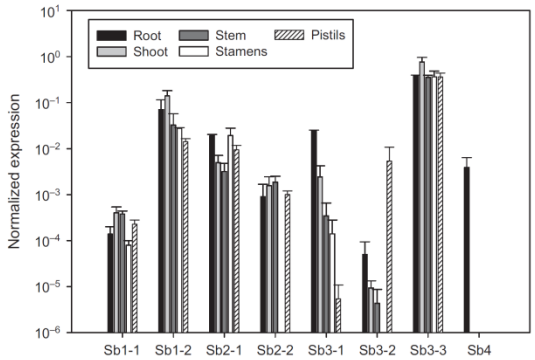

Fig. 4 Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of the eight *Sorghum bicolor* ammonium transporter (AMT) genes in different tissues from field. The values are the means of three replicates, and error bars represent the SD. Ubiquitin was used as the reference transcript.

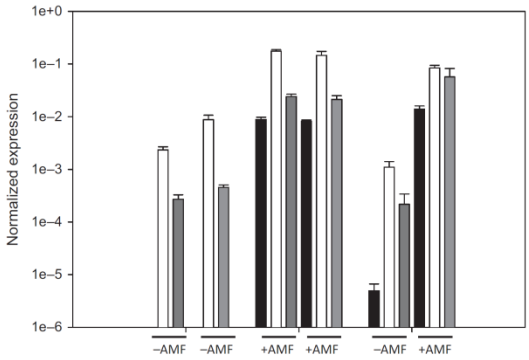

Fig. 5 Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *SbAMT3;1*, *SbAMT4* and *SbPht11* in split roots where roots are either noncolonized or colonized by the arbuscular mycorrhizal (AM) fungus *Glomus mosseae*. Three different AM fungal treatments were applied: AM fungi on both sides of the split-root system (+AMF/+AMF); AM fungi on one side and no AM fungi on the other side of the split-root system (+AMF/−AMF); and no AM fungi on both sides of the split-root system (−AMF/−AMF). Plants were grown for 13 wk. The values are the means of four replicates. Ubiquitin was used as the reference transcript. Missing bars, values below the detection limit. Error bars represent the SD.

SbAMT3;1 was predominantly expressed in the roots but could also be found in the other tissues. *SbAMT4* was exclusively expressed in the roots.

Nonsystemic expression of *SbAMT3;1* and *SbAMT4* in *S. bicolor* plants

A split-root experiment was set up to study the expression pattern of the two mycorrhizal-induced genes, *SbAMT3;1* and *SbAMT4*, at the whole-plant level. Plants of *S. bicolor* were grown with two parts of the root system under different conditions, colonized or not by *G. mosseae*, and additionally subjected to different N regimes. In all cases, root biomass was equally distributed between the two root compartments (Table S6). There was no mycorrhiza formation in root compartments without AM fungi. In the presence of *G. mosseae*, colonization of the roots in the root compartments varied between 26 and 48% (Table S8) without any significant differences between the treatments. Transcripts were quantified in each compartment of each split-root system (Fig. 5). In all samples, the genes coding for ubiquitin and for all AMTs were amplified by RT-qPCR (Table 2). In the different AMT genes, no significant differences were observed between the different N treatments.

Root colonization had no significant effect on gene expression, except for *SbAMT3;1* and *SbAMT4*. The genes coding for *SbAMT3;1* and *SbAMT4* were only induced in mycorrhizal root halves (Table 2). In the treatment +AMF/−AMF, *SbAMT3-1* and *SbAMT4* were only induced in the mycorrhizal part of the root system, indicating local but not systemic regulation (Fig. 4). By contrast, a mycorrhiza-induced phosphate transporter, *SORbiPht1;11* (*SbPt11*) from *S. bicolor*, was also expressed in nonmycorrhizal root halves in the +AMF/−AMF systems. A gradient of expression of *SbPt11* was observed (Fig. 5), varying from no expression in both parts of the split-root system of the −AMF/−AMF treatment, to a low but significant expression in the nonmycorrhized part of the root system of the treatment +AMF/−AMF, to a high expression in the mycorrhized part of the root system of the treatment +AMF/−AMF and in both parts of the root system of the +AMF/+AMF treatment. This indicates that this transporter is systematically induced, but the transcript abundances were relatively low in the nonmycorrhizal root halves.

Cell-specific expression of SbAMT3;1 and SbAMT4

The distribution of the *SbAMT3;1* and *SbAMT4* transcripts was studied after laser-dissection and collection of three types of cells: arbuscule-containing cells, noncolonized cortical cells from mycorrhizal roots, and cortical cells from nonmycorrhizal roots. In all samples, the gene coding for ubiquitin and for all AMTs was amplified after RT-qPCR analysis. Transcripts of *SbAMT3;1* and *SbAMT4* could be detected in all three cell types but were significantly higher in both arbuscule-containing and noncolonized cortical cells from mycorrhizal roots (Fig. 6). Samples were validated by analyzing the expression of a gene coding for an AM-inducible *S. bicolor* phosphate transporter, *SbPT11*, and for a fungal specific gene, *GmEIF* (elongation factor of *G. mosseae*): *SbPT11* was induced in both arbuscule-containing and noncolonized cortical cells from mycorrhizal roots. Transcripts of *GmEIF* were only detected in arbuscule-containing cells, where *G. mosseae* only is present (Fig. 6).

Western blot and immunolocalization of *SbAMT3;1*

SbAMT3;1 was detected in the insoluble protein fraction prepared from the mycorrhizal plant roots but not in the corresponding fraction from nonmycorrhizal roots (Fig. 7a). No signal

Table 2 Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundance of *SbAMT3;1* and of *SbAMT4* in split roots either noncolonized or colonized by the arbuscular mycorrhizal fungus *Glomus mosseae* at 9 wk postinoculation (second harvest) in the different N treatments (−N and 1 × NO_3^-)

	Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
N treatment	−N	−N	−N	−N	−N	−N	+N	+N	+N	+N
AMF treatment	−AMF	−AMF	+AMF	+AMF	−AMF	+AMF	−AMF	−AMF	+AMF	+AMF
SbAMT3-1	1.7 ± 1.4	1.9 ± 1.6	**29.8 ± 9.2**	**43.7 ± 8.9**	2.7 ± 1.5	**40.5 ± 15.9**	1 ± 0	1 ± 0	**46.9 ± 11.6**	**47.1 ± 11.4**
SbAMT4	0.5 ± 0.3	0.2 ± 0.2	**11.5 ± 5.6**	**18.1 ± 4.4**	1.4 ± 1.3	**15.4 ± 3.4**	1 ± 0	1 ± 0	**19.4 ± 3.1**	**20.8 ± 4.4**

	Treatment 6		Treatment 7		Treatment 8		Treatment 9		Treatment 10	
N treatment	+N	+N	−N	+N	−N	+N	−N	+N	+N	−N
AMF treatment	−AMF	+AMF	−AMF	−AMF	+AMF	+AMF	−AMF	+AMF	−AMF	+AMF
SbAMT3-1	1.3 ± 1.8	**28.6 ± 5.4**	0.6 ± 0.4	1.6 ± 1.8	**33.7 ± 13.4**	**44.7 ± 16.8**	0.5 ± 0.6	**22.2 ± 9.2**	1.0 ± 0.7	**20.1 ± 6.9**
SbAMT4	0.9 ± 0.7	**21.2 ± 14.6**	0.1 ± 0.1	0.0 ± 0.0	**14.6 ± 9.5**	**32.9 ± 18.9**	0.1 ± 0.1	**39.7 ± 12.9**	0.4 ± 0.2	**27.8 ± 6.5**

The values are the means and SD of four replicates. Ubiquitin was used as the reference transcript. Gene expression was normalized according to the '−AMF, 1×NO_3^-' treatment (= treatment 4). Differences in relative gene expression between the treatments were performed with a one-way ANOVA (Scheffe's *F*-test). Bold values indicate a *P*-value < 0.05.

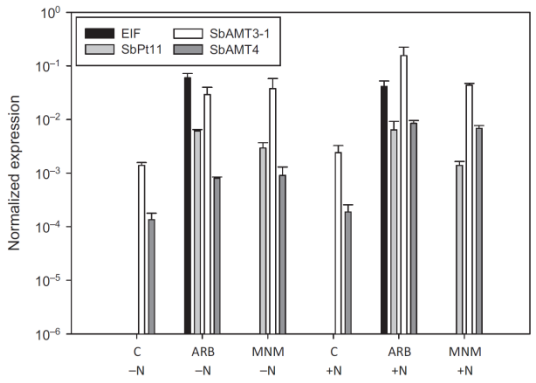

Fig. 6 Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *SbAMT3;1*, *SbAMT4*, *SbPht11* after laser microdissection of different cell populations of *Sorghum bicolor* roots colonized by *Glomus mosseae* under different nitrogen treatments (−N and +N). Different cell populations were investigated: arbuscule-containing cells (ARB), noncolonized cortical cells from mycorrhizal roots (MNM), and cortical cells from nonmycorrhizal roots (C). To quantify the presence of the mycorrhizal fungus in the selected samples, expression of the elongation factor of *G. mosseae* (EIF) was also measured (black columns). Missing bars correspond to measurements with values below the detection limit. Plants were grown for 13 wk. The values are the means of four replicates, and error bars represent the SD. Ubiquitin was used as the reference transcript.

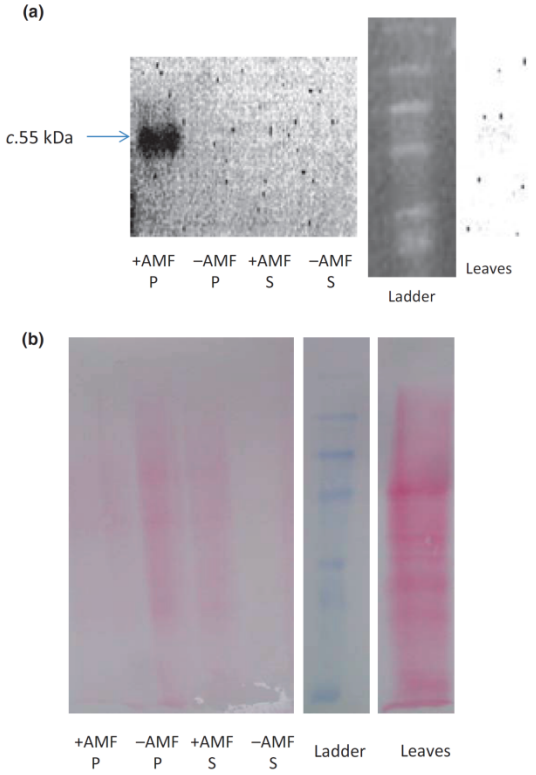

Fig. 7 Expression of SbAMT3;1 is up-regulated in response to arbuscular mycorrhizal (AM) symbiosis. (a) Western blot of insoluble, pelleted (P) and soluble (S) proteins from *Sorghum bicolor* roots and leaves. Roots were either noncolonized or colonized by the AM fungus *Glomus mosseae*. Size of the expected product = 55 kDa. (b) Ponceau staining of *S. bicolor* leaves and roots extracts.

was detected in the fractions containing soluble proteins (Fig. 7a). A very low amount of SbAMT3;1 was present in the plant leaves (Fig. 7a).

Confocal laser scanning microscopy was performed to localize SbAMT3;1 in the roots. The cells containing active arbuscules showed a strong red fluorescence arising from the wheat germ

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agglutinin – AlexaFluor 594 conjugate – and a green fluorescent signal, indicating the presence of SbAMT3;1 in the arbuscule-containing cells. The SbAMT3;1 signal colocalized with the arbuscules surrounding the individual branches (Fig. 8). No signal was detected on hyphae beside the arbuscule or on the vesicules (Fig. 8). Cortical cells with developing arbuscules showed SbAMT3;1 staining, whereas in cortical cells with collapsed arbuscules, no staining was detectable. Our data indicate that the protein SbAMT3;1 occurred only in plant cells containing developing or mature arbuscules, and that it was localized there at the periarbuscular membrane.

Discussion

Nitrogen is an essential, often limiting element for plant growth. For this reason, the use of N fertilizers in agriculture has greatly increased in the past decades (Tilman *et al.*, 2002). AM fungi may help plants in acquiring N by taking up inorganic and organic N sources (Lopez-Pedrosa *et al.*, 2006) with their extraradical mycelium foraging the soil, and thereby have a great potential in a sustainable agriculture. It has been established that the AM fungus *G. intraradices* has a functional AMT (Lopez-Pedrosa *et al.*, 2006), allowing the uptake of soil

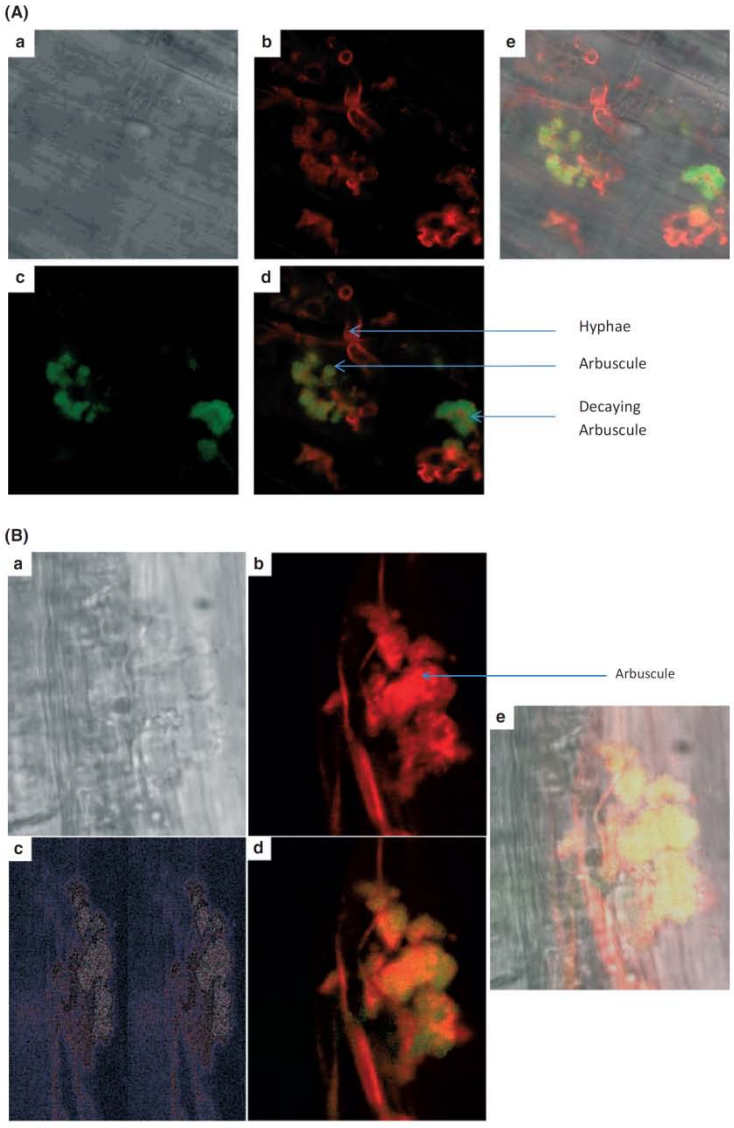

Fig. 8 Immunolocalization of SbAMT3;1 in symbiotic structures in root cortical cells of *Sorghum bicolor* colonized by the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae*. Confocal laser scanning micrographs of mycorrhizal sorghum roots show different mycorrhizal structures (A, hyphae, mature arbuscule, decaying arbuscule; B, arbuscule). Transparency and two types of staining are shown separately and as overlay: fungal structures visualized by WGA-Alexa Fluor 594 (red channel); and SbAMT3;1 protein detected by anti-rabbit IgG-Alexa Fluor 488 (green channel). Green and red colors together are showing the SbAMT3;1-related fluorescence signal colocalized with the *G. mosseae* symbiotic structures filling the cells. (a) Bright field; (b) fluorescence in the red channel; (c) fluorescence in the green channel; (d) overlay of panels (b) and (c); (e) overlay of panels (a), (b) and (c).

ammonium. Afterwards, AM fungi transfer the absorbed N from the extraradical mycelium to the intraradical mycelium in the form of arginine, and it is thought that their arbuscules deliver N in the form of ammonium to the plant (Tian *et al.*, 2010).

SbAMT3;1 and SbAMT4 are two AM-inducible AMTs

In our study, we identified eight genes coding for AMTs in the genome of *S. bicolor*. A similar number was described in *O. sativa* (10) or *A. thaliana* (six). A higher number was reported for poplar (14) (Couturier *et al.*, 2007). Interestingly, poplar and *Arabidopsis* have a higher number of AMT genes assigned to the AMT1 subfamily (six and five, respectively) than *S. bicolor* (two) and *O. sativa* (three) (Sonoda *et al.*, 2003), indicating a different organization of AMT genes in monocots and dicots.

Sorghum AMT genes had varying expression levels in the different tissues (Fig. 4) of the plant, confirming previous studies showing distinct roles of the different AMTs in the plant (Yao *et al.*, 2008). Sonoda *et al.* (2003) described that *OsAMT1;1* was constitutively expressed in rice shoots and roots, similarly to its sorghum homologous *SbAMT1;1*. By contrast, *OsAMT1;2* from rice was root-specific (Sonoda *et al.*, 2003) compared wth its sorghum ortholog, *SbAMT1;2*, which is constitutively expressed in the plant with the highest level in the shoots. Transcripts of *SbAMT3;1* were found in roots, shoots, stem, pistils and stamens while transcripts of *SbAMT4* were exclusively found in roots of the plants (Fig. 4). Additionally, the relative gene expression of *SbAMT3;1* and *SbAMT4* were significantly (70 and 20 times, respectively) higher in roots colonized by AM fungi than in nonmycorrhized roots (Fig. 2), indicating that SbAMT3;1 and SbAMT4 are AM-inducible AMTs. Functionality of both transporters was confirmed by yeast complementation (Fig. 3). Moreover, growth of the transformed yeast on media with low N concentrations (< 1 mM NH_4^+) indicate that SbAMT3;1 and SbAMT4 are both high-affinity AMTs compared with AtAMT1;3 from *A. thaliana* (Gazzarrini *et al.*, 1999), which has been described as a low-affinity transporter. In the phylogenetic analysis (Fig. 1), *SbAMT3;1* is clustering with the AM-inducible AMTs *GmAMT3;1* and *GmAMT4;4* (Kobae *et al.*, 2010) from soybean and belongs to the AMT3 subfamily. The closest homologs are *OsAMT3;1* from rice and *ZmAMT3;1* from maize; however, to our knowledge, no study has been done on the induction of these genes by AM fungi. *SbAMT4* is clustering with the AM-inducible AMTs *GmAMT4;1* (Kobae *et al.*, 2010) from soybean and *LjAMT2;2* (Guether *et al.*, 2009b) from *L. japonicus*, and belongs to the AMT4 subfamily. The closest homologs are *OsAMT4* from rice and *ZmAMT4* from maize. Here, also, the induction of these genes by AM fungi has, to our knowledge, not yet been studied. In contrast to the phosphate transporter gene family in which most AM-inducible transporters are clustering in one group (Nagy *et al.*, 2005), AM-inducible AMTs are distributed among different AMT subfamilies (Fig. 1). Other AM-inducible AMTs were described in the AMT1 subfamily: *PtAMT1;2* from poplar (Couturier *et al.*, 2007) and *GmAMT1;4* from soybean (Kobae *et al.*, 2010).

No effect of N nutrition on AMT gene expression

Our data show that the percentage of roots colonized with *G. mosseae* and *G. intraradices* is not affected by the N nutrition in the time-course experiment (Fig. S1). These data contradict the findings of Blanke *et al.* (2005), who reported in a field experiment that the colonization of *Artemisia vulgaris* was higher in N-deficient plots than in N-high plots, but under high P concentration. One potential caveat might be the growth of sorghum plants for up to 13 wk in the relatively small soil-volume of 500 ml. Quantitative PCR analyses on sorghum roots revealed that N nutrition had no significant effect on the abundance of AMT transcripts in the roots. This finding was unexpected, as previous studies on rice (Sonoda *et al.*, 2003), *L. japonicus* (D'Apuzzo *et al.*, 2004) and citrus (Camanes *et al.*, 2009) showed the effect of N nutrition on AMT gene expression. However, we measured the AMT gene expression after 5, 9 and 13 wk under different N nutrition conditions, in contrast to previous studies where expression was measured in min or h after N exposure. Based on our data, we cannot exclude a fine-tuning of sorghum AMT expression immediately after contact with different N sources. However, we studied the regulation of AMTs when plants had access to different N sources over a long period.

Local but not systemic induction of *SbAMT3;1* and *SbAMT4* in the root system

The results of the split-root experiment did not show any systemic induction of *SbAMT3;1* and *SbAMT4* in the whole root system. The expression of both transporters was higher in the AM-colonized parts of the split-root system, but not in the noncolonized parts (Fig. 5). The analysis of the expression level of the AM-induced phosphate transporter *SbPt11* (F. Walder *et al.*, unpublished) revealed a different picture: no expression when the two parts of the split-root system were not colonized; high and low levels of expression, respectively, in the mycorrhizal and nonmycorrhizal parts of the split-root system; and a high level of expression when the two parts of the split-root system were colonized. Thus, *SbPt11* was slightly, but significantly, induced systemically, indicating that in this case a signal might be transferred to the noncolonized roots, preparing the root for a potential future colonization as described by Gaude *et al.* (2011).

Root cell-specific expression of *SbAMT3;1* and *SbAMT4*

The exploration of the cell-specific expression pattern of *SbAMT3;1* and *SbAMT4* revealed that both genes were up-regulated in arbuscule-containing and noncolonized cortical cells from mycorrhizal roots, indicating that specific AMTs might have, like phosphate transporters (Javot *et al.*, 2007), an important role in symbiotic processes. High transcript abundances of both *SbAMT3;1* and *SbAMT4* were also found in noncolonized cells of *M. truncatula* roots in the survey of Gaude *et al.* (2011). As mentioned by Balestrini *et al.* (2007) in tomato, the fungal structures (senescent or young arbuscules) might be present in the part of the cell which has been removed after sectioning.

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Using a *G. mosseae* endogenous gene (elongation initiation factor, EIF), we have shown that we could only detect the AM fungus in arbuscule-containing cells.

Interestingly, using immunolocalization, we found the protein SbAMT3;1 only in the arbuscule-containing cells, although the corresponding mRNA was induced in arbuscule-containing and noncolonized cortical cells from mycorrhizal roots, indicating a degree of post-transcriptional regulation (Fig. 8). This post-transcriptional regulation was also clearly apparent in the difference between transcript and protein accumulation of SbAMT3;1 in shoots (Figs 4, 7a). More precisely, our data indicate that SbAMT3;1 is localized at the periarbuscular membrane, where Harrison *et al.* (2002) already described the presence of the AM-induced phosphate transporter MtPT4. SbAMT3;1, similarly to MtPT4, was surrounding mature arbuscules, but not young or collapsed arbuscules. It makes sense that the expression of several transporter genes is induced upon AM colonization, as the exchange of mineral nutrients and carbohydrates between host plant and AM fungus is the key element of a functioning AM symbiosis (Smith & Read, 2008). This transfer takes place at the periarbuscular membrane, an extension of the plasma membrane of the cell retaining many of the characteristics of the plasma membrane (Perotto *et al.*, 1994) but where AMTs and phosphate transporters regulated by AM symbiosis are localized (Harrison *et al.*, 2002; Pumplin & Harrison, 2009). The expression pattern of SbAMT3;1 also correlates with a previous report in which H^+-ATPase activity staining was noted to disappear from the periarbuscular membrane as the arbuscule aged (Gianinazzi-Pearson *et al.*, 1991), indicating that ammonium transport, like phosphate transport, occurs principally in the mature arbuscule.

Our results support the hypothesis that mycorrhizal genes are activated, induced or overexpressed by a small-scale systemic induction before arbuscule development, which could be part of the prepenetration response as the cell reorganizes and cytoplasmic bridges are built (Genre *et al.*, 2008).

Conclusion

Here, we demonstrate that two AMTs of sorghum, *SbAMT3;1* and *SbAMT4*, are locally but not systemically strongly induced in roots in response to mycorrhizal colonization, in contrast to an AM-inducible phosphate transporter gene (*SbPt11*), which is induced both locally and systemically. Locally, *SbAMT3;1* and *SbAMT4* were induced in noncolonized cells neighbouring arbuscule-containing cells, perhaps conditioning those cells to accommodate a future arbuscule (prepenetration response), a process of considerable importance in view of the short life span of arbuscules (*c.* 6–10 d, depending on the species; Toth & Miller, 1984; Alexander *et al.*, 1989). Using immunolocalization, SbAMT3;1 was found to be present exclusively in arbuscule-containing cells within the periarbuscular membrane, highlighting a degree of post-transcriptional of regulation and a potentially important role of this transporter in the transfer of N from the fungus to the plant in the AM symbiosis. Our observations highlight the need for identifying the functions, substrate specificity and regulation of *S. bicolor* AMTs, as well as further studies of temporal variations in gene expression on *S. bicolor* associated with different AM fungal species in agricultural ecosystems or in associations with other plant species connected by a common mycorrhizal network.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Plant growth conditions, identification and characterization of sorghum AMT transporters, RNA isolation and qRT-PCR, Immunolocalization, isolation of plasma membrane and soluble proteins.

Fig. S1 Colonization rate *G. mosseae* (A) and *G. intraradices* (B).

Fig. S2 N content in Sorghum.

Fig. S3 Shoot and root DW (g).

Table S1 AMT isolated and partially characterized in different plant species

Table S2 Composition of Hoagland solutions

Table S3 Characteristics of the *S. bicolor* AMT gene family

Table S4 Specific primers for real-time quantitative PCR and for full-length gene amplification

Table S5 Amino acid sequence similarity and identity between the AMT of *S. bicolor: SbAMT1;1*, *SbAMT1;2*, *SbAMT2;1*, *SbAMT2;2*, *SbAMT3;1*, *SbAMT3;2*, *SbAMT3;3* and *SbAMT4*

Table S6 Repartition of root DW (%) between the two pots of the split-root experiment

Table S7 Mean and SD of the relative gene expression of the eight SbAMTs and of the glutamine synthase gene

Table S8 Root length colonization (%) in each pot of the split-root experiment

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Supplemental Materials and Methods S1

Plant growth conditions for tissue analysis

Five different Hoagland solutions, modified after Gamborg & Wetter (1975), were prepared to obtain different N sources or N concentrations: "-N" ($Ca(NO_3)_2 \cdot 4H_2O$, KNO_3 and $NH_4H_2PO_4$ from the original solution were replaced by $CaCl_2 \cdot 2H_2O$, KCl and KH_2PO_4), 1xNO_3^- ($NH_4H_2PO_4$ was replaced by KH_2PO_4), 0.3xNO_3^- and 0.1xNO_3 (mixtures of -N and 1xNO_3^-) and 1xNH_4^+ ($Ca(NO_3)_2 \cdot 4H_2O$, KNO_3 and $NH_4H_2PO_4$ from the original solution were replaced by $CaCl_2 \cdot 2H_2O$, KCl, NH_4Cl and KH_2PO_4). Additionally, for all the solutions, $(NH_4)_2MoO_4$ was replaced by Na_2MoO_4.

Identification and characterization of sorghum AMT transporters

Sequences from the *S. bicolor* genome (v1.1) database were: SbAMT1;1 (Sb06g022230), SbAMT1;2 (Sb04g026290), SbAMT2;1 (Sb09g023030), SbAMT2;2 (Sb03g038840), SbAMT3;1 (Sb03g041140), SbAMT3;2 (Sb01g001970), SbAMT3;3 (Sb04g022390), SbAMT4 (Sb01g008060). The accession numbers or gene models of UniProt data base were used: PtrAMT1;1 (B9HSW3), PtrAMT1;2 (B9IPE2), PtrAMT1;3 (B9HKW8), PtrAMT1;4 (B9GRB5), PtrAMT1;5 (B9GRB4), PtrAMT1;6 (B9HP47), PtrAMT2;1, (B9HCZ0), PtrAMT2;2 (B9IGE2), PtrAMT3;1 (B9GHA5), PtrAMT4;1 (B9GS88), PtrAMT4;2 (B9IKS2), PtrAMT4;3 (B9H8E7), PtrAMT4;4 (B9I5F0), PtrAMT4;5 (B9MX92). *Arabidopsis thaliana* (at NCBI): AtAMT1;1 (At4g13510), AtAMT1;2 (At1g64780), AtAMT1;3 (At3g24300), AtAMT1;4 (At4g28700), AtAMT1;5 (At3g24290), AtAMT2;1 (At2g38290), *Lycopersicon esculentum:* LeAMT1;1 (P58905), LeAMT1;2 (O04161), LeAMT1;3 (Q9FVN0), *Lotus japonicus:* LjAMT1;1 (Q9FSH3), LjAMT1;2 (Q7Y1B9), LjAMT1;3 (Q70KK9), LjAMT2;1 (Q93X02), *Oryza sativa:* OsAMT1;1 (Q7XQ12), OsAMT1;2 (Q6K9G1), OsAMT1;3 (Q6K9G3), OsAMT2;1 (Q84KJ7), OsAMT2;2 (Q8S230), OsAMT2;3 (Q8S233), OsAMT3;1 (Q84KJ6), OsAMT3;2 (Q851M9), OsAMT3;3 (Q69T29), OsAMT4;1 (Q10CV4), *Brassica napus:* BnAMT1;2 (Q9FUH7), *Populus tremula x tremuloides:* PttAMT1;2 (Q5K411), *Triticum aestivum:* TaAMT1;1 (Q6QU81), TaAMT1;2 (Q6QU80), TaAMT2;1 (Q6T8L6), *Nitrosomona europeae:* NeAMT/Rh1 (Q82X47), *Escherichia coli:* EcAMTB (P69681).

The accession numbers of the Phytozome 6.0 database were used for soybean (*Glycine max*) as follows: GmAMT1.1 (Glyma20g21030.1), GmAMT1;2 (Glyma10g26690.1), GmAMT1;3 (Glyma10g31080.1), GmAMT1;4 (Glyma10g31110.1), GmAMT1;5 (Glyma1031130.1), GmAMT1;6 (Glyma20g36390.1),

GmAMT2;1 (Glyma07g18670.1), GmAMT2;2 (Glyma18g43540.1), GmAMT2;3 (Glyma01g30920.1), GmAMT3;1 (Glyma05g33010.1), GmAMT4;1 (Glyma09g41810.1), GmAMT4;2 (Glyma20g00680.1), GmAMT4;3 (Glyma19g43380.1), GmAMT4;4 (Glyma02g04960.1), GmAMT4;5 (Glyma02g16200), GmAMT4;6 (Glyma10g03600.1).

PCR amplification of the full-length cDNAs, from the start to the stop codon, with primers designed using the nucleotide sequences of manually annotated gene models (Supplementary Table 1), was performed on a T3 thermocycler (Biometra, Labgene Scientific SA, Switzerland) using the Advantage 2 Polymerase Mix (Clontech). PCR reactions resulted in single bands on a 1% agarose gel (Promega, Madison, WI, USA) in 0.5% TAE (Tris Acetate-EDTA) stained with Midori Green according to manufacturer's instructions (Labgene, Chatel-St-Denis, Switzerland). Amplified products were purified with ExoSAP treatment (USB, Cleveland, Ohio, USA) and direct cDNA sequencing was performed on a 3500 Genetic Analyser (Applied Biosystems, Courtaboeuf, France).

RNA isolation and quantitative reverse transcription-PCR

Mycorrhizal and non-mycorrhizal roots, as well as shoots, stems, stamina and pistils were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Full-length doubled-stranded cDNAs corresponding to mRNAs expressed in plant roots were obtained using the SMART–PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Quantification of AMT transcripts was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in real time quantitative PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Supplementary Table S1). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C and a GC percentage > 50%. Target gene expression was normalized to the gene encoding the *S. bicolor* ubiquitin (Protein 5060159). Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. For data analysis, the

geometric mean of the biological replicates (*n* = 4) was calculated. The primer efficiency ranged between 90% and 110%.

Immunolocalization

Immunolocalization was performed essentially as described by Blancaflor et al. (2001) with minor modifications. Root pieces were fixed for 2 h in 4% formaldehyde and 5% (v/v) DMSO in PME buffer (50 mM Pipes, 5 mM MgSO4, and 10 mM EGTA, pH 6.9), and hand sectioned with a razor blade. The root segments were fixed temporarily to a cover slip with a thin layer of agar as described by Brown and Lemmon (1995) and then were digested in 1% cellulase RS, 0.01% pectolyase Y23 (Karlan Research Products, Santa Rosa, CA), and 0.1% BSA in PME buffer for 10 min. After digestion, the segments were washed three times for 5 min each with PME buffer and then incubated in 1% BSA in PBS (135 mM NaCl, 25 mM KCl, and 10 mM Na_2HPO_4), pH 7.5) for 30 min. The BSA was removed, and the segments were incubated overnight with SbAMT3;1 antibody (1:100) in PBS containing 0.5% (w/v) BSA. The segments were washed five times in PBS and incubated in the secondary antibody conjugate, a 1:800 dilution of donkey anti-rabbit IgG–AlexaFluor 488 conjugate (Molecular Probes, Eugene, OR) in PBS for 2 h. After five washes in PBS, the segments were incubated in 0.1 mg/mL wheat germ agglutinin–AlexaFluor 594 conjugate (Molecular Probes, Eugene, OR) in PBS for two hours to stain the fungus (Genre & Bonfante, 1997). After five more washes in PBS, the sections were mounted in 20% (w/v) Mowiol 4-88 containing 0.1% (w/v) phenylenediamine in PBS, adjusted to pH 8.5 with NaOH, and viewed using a confocal and an epi-fluorescence microscope.

Isolation of plasma membrane and soluble proteins

300 mg of either non-mycorrhizal or mycorrhizal roots were ground in liquid nitrogen with a mortar and mixed with grinding solution I (0.5 M Tris-HCl buffer, pH 7.5, 50 mM EDTA, 0.1M KCl, 2 % (v:v) β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.7 M sucrose, 10mM thiourea) at a ratio of 5 : 1 (volume/fresh weight). The mixture was centrifuged at 18000 g for 5 min at 4°C. The supernatant was collected to analyze soluble proteins and the pellet for cell wall proteins.

The supernatant was mixed with cold acetone (2:1, v:v) and soluble proteins were precipitated at -20°C for a minimum of 2 hours. The mixture was then centrifuged at 18000 *g* at 4°C for 15 min. The pellet was resuspended and centrifuged as previously. The supernatant was removed, and the pellet was air dried

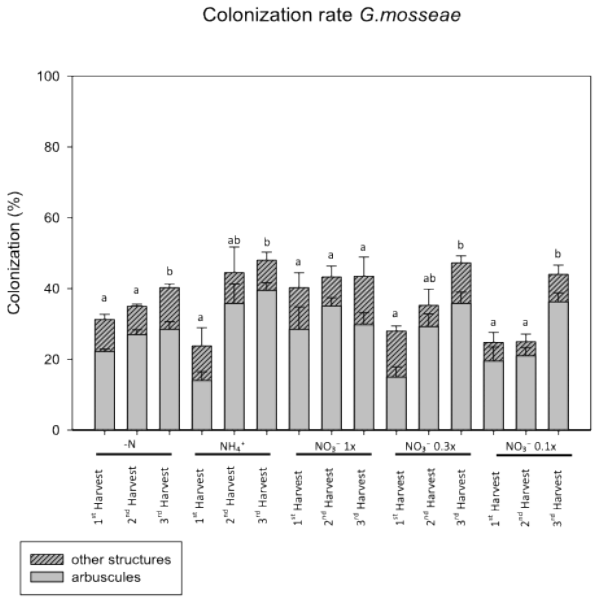
and then resuspended in 100 µl of Laemmli's buffer.

The pellet was resuspended in grinding solution II (330 mM sorbitol 2 mM Hepes, 10 mM KCl, 0.1 mM DTT, pH 7.8) and phenol (1:1, v:v) was added. After shaking, the aqueous phase and the phenol/water interface containing cell debris were kept for the extraction of plasma membrane proteins. Cell walls were recovered by mixing 800 µl of the above mentioned aqueous phase with 800 µl of Wessels solution (100 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride, 0.2 % (v:v) β-mercaptoethanol) (Wessels et al. 1991). The suspensions were then centrifuged at 15000 *g* for 15 min at 4°C. Pellets containing cell walls were washed three times with 800 µl of Wessels solution, twice with 800 µl of water, once in 800 µl of cold acetone, and dried. Noncovalently bound proteins were extracted from cell walls by incubation of 5 mg of dried cell walls in 100 µl of Laemmli's buffer (Laemmli 1970) at 100°C for 10 min.

Supplemental References

Genre A, Bonfante P (1997) A mycorrhizal fungus changes microtubule orientation in tobacco root cells. Protoplasma **199:** 30-38

A



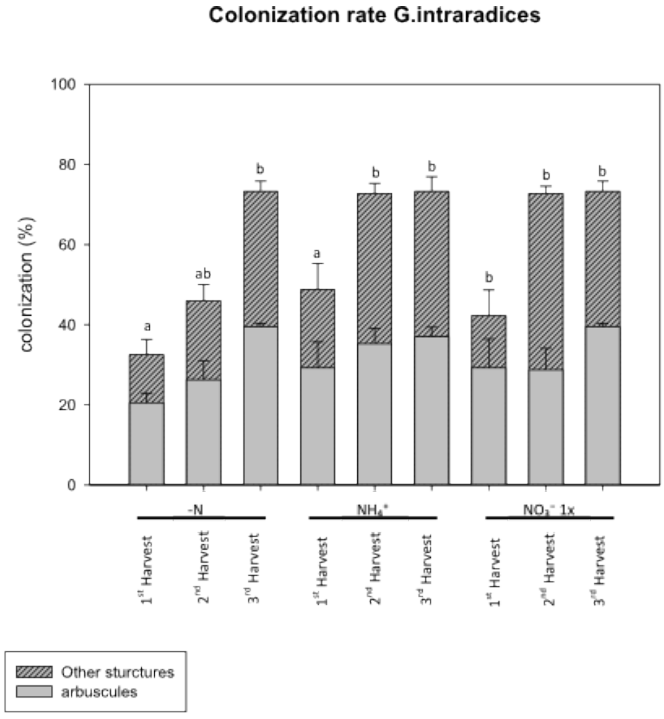


Figure S1: Percentage of *S.bicolor* roots colonized by *G.mosseae* (a) and *G.intraradices* (b) at the first (5 weeks post-inoculation), second (9 weeks post-inoculation) and third (13 weeks post inoculation) harvest in the different N treatments (-N, 1× NH_4^+, 1× NO_3^-, 0.3× NO_3^- and 0.1× NO_3^-). A total of 156 samples were analyzed. Differences in colonization between the three harvests for each treatment were tested with ANOVA.

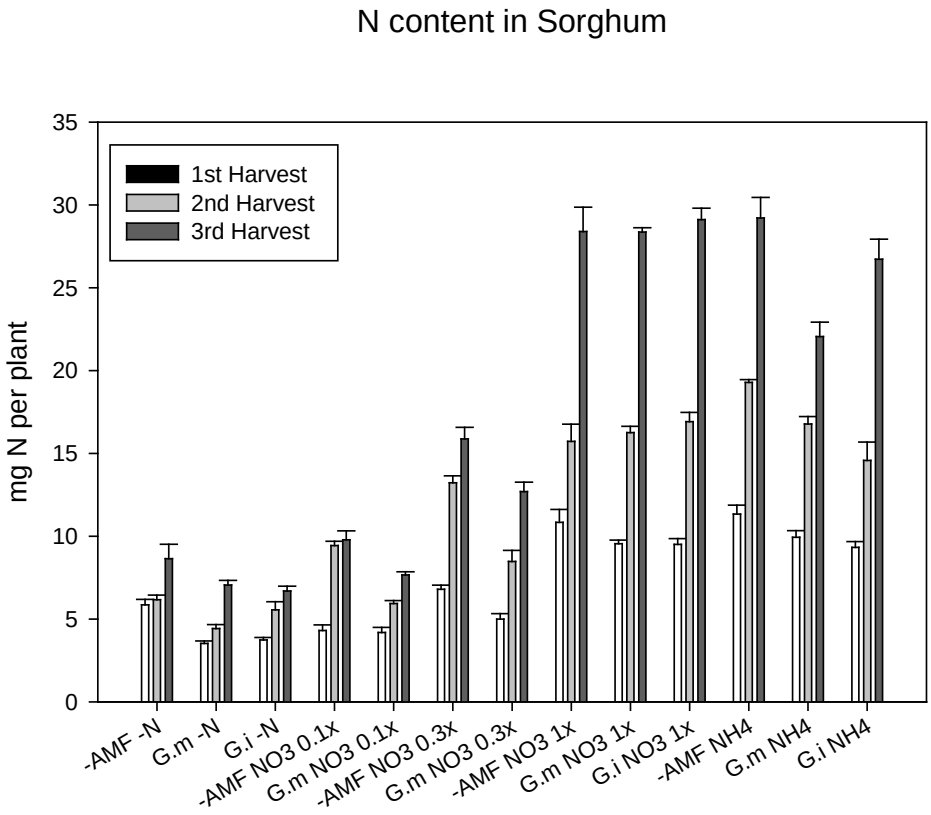


Figure S2: Total N content in plants at the first (5 weeks post-inoculation), second (9 weeks post-inoculation) and third (13 weeks post inoculation) harvest in plants either non-colonized or colonized by arbuscular mycorrhizal fungi (Gi; *Glomus intraradices*, Gm; *Glomus mosseae*) and in the different N treatments (-N, 1× NH_4^+, 1× NO_3^-, 0.3× NO_3^- and 0.1× NO_3^-).

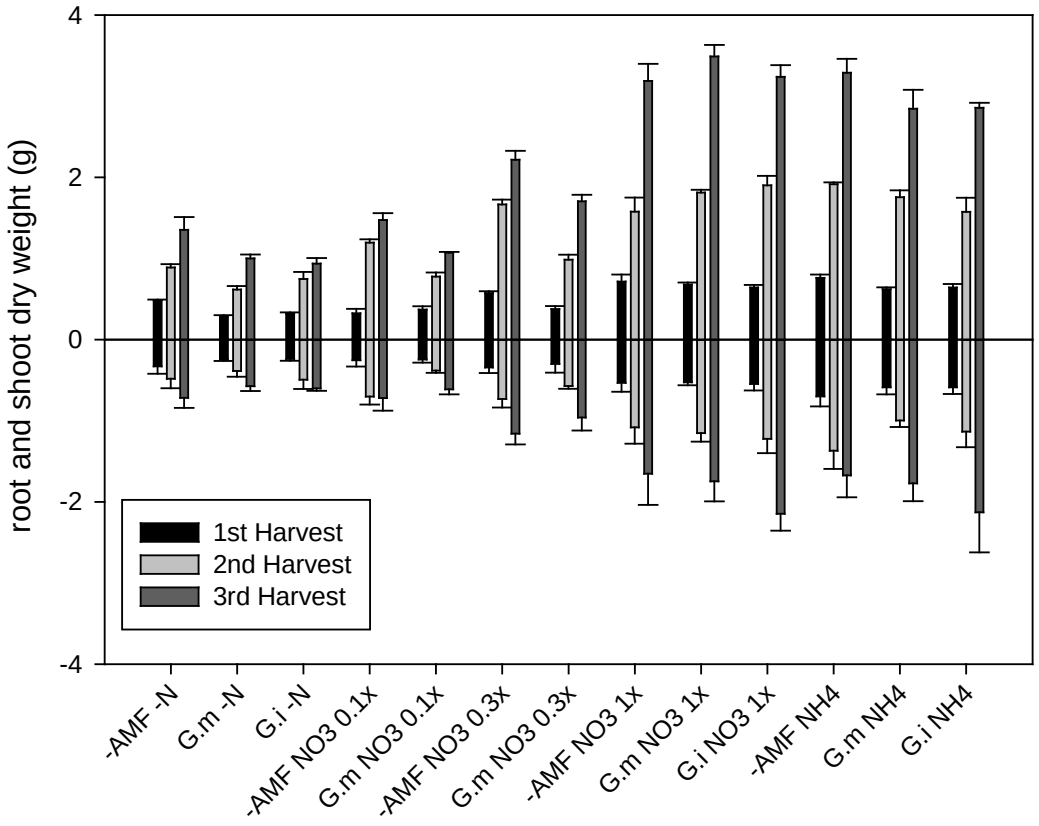


Figure S3: Shoot (up) and root (down) dry weight (g) at the first (5 weeks post-inoculation), second (9 weeks post-inoculation) and third (13 weeks post inoculation) harvest in plants either non-colonized or colonized by arbuscular mycorrhizal fungi (Gi; *Glomus intraradices*, Gm; *Glomus mosseae*) and in the different N treatments (-N, 1× NH_4^+, 1× NO_3^-, 0.3× NO_3^- and 0.1× NO_3^-).

Primer	Sequence 5'→ 3'
SbAMT1;1Fw	GCTGTGGTTCGGCTGGTA
SbAMT1;1R	GGACTTGAGGATGGTGGTGAA
SbAMT1;2Fw	TCCATTGCTCCTCGTTGC
SbAMT1;2R	GGCTTTGCTCCCTCTTCC
SbAMT2;1Fw	TCCCGCCCGCCTACAGCT
SbAMT2;1R	GTCACCATTCAGCTGTAG
SbAMT2;2Fw	GCGGCTTCCTCTACCAGTG
SbAMT2;2R	CCTCTCCCTGTCGCTCTTC
SbAMT3;1Fw	GGCCTCGTCTGCATCACT
SbAMT3;1R	GGGTGTCGTCCACTTGCT
SbAMT3;2Fw	CCGCACGCACTCTATCTGTA
SbAMT3;2R	TCGCTGCTTATTGGGGTTAG
SbAMT3;3Fw	CGTCATTGCCTGGAACATC
SbAMT3;3R	AGCATCATCCCCGATAAGC
SbAMT4Fw	CGAACAACATTCTCCTGACG
SbAMT4R	CCCGAACACGAAGCAGTC
SbUbiFw	CAAGGAGTGCCCCAACAC
SbUbiR	GGTAGGCGGGTAAAGCAAA
EIF Fw	CGTCGGTCTCACCACTGAA
EIF R	GACCTGAGCTTGGAAGGAAG
SbPt11Fw	CGTGGTTCCTTCTGGACATA
SbPt11R	TCTCGAACACCTCCTTGAGT
Sb1;1Fw full-length	CCCAAGATGTCGACGTGC
Sb1;1R full-length	TCACCTGTAGCCGGCCGCC
Sb1;2Fw full-length	CCGGTGGAGATGGCGACG
Sb1;2R full-length	TATGCCCCACTAGAACTC
Sb2;1Fw full-length	CGAGCTCGATGGCGGCGT
Sb2;1R full length	GTCACCATTCAGCTGTAG
Sb 2-2Fw full length	CCACCGGAATGGCGTCGC
Sb2;2R full length	CGGAGAGGAGCTACAGCT
Sb 3;1Fw full length	CCGCGAGGATGGCGAGCC
Sb3;1R full length	CGCCGTGCACGGCGTCGTCGCC
Sb 3;2Fw full length	CGTCGGCAACTAACATG
Sb 3;2R full length	TCAATTCGGGGTCGTGGACAC
Sb 3;3Fw full length	GTGCCAGCCATGGCAGCA
Sb3;3R full lenght	TCAAACATTCTGTGTAACTC
Sb4Fw full length	CCTCCGAGCTGAGATGGC
Sb4R full length	TCATATCATCTCGTCGGCTTTG

Table S1: Specific primers for real-time quantitative PCR (grey) and for full-length gene amplification (white)

Model name	Gene name	Protein ID	Strand	Introns	CDS (bp)	Legth of the AA sequence	Supporting EST	Location	Best blast hit with *AMTs* (%)	Sequence logo of AMT1 and AMT2 signal
Sb06g022230	SbAMT1-1	5057502	+	1	1602	496	+	6:51467178-51475592	*Zea mays* NM_001147356.1 (96)	DFAGSGVVHMVGGIAGLWGALIEGPR
Sb04g026290	SbAMT1-2	5055467	+	0	1470	489	+	4:56058065-56059793	*Zea mays* NM_001136984.1 (95)	DFAGSSVVHMVGGIAGLWGALIEGPR
Sb09g023030	SbAMT2-1	5060664	+	2	1476	491	+	9:52669835-52672606	*Zea mays* NM_001154311.1 (94)	DYSGGYVIHLSSGVAGFTAAYWVGPR
Sb03g038840	SbAMT2-2	5036873	-	2	1497	498	+	3:66640934-66642611	*Oryza sativa* NM_001051237.1 (90)	DYSGGYVIHLSSGIAGFTAAYWVGPR
Sb03g041140	SbAMT3-1	5036989	+	1	1467	488	+	3:68682524-68685235	*Zea mays* NM_001152541.1 (95)	DYSGGYVIHLSSGVAGFTAAYWVGPR
Sb01g001970	SbAMT3-2	5047758	-	0	1452	483	+	1:1689233-1693195	*Zea mays* NM_001174872.1 (93)	DYCGGYVIHLSAGFAGFTAAYWVGPR
Sb04g022390	SbAMT3-3	5055240	+	1	1455	484	+	4:51883452-51888136	*Zea mays* NM_001165846.1 (93)	DYSGGYVIHLSSGIAGLTAAYWVGPR
Sb01g008060	SbAMT4	5028300	-	1	1425	474	+	1:6978013-6979495	*Oryza sativa* AC091811.7 (86)	DFAGGYVIHLSSGIAGFTAAYWKKLI

Table S2: Characteristics of the *S. bicolor* AMT gene family.

	SbAMT1;1	SbAMT1;2	SbAMT2;1	SbAMT2;2	SbAMT3;1	SbAMT3;2	SbAMT3;3	SbAMT4
SbAMT1;1		78.3	24.1	20.1	22.4	24.1	24.0	24.2
SbAMT1;2	84.8		25.7	20.6	24.7	24.5	24.3	27.1
SbAMT2;1	41.1	45.2		58.0	66.2	66.2	64.0	58.4
SbAMT2;2	35.3	37.6	68.5		46.7	46.3	47.0	41.0
SbAMT3;1	40.3	43.8	80.0	61.2		72.1	68.8	58.9
SbAMT3;2	39.4	44.6	78.2	59.0	83.6		69.6	60.7
SbAMT3;3	40.0	44.0	80.0	60.2	83.6	84.9		58.5
SbAMT4	43.2	46.6	73.5	56.6	74.6	77.4	77.1	

Tab. S3: Amino acid sequence similarity (grey) and identity (white) between the AMT of *S. bicolor: SbAMT1;1, SbAMT1;2, SbAMT2;1, SbAMT2;2, SbAMT3;1, SbAMT3;2, SbAMT3;3* and *SbAMT4.*

		Harvest 1				Harvest 2				Harvest 3			
		-N	NH4+	NO3-1x	NO3- 0.3x	NO3- 0.1x	-N	NH4+	NO3-1x	NO3- 0.3x	NO3- 0.1x	-N	NH4+	NO3-1x	NO3- 0.3x	NO3- 0.1x
SbAMT1-1	-AMF	1.4 +/- 1.00	1.3 +/- 0.54	1	1.5 +/- 1.11	1.3 +/- 0.77	7.9 +/- 2.46	1.8 +/- 0.65	1	2.0 +/- 0.97	2.2 +/- 1.82	3.3 +/- 0.85	1.8 +/- 0.82	1	1.3 +/- 0.68	2.1 +/- 0.98
	G:m	1.0 +/- 0.50	0.4 +/- 0.19	1.0 +/- 0.50	0.4 +/- 0.20	0.5 +/- 0.33	1.5 +/- 0.42	3.8 +/- 2.54	1.50 +/- 0.42	1.1 +/- 1.10	2.1 +/- 1.77	1.7 +/- 1.87	0.5 +/- 0.22	1.7 +/- 0.86	0.9 +/- 0.96	1.3 +/- 1.55
	G:i	0.7 +/- 0.48	0.3 +/- 0.13	0.7 +/- 0.48	-	-	3.6 +/- 1.42	0.7 +/- 0.88	1.0 +/- 0.91	-	3.1 +/- 3.29	1.6 +/- 0.80	0.6 +/- 0.48	-	-	-
SbAMT1-2	-AMF	1.7 +/- 0.71	1.4 +/- 0.61	1	1.1 +/- 0.43	1.3 +/- 0.15	9.4 +/- 3.10	7.2 +/- 3.78	1	3.2 +/- 1.21	3.2 +/- 1.42	2.4 +/- 1.03	2.3 +/- 0.96	1	2.1 +/- 0.90	3.1 +/- 1.03
	G:m	0.6 +/- 0.16	0.3 +/- 0.12	0.6 +/- 0.16	0.3 +/- 0.13	0.4 +/- 0.18	2.9 +/- 1.45	3.0 +/- 1.47	2.9 +/- 1.45	2.6 +/- 2.05	1.4 +/- 1.08	1.3 +/- 0.69	0.7 +/- 0.28	1.3 +/- 0.69	0.6 +/- 0.45	0.5 +/- 0.39
	G:i	0.6 +/- 0.23	0.7 +/- 0.31	0.8 +/- 0.33	-	-	2.7 +/- 1.53	1.3 +/- 0.78	1.0 +/- 0.53	-	-	2.0 +/- 1.22	1.4 +/- 0.65	1.9 +/- 1.64	-	-
SbAMT2-1	-AMF	3.5 +/- 1.16	1.09 +/- 1.05	1	2.3 +/- 1.31	1.4 +/- 0.84	5.4 +/- 1.16	1.5 +/- 0.47	1	1.8 +/- 0.36	1.6 +/- 1.49	1.5 +/- 0.79	0.8 +/- 0.63	1	0.6 +/- 0.21	1.0 +/- 0.76
	G:m	1.2 +/- 0.35	0.6 +/- 0.31	0.7 +/- 0.26	1.0 +/- 1.25	1.8 +/- 1.58	1.7 +/- 1.15	5.6 +/- 3.71	1.4 +/- 1.31	1.3 +/- 1.25	1.5 +/- 1.32	0.6 +/- 0.7	1.5 +/- 2.05	0.7 +/- 0.96	0.4 +/- 0.18	0.7 +/- 0.35
	G:i	0.8 +/- 0.36	0.5 +/- 0.51	1.3 +/- 0.45	-	-	3.1 +/- 1.93	1.1 +/- 0.64	1.7 +/- 0.97	-	-	2.7 +/- 1.85	0.7 +/- 0.67	1.3 +/- 1.01	-	-
SbAMT2-2	-AMF	1.4 +/- 1.12	0.6 +/- 0.34	1	4.5 +/- 2.01	2.5 +/- 1.83	5.8 +/- 2.32	0.2 +/- 0.01	1	1.7 +/- 2.97	1.4 +/- 1.10	0.3 +/- 0.33	0.0 +/- 0.00	1	0.1 +/- 0.13	0.1 +/- 0.05
	G:m	7.1 +/- 5.06	1.3 +/- 1.43	1.6 +/- 1.42	1.5 +/- 2.48	5.3 +/- 3.32	0.6 +/- 0.69	5.0 +/- 3.08	1.2 +/- 1.65	3.9 +/- 2.07	2.0 +/- 1.47	0.9 +/- 1.51	0.2 +/- 0.18	1.2 +/- 1.75	0.1 +/- 0.07	0.4 +/- 0.51
	G:i	1.4 +/- 0.88	0.3 +/- 0.14	3.0 +/- 2.43	-	-	2.4 +/- 1.18	1.0 +/- 0.67	3.7 +/- 2.11	-	-	0.7 +/- 0.45	0.2 +/- 0.35	1.2 +/- 1.16	-	-
SbAMT3-1	-AMF	2.2 +/- 0.41	1.5 +/- 1.00	1	1.2 +/- 0.37	1.5 +/- 0.52	2.2 +/- 1.14	2.2 +/- 2.04	1	1.2 +/- 1.47	1.1 +/- 0.88	4.8 +/- 2.57	1.6 +/- 1.37	1	1.4 +/- 0.90	1.5 +/- 0.55
	G:m	65.4 +/- 22.5	23.4 +/- 9.01	64.4 +/- 17.13	53.9 +/- 14.9	51.4 +/- 13.8	59.3 +/- 19.4	43.1 +/- 16.6	47.2 +/- 28.2	38.8 +/- 16.5	35.5 +/- 7.42	33.7 +/- 10.7	33.6 +/- 9.4	33.8 +/- 15.9	37.7 +/- 11.63	53.5 +/- 22.1
	G:i	44.1 +/- 13.3	28.6 +/- 6.80	26.9 +/- 14.5	-	-	66.7 +/- 19.9	87.8 +/- 25.0	53.8 +/- 16.5	-	-	121.6 +/- 13.0	77.2 +/- 22.6	41.2 +/- 9.2	-	-
SbAMT3-2	-AMF	1.4 +/- 1.04	0.7 +/- 0.44	1	1.1 +/- 0.73	1.0 +/- 0.67	8.3 +/- 4.33	0.3 +/- 0.06	1	1.1 +/- 0.73	0.4 +/- 0.42	0.6 +/- 0.66	0.1 +/- 0.11	1	0.7 +/- 0.71	0.5 +/- 0.41
	G:m	2.5 +/- 1.92	0.9 +/- 0.87	0.5 +/- 0.55	0.8 +/- 0.68	6.9 +/- 2.93	0.4 +/- 0.14	0.8 +/- 0.51	1.1 +/- 0.89	2.3 +/- 2.16	1.6 +/- 0.70	0.4 +/- 0.55	0.3 +/- 0.26	1.1 +/- 0.89	0.3 +/- 0.31	0.5 +/- 0.45
	G:i	0.9 +/- 0.41	0.3 +/- 0.21	1.2 +/- 0.87	-	-	2.7 +/- 1.33	1.0 +/- 0.84	1.4 +/- 1.12	-	-	0.4 +/- 0.26	1.0 +/- 0.66	0.5 +/- 0.45	-	-
SbAMT3-3	-AMF	1.6 +/- 1.16	0.7 +/- 0.46	1	2.1 +/- 1.68	1.2 +/- 0.99	5.7 +/- 2.14	1.5 +/- 0.54	1	1.3 +/- 0.69	1.9 +/- 1.16	0.6 +/- 0.32	0.7 +/- 0.77	1	0.6 +/- 0.38	1.4 +/- 0.86
	G:m	1.8 +/- 1.26	0.4 +/- 0.25	0.9 +/- 0.54	1.3 +/- 0.91	2.5 +/- 1.11	1.6 +/- 0.98	2.2 +/- 1.82	1.0 +/- 0.44	2.3 +/- 1.90	2.3 +/- 0.50	0.7 +/- 0.46	0.5 +/- 0.29	1.1 +/- 1.59	0.6 +/- 0.49	1.0 +/- 0.64
	G:i	1.1 +/- 0.37	0.6 +/- 1.18	1.0 +/- 0.70	-	-	2.6 +/- 1.65	1.4 +/- 0.57	1.7 +/- 0.86	-	-	1.8 +/- 0.64	1.3 +/- 0.50	0.7 +/- 0.45	-	-
SbAMT4	-AMF	2.5 +/- 1.46	0.2 +/- 0.20	1	2.4 +/- 1.65	0.7 +/- 0.75	0.1 +/- 0.03	0.0 +/- 0.01	1	0.0 +/- 0.01	0.5 +/- 0.95	0.6 +/- 0.68	0.1 +/- 0.16	1	0.3 +/- 0.15	0.4 +/- 0.40
	G:m	4.8 +/- 2.0	0.6 +/- 0.36	4.2 +/- 1.40	4.1 +/- 2.98	1.2 +/- 0.87	2.6 +/- 1.38	11.9 +/- 5.07	5.7 +/- 5.45	6.9 +/- 6.66	3.7 +/- 2.41	0.9 +/- 0.33	1.7 +/- 0.51	1.5 +/- 0.65	3.2 +/- 2.34	2.2 +/- 1.41
	G:i	0.8 +/- 0.26	1.0 +/- 0.31	1.4 +/- 0.55	-	-	5.2 +/- 3.76	0.1 +/- 0.08	0.1 +/- 0.05	-	-	5.7 +/- 4.12	1.2 +/- 0.66	6.4 +/- 4.07	-	-
SbGln	-AMF	1.4 +/- 0.58	4.8 +/- 1.81	1	1.1 +/- 0.61	4.8 +/- 1.81	0.1 +/- 0.06	1.7 +/- 1.06	1	0.7 +/- 0.44	1.5 +/- 0.56	0.9 +/- 0.66	1.6 +/- 1.06	1	0.5 +/- 0.16	0.7 +/- 0.46
	G:m	0.2 +/- 0.07	1.3 +/- 0.46	0.5 +/- 0.05	0.3 +/- 0.07	0.2 +/- 0.12	0.1 +/- 0.04	1.0 +/- 1.10	0.6 +/- 0.35	0.2 +/- 0.05	0.1 +/- 0.06	0.3 +/- 0.10	0.3 +/- 0.34	0.4 +/- 0.14	0.2 +/- 0.12	0.3 +/- 0.23
	G:i	0.4 +/- 0.08	0.9 +/- 0.16	0.7 +/- 0.21	-	-	0.3 +/- 0.34	4.9 +/- 1.93	3.9 +/- 0.68	-	-	0.4 +/- 0.10	0.7 +/- 0.20	0.2 +/- 0.08	-	-

Table S4: Mean and SD of the relative gene expression of the eight SbAMTs and of the glutamine synthase gene. Reference condition: without AMF with NO_3^- 1x. Reference gene: Ubiquitin

	Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
	-N -AMF	-N -AMF	-N +AMF	-N +AMF	-N -AMF	-N +AMF	+N -AMF	+N -AMF	+N +AMF	+N +AMF
Root dry weight %	49.4 +/- 5.44	50.6 +/- 5.44	52.75 +/- 3.47	47.25 +/- 3.47	56.18 +/- 6.05	43.81 +/- 6.05	55.87 +/- 8.20	44.14 +/- 8.20	49.01 +/- 19.37	50.99 +/- 19.37
	Treatment 6		Treatment 7		Treatment 8		Treatment 9		Treatment 10	
	+N -AMF	+N +AMF	-N -AMF	+N -AMF	-N +AMF	+N +AMF	-N -AMF	+N +AMF	+N -AMF	-N +AMF
Root dry weight %	62.46 +/- 18.33	37.53 +/- 18.33	49.86 +/- 9.37	50.13 +/- 9.37	42.32 +/- 7.85	57.67 +/- 7.85	55.46 +/- 14.57	44.53 +/- 14.57	51.37 +/- 20.37	48.63 +/- 20.37

Tab.S5: repartition of root dry weight between the two pots of the split-root experiment in percent

	Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
	-N -AMF	-N -AMF	-N +AMF	-N +AMF	-N -AMF	-N +AMF	+N -AMF	+N -AMF	+N +AMF	+N +AMF
Root dry weight %	-	-	30.33 +/- 7.37	34.66 +/- 9.07	-	44.25 +/- 6.65	-	-	36.66+/- 3.51	31.33 +/- 4.73
	Treatment 6		Treatment 7		Treatment 8		Treatment 9		Treatment 10	
	+N -AMF	+N +AMF	-N -AMF	+N -AMF	-N +AMF	+N +AMF	-N -AMF	+N +AMF	+N -AMF	-N +AMF
Root dry weight %	48.33 +/- 18.33	37.53 +/- 11.26	-	-	26.5 +/- 9.19	36 +/- 2.83	-	60.66+/- 8.73	-	34 +/- 2.16

Tab.S6: Root length colonization in each pot of the split-root experiment in percent

Hoagland solution after Gabor & Wetter (1975) Solution D	"-N"	"1xNO_3^-"	"1xNH_4^+"	"0.3xNO_3^-"	"0.1xNO_3^-"
39.805 mMol $Ca(NO_3)_2 \cdot 4H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $Ca(NO_3)_2 \cdot 4H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	11.941 mMol $Ca(NO_3)_2 \cdot 4H_2O$	3.9805 mMol $Ca(NO_3)_2 \cdot 4H_2O$
20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	6.085 mMol $MgSO_4 \cdot 7H_2O$	2.0286 mMol $MgSO_4 \cdot 7H_2O$
65.273 mMol KNO_3	75.70 mMol KCl	65.273 mMol KNO_3	75.70 mMol KCl	19.582 mMol KNO_3	6.5273 mMol KNO_3
10.433 mMol $NH_4H_2PO_4$	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	3.130 mMol KH_2PO_4	1.0433 mMol KH_2PO_4
			155.318 mMol NH_4Cl		

Table S7: Modifications made to the Hoagland solutions after Gabor & Wetter (1975) to obtain the treatments "-N", "1xNO_3^-", "0.3xNO_3^-", "0.1xNO_3^-" and "1xNH_4^+".

3. Evolution and functional characterization of two ammonium transporters present in grasses and induced by arbuscular mycorrhizal fungi

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In preparation

3.1 Abstract

Previously, we have described the family of ammonium transporters in sorghum (*Sorghum bicolor*) and have shown that two of them, AMT3;1 and AMT4 are induced in roots upon colonization by arbuscular mycorrhizal (AM) fungi. In the present work, we have characterized the orthologues of AMT3;1 and AMT4 in various grasses (maize, rice, sorghum, *Setaria italica* and *Brachipodium distachyon*). Using quantitative PCR, we studied the expression of both transporters in plant roots grown in the presence or absence of nitrogen (NH_4^+) supply and in the presence or absence of AM fungi. In none of the plant species, expression of the two AMT genes was affected by the presence or absence of NH_4^+. However, we found a strong induction of both AMT genes in response to colonization by AM fungi in all plant species studied. This finding indicates that both AMT3;1 and AMT4 were present in a common ancestor of these four plants. Additionally, the products of the AMT3;1 genes were confirmed to function as ammonium transporters by yeast mutant complementation assays. Our homology models of the AMT3;1 ammonium transporters indicate that the these transporters, like the already characterized bacterial AmtB, are more likely to catalyze the uptake of charged ammonium (NH_4^+) rather than the neutral form (NH_3) as hypothesized before.

3.2 Introduction

In a previous publication we described the expanded family of sorghum (*Sorghum bicolor*) ammonium transporters (AMT) including two mycorrhizal induced one (AM inducible AMTs), SbAMT3;1 and SbAMT4. SbAMT3;1 was clustering together with an AM inducible AMT from soybean: GmAMT3;1 (Kobae *et al.*, 2010). SbAMT4 was clustering with the AM inducible AMTs from lotus: LjAMT2;2 (Guether *et al.*, 2009), and soybean: GmAMT4;1 (Kobae *et al.*, 2010). However, other AM-inducible AMTs like poplar PtAMT1;2 or soybean GmAMT1;4 cluster separately, suggesting that AM-inducible AMTs evolved independently in different plant species.

In this study, we wanted to test whether the two AM inducible AMTs of sorghum are conserved within the plant family of the Poaceae. The family of the Poaceae include approx. 1000 species (Glémin & Bataillon, 2009). In its evolution, the core Poaceae split into two major clades the BEP and PACCMAD clades (BEP: Bambusoideae, Ehrhartoideae, Pooideae and PACCMAD: Panicoideae, Arundinoideae, Centothecoideae, Chloridoideae, Aristidoideae, Danthonioideae) (Bouchenak-Khelladi *et al.*, 2008), which diverged around or even before -55 million years ago (Mya) (Prasad *et al.*, 2005). The five grasses we studied belong to these two clades (BEP and PACCMAD) and are distributed in three subfamilies: rice (*Oryza sativa*) in the subfamily of the Ehrartoideae and *Brachypodium distachyon* in the subfamily of the Pooideae both clustering in the BEP clade; and maize(*Zea mays*), sorghum (*Sorghum bicolor*) and *Setaria italica* in the subfamily of the Panicoideae clustering in the PACCMAD clade. These five plants were chosen because of their character as model plants, their agronomical importance (except for *B. distachyon*) and their phylogenitic relationship. Additionally, the genome of these plants was sequenced and annotated (Matsumoto, 2005; Paterson *et al.*, 2009; Schnable *et al.*, 2009; Initiative, 2010; Zhang *et al.*, 2012).

The orthologs of the sorghum AMT3;1 and AMT4 in the five plants studied cluster together in two clusters. We verified by qPCR that all genes were induced in response to colonization by AM fungi. This indicates that the common ancestor of all five plants already possessed both AM-inducible AMTs. Additionally, SbAMT3;1, ZmAMT3;1, OsAMT3;1, SiAMT3;1 and BdAMT3;1 were characterized in yeast to test their functionality. To complement the characterization we built homology models for the five proteins. By comparison with the AmtB transporter from E.coli for which a detailed computer simulation based on quantum chemistry has been performed (Wang, S *et al.*, 2012), we suggest that all three

transporters bind the ionic form of ammonium (NH_4^+) with high affinity and promote the uptake of NH_4^+ rather than the neutral NH_3 as previously hypothesized by Guether *et al.* (2009).

3.3 Materials and Methods

Plant growth conditions for tissue analysis

Seeds from *Sorghum bicolor* (L.) Moench (cultivar Pant-5), *Zea mays* (cultivar Maeva), *Oryza sativa* (cultivar Baviva), *Setaria italica* (cultivar Manta) and *Brachypodium distachyon* (wild type Bd21) were surface sterilized (10min in 2.5% KClO), rinsed with sterile deionized water several times during one day) and soaked in sterile deionized water over night. Seeds were pre-germinated on autoclaved Terra Green (Oil Dri US-special, American aluminiumoxide, type III/R; Lobbe Umwelttechnik, Iserlohn, Germany) at 25°C for 24h and then grown in the dark at room temperature for 72h. The fungal strain *G. mosseae* ISCB13 (Botanical Institute, Basel, Switzerland) was propagated by trap cultures set up as previously described (Oehl *et al.*, 2004). To establish AM symbiosis, pre-germinated seeds were individually inoculated in pots containing a mixture of acid-washed Terragreen, sand and loess soil (5:4:1 w/w/w). About 100 spores were added to the mixture. For the controls (non-mycorrhizal plants), the same amount of autoclaved inoculum was added to the mixture. To correct for possible differences in microbial communities, each pot received one ml of filtered washing of AM fungal inoculum (van der Heijden *et al.,* 1998). Plants were grown in a greenhouse with day-night temperatures of 28 and 15°C, respectively.

Plants were watered twice a week during experiments. From the first week on, 8 ml of modified Hoagland solutions were applied weekly. Two different Hoagland solutions, modified after Gamborg & Wetter (1975), were prepared to obtain different N sources (Table.3.1): "-N" and "1xNH_4^+".

Staining of AM fungi in plant roots

From each analyzed plant, one subsample of 100mg of fresh roots was used to determine the degree of AM fungal colonization, as follows. Root subsamples were stained with trypan blue (0.005% w/v in lactic acid, glycerol, water, 1:1:1, w:w:w) at 60°C for 10 minutes in 15ml tubes in a water bath and destained 24h in glycerol: 1%HCl (w:w). Root colonization was quantified according to the grid intersection method as described by Brundrett et al. (1984). Total percentage colonization comprised root intercepts containing hyphae, vesicles, spores and arbuscules. Differences between means of variables were analyzed by ANOVA ($p \leq 0.05$), using IBM SPSS 18.0 (Chicago, Il, USA).

AMT genes

Sequencing, assembly and annotation of the plant genomes were described (Matsumoto, 2005; Paterson *et al.*, 2009; Schnable *et al.*, 2009; Initiative, 2010; Zhang *et al.*, 2012). Complete *S. bicolor, Z. mays, O. sativa, S. italica* and *B. distachyon* DNA and protein sequences are available at the Phytozome website (www.phytozome.org). All sequences have been deposited at GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan. Using BLAST search at the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) we identified orthologs of SbAMT3;1 and SbAMT4 in the genome of the different plants based on conserved domains, identities and E-values. Sequences of the cDNAs described here are available at the NCBI database under accession numbers JX29485, JX294859 and KC997569 to KC997576.

Phylogenetic analyses

For phylogenetic analysis, the AMTs amino acid sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbor joining trees were constructed using Poisson correction model for distance computation in MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was carried out with 1000 replicates. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Gene accession numbers of amino acids sequences are given in the Table.3.2.

Samples, RNA isolation and quantitative reverse transcription-PCR

The procedure used for RNA extraction and cDNA synthesis was as described by Courty et al. (2009) using the following conditions: mycorrhizal roots were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Full-length doubled-stranded cDNAs corresponding to mRNAs expressed in plant roots were obtained using the SMART–PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). Quantification of AMT transcripts was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the

iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in real time quantitative PCR reactions with gene-specific primers from designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Table 3). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C and a GC percentage > 50%. Primers used as controls or for analysis had an efficiency ranging between 90% and 110%. Target gene expression was normalized in each plant to the expression of ubiquitin (Sb: gene Sb01g005570, Zm:gene AAC49014, Os: gene AAL77200, Si: gene ADE62029, Bd: gene XP003569182). Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. For data analysis, the geometric mean of the biological replicates (n = 4) was calculated.

Primers used as controls or for analysis had efficiency ranged between 90% and 110%. Primers for sorghum AMTs were design by Koegel *et al.* (2013). All used primers are listed in Tab.3.3.

From each of the three *S. bicolor, Z. mays, O. sativa, S. italica, and B. distachyon* plants harvested three subsamples (about 100mg) of carefully washed roots, and of shoots, stem, pistils and stamina were snap frozen and stored at -80°C for further gene expression analysis.

All full-length cDNAs were sequenced by cDNA walking PCR: amplification of the full-length cDNAs, from the start to the stop codon, with primers designed using the nucleotide sequences of manually annotated gene models (Table 2), was performed on a T3 thermocycler (Biometra, Labgene Scientific SA, Switzerland) using the Advantage 2 Polymerase Mix (Clontech). PCR reactions resulted in single bands on a 1% agarose gel (Promega, Madison, WI, USA) in 0.5% TAE (Tris Acetate-EDTA) stained with Midori Green according to manufacturer's instructions (Labgene, Chatel-St-Denis, Switzerland). Amplified products were purified with ExoSAP treatment (USB, Cleveland, Ohio, USA) and direct cDNA sequencing was performed on a 3500 Genetic Analyser (Applied Biosystems, Courtaboeuf, France).

C and N analysis

The remainder of the root samples and the shoot material were dried at 80°C for 72h and weighed. These samples were ground in 1.5 ml Eppendorf® tubes using 1.1 mm diam. tungsten carbide balls (Biospec Products, Inc., Bartlesville, Oklahoma, USA) in a Retch MM301 vortexer (Retch Gmbh and Co.,

Haan, Germany). Total N and C were measured using an on-line continuous flow CN analyzer coupled with an isotope ratio mass spectrometer (ANCA-SL MS 20-20 system, Sercon Ltd, Crewe, UK).

Heterologous complementation of a yeast mutant defective in ammonium uptake

The full length *SbAMT3;1, ZmAMT3;1*, *OsAMT3;1*, and *BdAMT3;1* cDNA were cloned in pDR196 using Gateway technology (Invitrogen), as described earlier (Wipf *et al.*, 2003). The resulting plasmids were called pDR196-*SbAMT3;1,* pDR196-*ZmAMT3;1,* pDR196-*OsAMT3;1,* and pDR196-*BdAMT3;1*. The yeast strain 31019b (*MATa ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) (Marini *et al.*, 1997) was transformed with pDR196-*SbAMT3;1,* pDR196-*ZmAMT3;1,* pDR196-*OsAMT3;1,* and pDR196-*BdAMT3;1,* according to (Dohmen *et al.*, 1991). As control, we also cloned and transformed similarly the low-affinity transporter AtAMT1;3 from *Arabidopsis thaliana* described by Gazzarrini et al.(1999).

For *S. cerevisiae* uptake studies, yeast cells were grown to logarithmic phase. Cells were harvested at an OD600 of 0.9, washed twice in water, and resuspended in buffer A (0.6 M sorbitol, 50 mM potassium phosphate, at the desired pH) to a final OD600 of 5. Prior to the uptake measurements, the cells were supplemented with 100 mM glucose and incubated for 5 min at 30°C. To start the reaction, 100 µl of this cell suspension was added to 100 µl of the same buffer containing at least 18.5 kBq [14C]-aspartate, specific activity 7.66 GBq/mmol (Amersham) and unlabeled amino acid to the concentrations used in the experiments. Sample aliquots of 45 µl were removed after 15, 60, 120, and 240 s, transferred to 4 ml of ice-cold buffer A, filtered on glass fiber filters, and washed twice with 4 ml of buffer A. The uptake of carbon-14 was determined by liquid scintillation spectrometry. Competition for aspartate uptake was performed by adding a five-fold molar excess of the respective competitors to 150 µM aspartate. Transport measurements were repeated independently and represent the mean of at least three experiments.

Statistical analyses

An analysis of variance (ANOVA) was performed on the total biomass, on the C and N content, and on the total and arbuscular colonization for each treatment separately, where the two latter parameters were arcsine-transformed to fit the assumption of normal distribution. The ANOVA was based on N treatments and AMF treatments. Pairwise comparisons between the treatments were done with planned contrast analysis. Independent paired t-tests were performed. A probability of $P \leq 0.05$ was

considered as representing a significant difference.

Modeling

Homology models were built using swiss-model web server. Sequences were aligned to the sequence of EcAmtB using ClustalW. The X-ray structure of EcAmtB (Pdb Id:1U7G) was used as a template for homology modeling. Pairwise sequence identities were calculated from sequence alignments using Jalview Program. VMD program was used to analyze the modeled structures.

3.4 Results

From the BLAST search at the NCBI website, highly homologous sequences to SbAMT3;1 and SbAMT4 in maize, rice, *S. italica* and *B. distachyon* were identified. Phylogenetic analysis based on protein - sequence alignment shown that *SbAMT3;1, ZmAMT3;1, OsAMT3;1, SiAMT3;1* and *BdAMT3;1* as well as *SbAMT4, ZmAMT4, OsAMT4, SiAMT4* and *BdAMT4* cluster together, with Bootstrap values of 85 and 59 respectively (Fig.3.1). These genes are belonging to the AMT2 subfamily which has been split into four subclades, AMT2, AMT3 and AMT4 (Fig.3.1). AMT2 genes contain introns with size and splicing location roughly conserved in each subclade. The transmembrane prediction program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) shows that *SbAMT3;1, ZmAMT3;1, OsAMT3;1, SiAMT3;1* and *BdAMT3;1* as well as *SbAMT4, ZmAMT4, OsAMT4, SiAMT4* and *BdAMT4* are coding for proteins predicted to have eleven transmembrane domains with an extracellular N-*terminus* and a cytosolic C-*terminus* like other plant AMT members (Marini & Andre, 2000; Thomas *et al.*, 2000).

AM fungal colonization

Average AM fungal colonization was between 35 and 40% after three month and was not significantly different between plant species (P=0.39). N treatment had no significant effect on AM fungal colonization (Fig.3.2).

Plant growth and N nutrition

Mycorrhized and non-mycorrhized sorghum, rice, *S. italica* and *B. distachyon* plants had a significant higher dry weight in the +N treatments compared to the –N treatments (Tab. 3.4). No significant difference was found in the maize plants between +N and –N treatments.

Additionally, we could see a significantly higher N concentration in the +N treatments compared to the –N treatments in sorghum, *S.italica* and *B. distachyon* in mycorrhized and non-mycorrhized plants (Tab.3.5). In maize, N concentration was higher in the +N treatment compared to the –N treatment only in the mycorrhized plants. In rice, we found a similar tendency (Tab.3.5).

Gene expression in different tissues

Quantitative PCR analyses on the different plant tissues shown that *SbAMT3;1, ZmAMT3;1*, and

OsAMT3;1 were expressed in all the studied tissues (mycorrhized and non-mycorrhized roots, leaves, stem, stamina and pistils). *BdAMT3;1* was expressed only in the mycorrhized and non-mycorrhized roots as well as in the stamina. In all species, the highest expression of the AMT3;1 orthologs was measured in the roots of mycorrhized plants. The lowest expression of *SbAMT3;1*, *OsAMT3;1* and SiAMT3;1 was measured in the pistils, the lowest expression of *ZmAMT3;1* was measured in the stamina and the lowest expression of *BdAMT3;1* was measured in the non-mycorrhized roots (Fig.3.3).

The highest expression of the AMT4 transporters orthologs of all five plants was measured in the mycorrhized roots (Fig.3.3). Additionally, all five AMT4 were detected in the non-mycorrhized roots. Expression in the other tissues varied between plants (Fig.3.3).

Effect of N on root gene expression

We quantified the level of expression of *ZmAMT3;1, OsAMT3;1, SbAMT3;1, SiAMT3;1* and *BdAMT3;1* as well as *ZmAMT4, OsAMT4, SbAMT4, SiAMT4* and *BdAMT4* under two different N conditions in mycorrhized and non-mycorrhized plants (Fig. 3.4a+b). Plants were feed with Hoagland solution either without N or with NH_4^+ (Tab.3.1) during three months. Afterward gene expression was studied in the plant roots. We could not see any significant difference in the expression of *ZmAMT3;1, OsAMT3;1, SbAMT3;1, SiAMT3;1* and *BdAMT3;1* between the treatments with and without N. The was true for *ZmAMT4, OsAMT4, SbAMT4, SiAMT4* and *BdAMT4*. N treatment had no effect on the expression of these genes in the different plants. Only the mycorrhization had a significant effect on gene expression: mycorrhized plants had a significant higher gene expression compared to non-mycorrhized plants independently of the nitrogen treatment.

Effect of mycorrhization on gene expression

We found a very low expression of *OsAMT3;1* in non-mycorrhized roots and in other plant tissues like leaves, stem, stamina and pistils confirming previous results. Similarly *ZmAMT3;1, SbAMT3;1, SiAMT3;1* and *BdAMT3;1* had also a very low expression level in other tissues than mycorrhized roots. The expression pattern of *SbAMT3;1, OsAMT3;1* and *ZmAMT3;1* were very similar to each other. In mycorrhized roots, all five *AMT3;1* studied were highly up-regulated compared to non-mycorrhized roots independently of the N treatment: maize was up to 18 times up-regulated, rice up to 40 times, purple false brome up to 60 times and sorghum and foxtail millet more than 60 times (Fig.3.4a)

In the five Poaceae species studied, *AMT4* was also predominantly expressed in mycorrhized roots (Fig.3.3). Compared to the plants without mycorrhiza, this up-regulation was reaching 15 times by sorghum and maize, 40 times by rice and *S. italica* and 60 times by *B. distachyon* (Fig.3.4b).

Yeast complementation

A yeast mutant complementation test was used to demonstrate the NH_4^+ transport function and to biochemically characterize *ZmAMT3;1, OsAMT3;1, SbAMT3;1, SiAMT3;1* and *BdAMT3;1*. *AtAMT3;1* was used as control. All five transporters were expressed through the yeast expression vector pDR196 (Wipf *et al.*, 2003) in a mutant yeast strain, 31019b, which lacked the three endogenous NH_4^+ transporter genes (MEP1, MEP2, MEP3) and was unable to grow on a medium containing <3mM NH_4^+ as the sole N source (Marini *et al.*, 1997). ZmAMT3;1cDNA, OsAMT3;1cDNA, SbAMT3;1cDNA, SiAMT3;1 cDNA and BdAMT3;1cDNA functionally complemented the yeast mutant efficiently, when 1 and 2 mM NH_4^+ were supplied to the agar medium (Fig.3.5). As expected, AtAMT1;3 complemented as well, but the cells expressing the purple false brome transporter grew more vigorously.

Preliminary results on the uptake capabilities: SbAMT3;1 had a K_m of 87.65 µM and SbAMT4 a K_m of 18.99 µM indicating a higher affinity of SbAMT4.

Modeling

In order to acquire information on the transport mechanism in SbAMT3;1, OsAMT3;1, BdAMT3, SiAMT3 and ZmAMT3;1 we built homology models of these proteins based on the X-ray structure of E.Coli ammonium transporter (EcAmtB). Since there is plenty of experimental and computational study on EcAmtB, comparison of modeled structures with the EcAmtB structure provides us important insight in the transport mechanisms of AMT3:1 transporters in plants. Sequence similarity between SbAMT3;1, OsAMT3;1, BdAMT3, SiAMT3, ZmAMT3;1 and EcAmtB are reported in Tab.6. Most of the amino acids important for the transport of ammonium in EcAmtB are also found along the pore of the five plant species (Fig. 3.6 and Tab. 3.6). Mutations with respect to EcAmtB are Ala162Ser, Ser219Asp, Met23Gln and Phe31Leu. The biggest impact would come from the mutation of a neutral serine to a negatively charged aspartic acid, which may increase the ammonium binding affinity.

3.5 Discussion

The five grass species studied had an ortholog of AMT3;1 and AMT4 in their genome. As rice and *B. distachyon* separated from sorghum, maize and *S. italica* about -55 Mya, we assume that both genes were present in a common ancestor of these plants. AMT3;1 and AMT4 orthologs were clustering in the AMT2 subfamily which can be further divided into three clades, AMT2, AMT3 and AMT4 (Suenaga *et al.*, 2003)(Fig. 3.1).

AM colonization

Percent colonization was shown to vary frequently with different plant-fungus combination and can be related among other things to differences in rate of root growth (Smith & Read, 2008). Plants like cereals, that have a rapid root growth, tend to have a lower plateau values for the percent root colonization than those with slower root growth like leek (Smith & Read, 2008). Indeed, we found here colonization values between 32 and 50% in the different cereal plants (Fig.3.2), what is clearly less than for example 80% in leek (Sorensen *et al.*, 2005). However, the level of colonization can't be related to the AM fungal responsiveness (Alberton *et al.*, 2005) meaning that plants with a lower colonization level might profit similarly to the AM symbiosis that plants with a higher colonization level.

The expression of AMT3;1

The expression of the five orthologs of AMT3;1 was very low in other tissues than mycorrhized roots confirming the results from Suenaga *et al.* (2003) on *OsAMT3;1*. Interestingly, *ZmAMT3;1, OsAMT3;1, SbAMT3;1* and *SiAMT3;1* were expressed in all the different plant tissues studied (mycorrhized and non-mycorrhized roots, leaves, stems, stamina and pistils) in contrary to *BdAMT3;1* expressed only in the mycorrhized and non-mycorrhized roots as well as in the stamina. This result is surprising as rice and *B. distachyon* cluster together in the BEP group (Glémin & Bataillon, 2009) and we expected a similar expression of AMT3;1 in both plants.

The five orthologs of AMT3;1 were highly induced upon colonization by AM fungi: maize up to 18 times, rice up to 40 times, *B. distachyon* up to 60 times, *S. italica* and sorghum more than 60 times (Fig.3.3). This finding shown clearly a conserved induction of the five AMT3;1 orthologs upon colonization by AM fungi and indicated that the induction of AMT3;1 upon colonization by AM fungi might have been already present in a common ancestor of the five grasses studied.

Interestingly, the five orthologs of AMT3;1 studied here were clustering with an AM-inducible AMT from soybean *GmAMT3;1* (Kobae *et al.*, 2010). This result seems to indicate that the induction of AMT3;1 upon colonization by AM fungi could even has been present in a common ancestor of the monocots and the dicots. Contrasting with this, the ortholog of AMT3;1 in poplar (*PtAMT3;1*) which also clustered with *GmAMT3;1*, *SbAMT3;1, ZmAMT3;1, OsAMT3;1, SiAMT3;1* and *BdAMT3;1* was detected specifically in senescing leaves (Couturier *et al.*, 2007) and was not up-regulated in poplar roots colonized with the ectomycorrhizal fungus *Paxillus involutus*. This could be explained by the fact that in poplar, gene expression can vary when plants are mycorrhized with ectomycorrhizal or with AM fungi as it was shown for the expression of phosphate transporters (Loth-Pereda *et al.*, 2011): *PtPT10* was only induced in plants mycorrhized with *G.mosseae* or *G.intraradices* and was not expressed in the non-mycorrhized roots or in the roots mycorrhized with *P. involutus* or *Laccaria bicolor*. Also *PtPT3, PtPT4/PtPT7* and *PtPT5* shown a higher expression in the roots mycorrhized with *G.mosseae* or *G.intraradices* compared to non-mycorrhized roots or roots mycorrhized with *P. involutus* or *L. bicolor*.

The expression of AMT4

The grass species studied here had only one AMT gene clustering in the AMT4 clade contrasting with poplar (5) or soybean (6) (Fig.3.1) and suggesting a different organization of AMT genes in different plant families.

The orthologs of AMT4 in the five grass species studied were highly induced upon colonization by AM fungi: sorghum and maize 15 times, rice and *S. italica* up to 40 times and *B. distachyon* up to 70 times. Interestingly, in addition to SbAMT4 we described in a former study (Koegel *et al.*, 2013), three other AM-inducible AMTs were already described in the AMT4 clade: *Lotus japonicas LjAMT2;2* (Guether *et al.*, 2009), and soybean *GmAMT4;1* and *GmAMT4;4* (Kobae *et al.*, 2010). However, the AMT4 clade also includes non AM-inducible AMTs (Fig. 3.1) suggesting a less conserved evolution than AMT3;1.

The transport of ammonium

Ammonium transporters proteins are present in all domains of life, notably in bacteria (Siewe *et al.*, 1996; Marini *et al.*, 2000), mammals and plants (Ninnemann *et al.*, 1994). The AMT genes in plants, as AMT3;1 and AMT4 encode protein containing eleven putative transmembrane domains similarly to AmtB from *E.coli* (EcAmtB) (Zheng *et al.*, 2004). Based on experimental studies of various proteins of the

AMT family, three transport mechanisms have been suggested: electroneutral NH_3 transport (Khademi *et al.*, 2004; Javelle *et al.*, 2008), NH_3/H^+ cotransport (Boeckstaens *et al.*, 2008; Javelle *et al.*, 2008) and NH_4^+ transport (Ninnemann *et al.*, 1994; Siewe *et al.*, 1996; Fong *et al.*, 2007). A recent study by Wang et al. (2012) shown a detailed molecular mechanism consistent with the transport of NH_4^+ in the EcAmtB transporter. It involves the binding of NH_4^+, the transfer of a proton from NH_4^+ to the histidine H168, and the subsequent diffusion of NH_3 followed by the release of the proton from H168 along a water wire formed in the pore. Here, we found apparent structural similarity between the pores of EcAmtB and the AMT3:1 (Fig.3.6). Only four amino acids varied between EcAmtB and the five orthologs of AMT3;1 studied (Tab. 3.7), which did not affect the transport capabilities of the pore, as suggested by our homology models (Fig. 3.6). In view of this, we propose that the same transport mechanism than the one described by Wang *et al.* (2012) takes place in the AMT3:1 ammonium transporters from sorghum, maize, rice, *S. italica* and *B. distachyon*.

AM inducible AMTs are conserved in the Poaceae

Our results support the hypothesis of Wang *et al.* (2010) that genes important for AM symbiosis were already present in a common ancestor of land plant and stayed conserved in most plants until today. The Poaceae split into two major clades the BEP and PACCMAD clades (Bouchenak-Khelladi *et al.*, 2008; Glémin & Bataillon, 2009) (Fig. 3.7), which diverged around or even before -55 Mya (Prasad *et al.*, 2005). Here we could show that AM inducible AMTs were already present in a common ancestor of both clades -55 Mya ago.

3.6 Acknowledgments

This project was supported by the Swiss National Science Foundation (grant nos. 130794 to A.W. and PZ00P3_136651 to P-E.C).

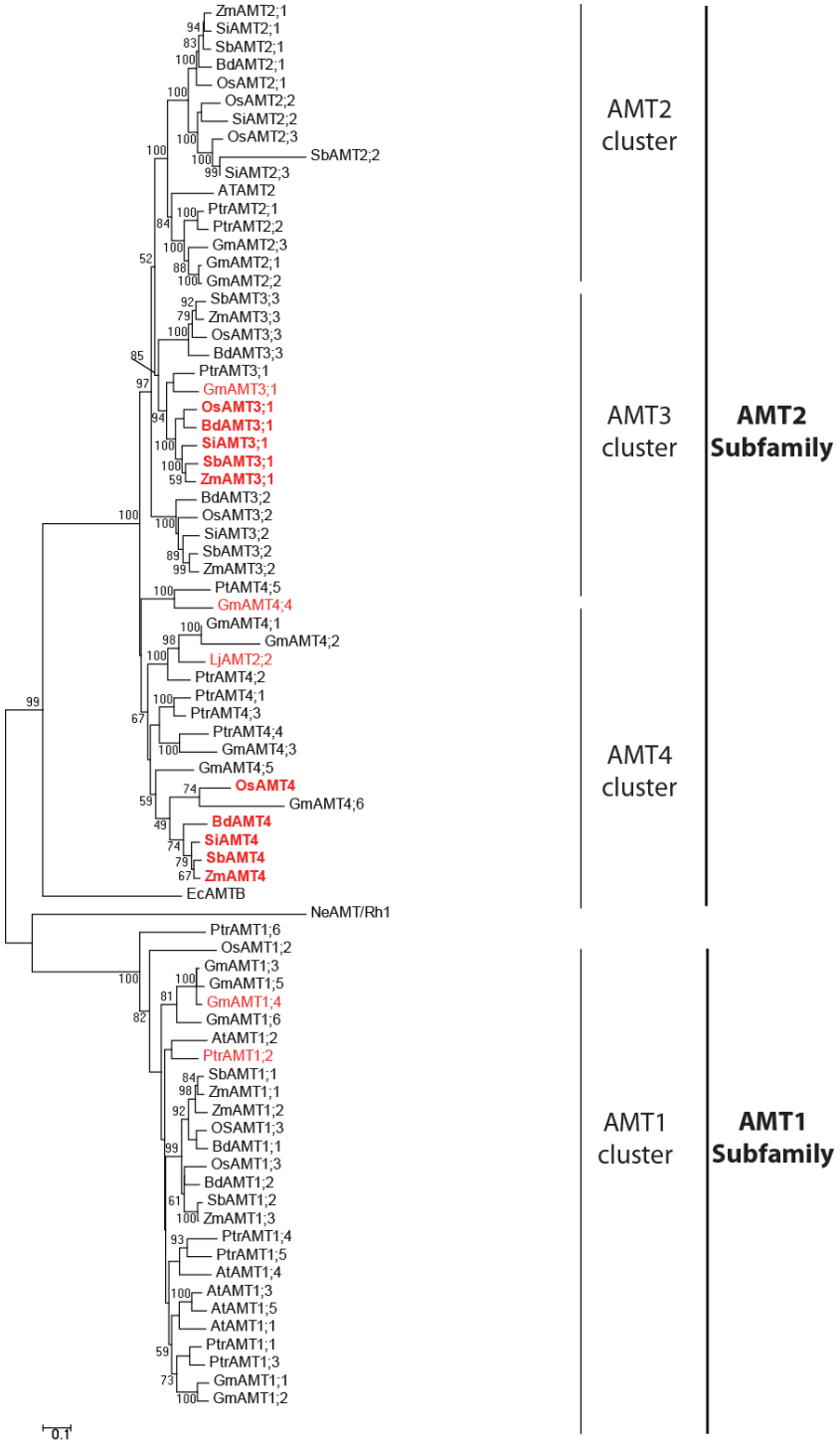


Figure 3.1. Neighbor joining tree of the ammonium transporter (AMT) family. Bootstrap values are from 1000 replications. Sequence names consist of species code (first letter of genus and first letter of species name) and the AMT number. The scale indicates a distance equivalent to 0.1 amino acid substitutions per site. Species codes: Ec, *Escherichia coli,* Ne, *Nitrosomonas europea,* At, *Arabidopsis thaliana,* Gm, *Glycine max,* Lj, *Lotus japonicus,* Os, *Oryza sativa,* Ptr, *Populus trichocarpa,* Sb, *Sorghum bicolor,* Zm, *Zea mays*, Bd, *Brachypodium distachyon,* Si, *Setaria italica.* AM inducible AMTs are in red and AM inducible AMTs from the Poaceae are in red bold front.

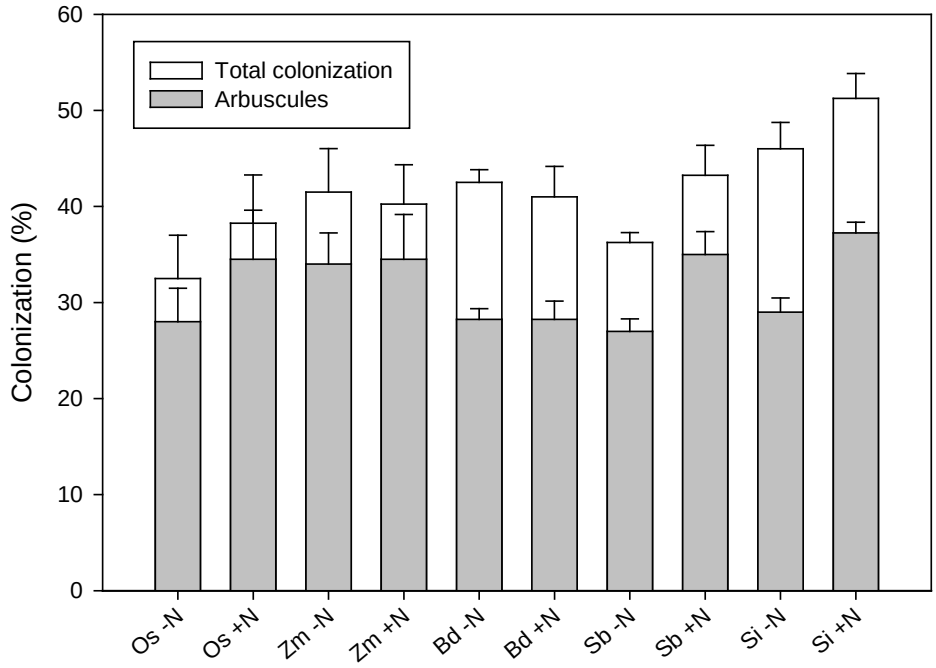


Figure 3.2: Percentage of the root length colonized by *G. mosseae* in the different plants under -N and NH_4^+ treatments. Grey color indicates the percentage of arbuscules and white color the percentage of total colonization including arbuscules, hyphae, and vesicles. There was no significant difference between the different N treatments (P=0.6) and the different plant (P=0.39). Abbreviations: rice, Os; maize, Zm; purple false brome, Bd; sorghum, Sb; foxtail millet, Si.

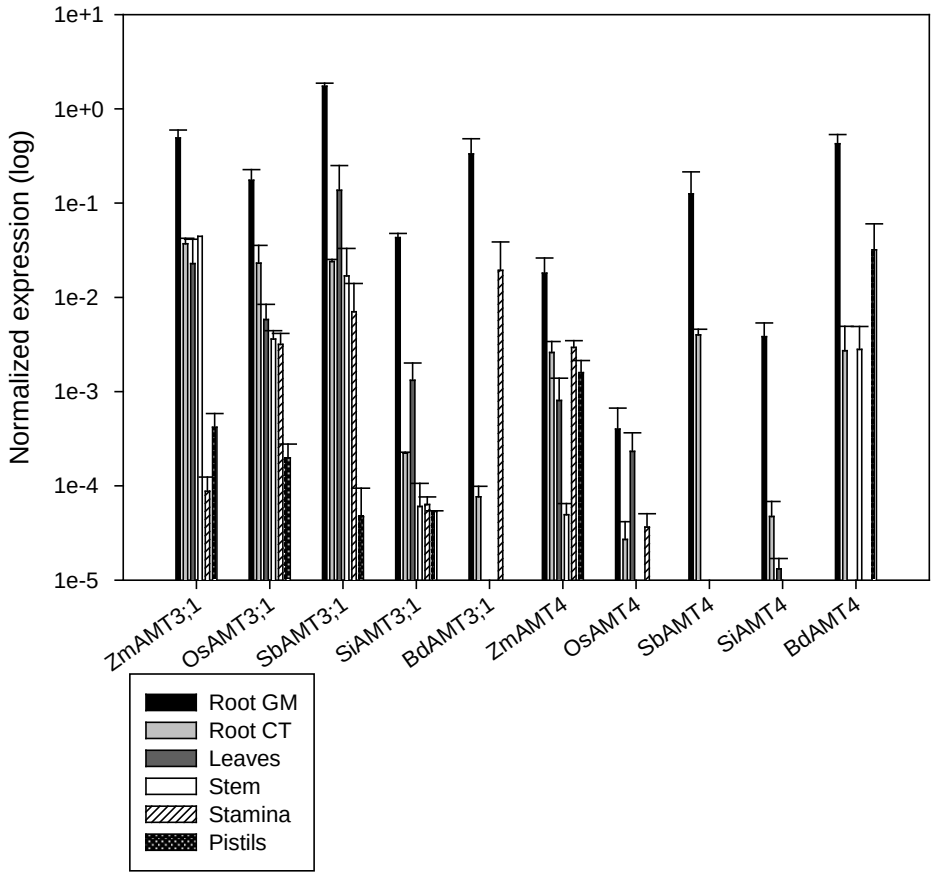


Figure 3.3. Quantification by qRT-PCR of the transcript levels of the AMT3;1 and AMT4 genes in the tissues of the different plants. The values are the means of three replicates. Ubiquitin was used as the reference transcript. Abbreviations: rice, Os; maize, Zm; purple false brome, Bd; sorghum, Sb; foxtail millet, Si; *G. mosseae*, GM; control without AM fungi, CT.

a)

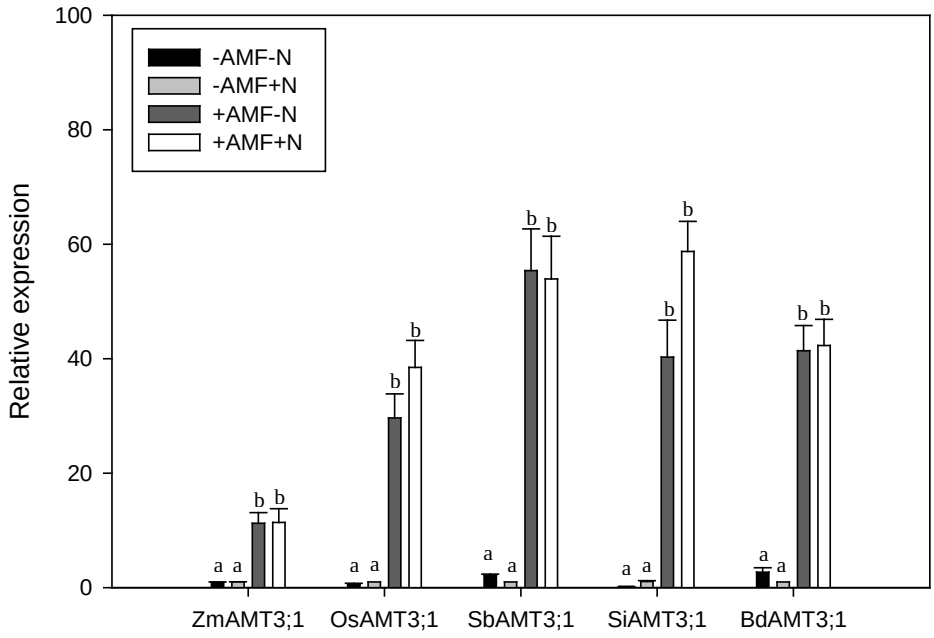
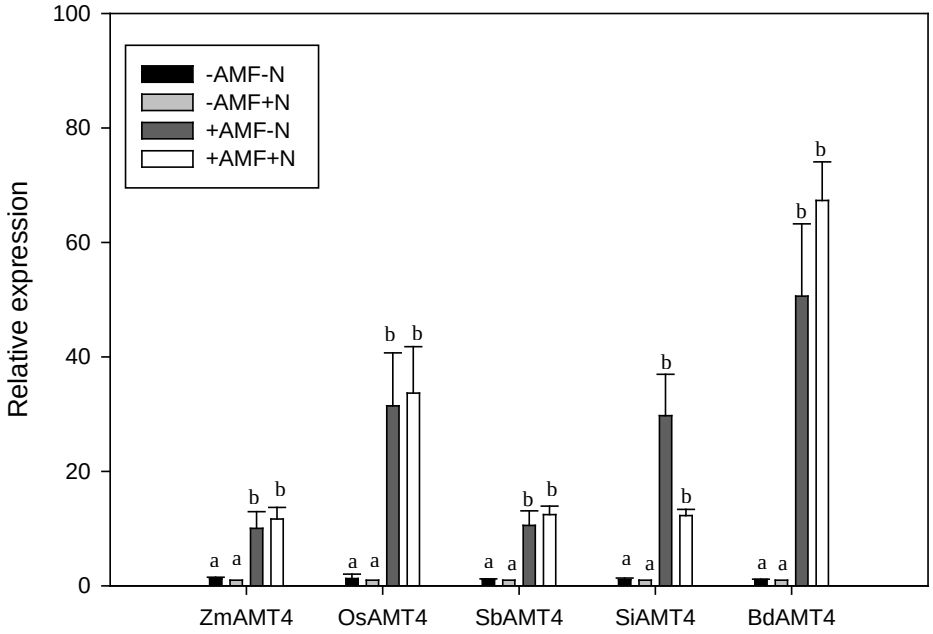


Figure 3.4. Quantification by qRT-PCR of the transcript levels of *ZmAMT3;1, OsAMT3;1, SbAMT3;1, SiAMT3;1* and *BdAMT3;1* (a) or *ZmAMT4,OsAMT4, SbAMT4, SiAMT4* and *BdAMT4* (b) in roots either non-colonized or colonized by arbuscular mycorrhizal fungi 13 weeks post-inoculation in the different N treatments (-N, +N (NH_4^+)).

The values are the means of four replicates. Ubiquitin was used as the reference transcript. Gene expression was normalized according to the "-AMF, 1×NH_4^+" treatment. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F test). Letters indicate a *p*-value < 0.05.

b)


100
80
60
40
20
0
Relative expression
-AMF-N
-AMF+N
+AMF-N
+AMF+N
a a b b
a a b b
a a b b
a a b b
a a b b
ZmAMT4
OsAMT4
SbAMT4
SiAMT4
BdAMT4

a)

b)

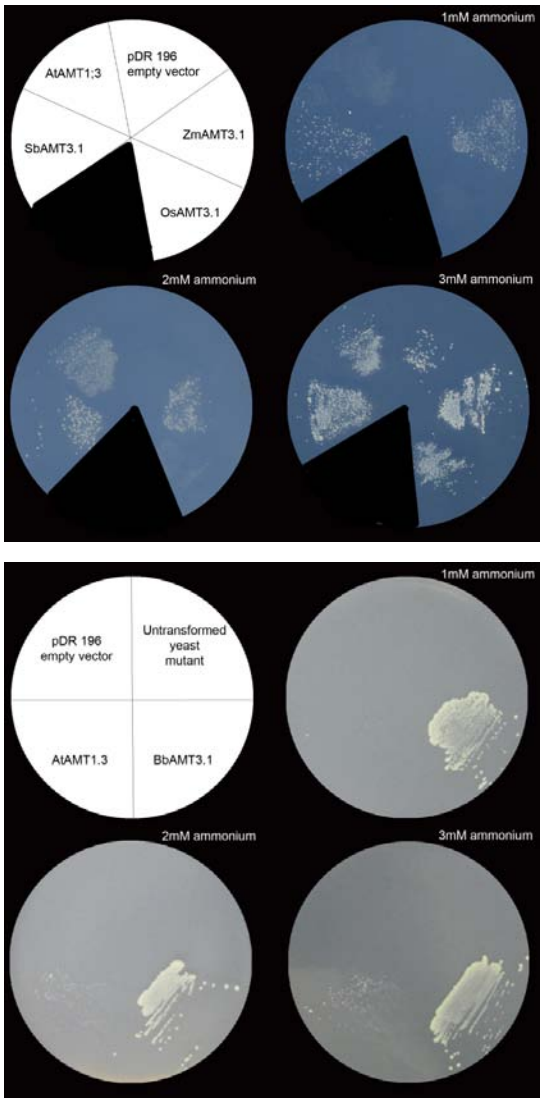


Figure 3.5. Complementation of a yeast mutant defective in ammonium uptake by (a) ZmAMT3;1, OsAMT3;1, SbAMT3;1, and (b) BdAMT3;1.

Growth of the yeast strain 31019b, transformed with various constructs, on minimal medium supplemented with various NH_4^+ concentrations (1, 2 or 3 mM) as a sole nitrogen source. All strains were incubated 5 days at 29°C. AtAMT2 from *Arabidopsis thaliana* was used as a control (Solenkamp *et al.,* 2000).

Abbreviations; pDR196 empty vector: *mep1Δmep2Δmep3Δ* + pDR196, AtAMT1;3: *mep1Δmep2Δmep3Δ* + pDR196-AtAMT2, SbAMT3;1: *mep1Δmep2Δmep3Δ* + pDR196-SbAMT3;1, OsAMT3;1: *mep1Δmep2Δmep3Δ* + pDR196-OsAMT3;1, ZmAMT3;1: *mep1Δmep2Δmep3Δ* + pDR196-ZmAMT3;1, BdAMT3;1: *mep1Δmep2Δmep3Δ* + pDR196-BdAMT3;1.

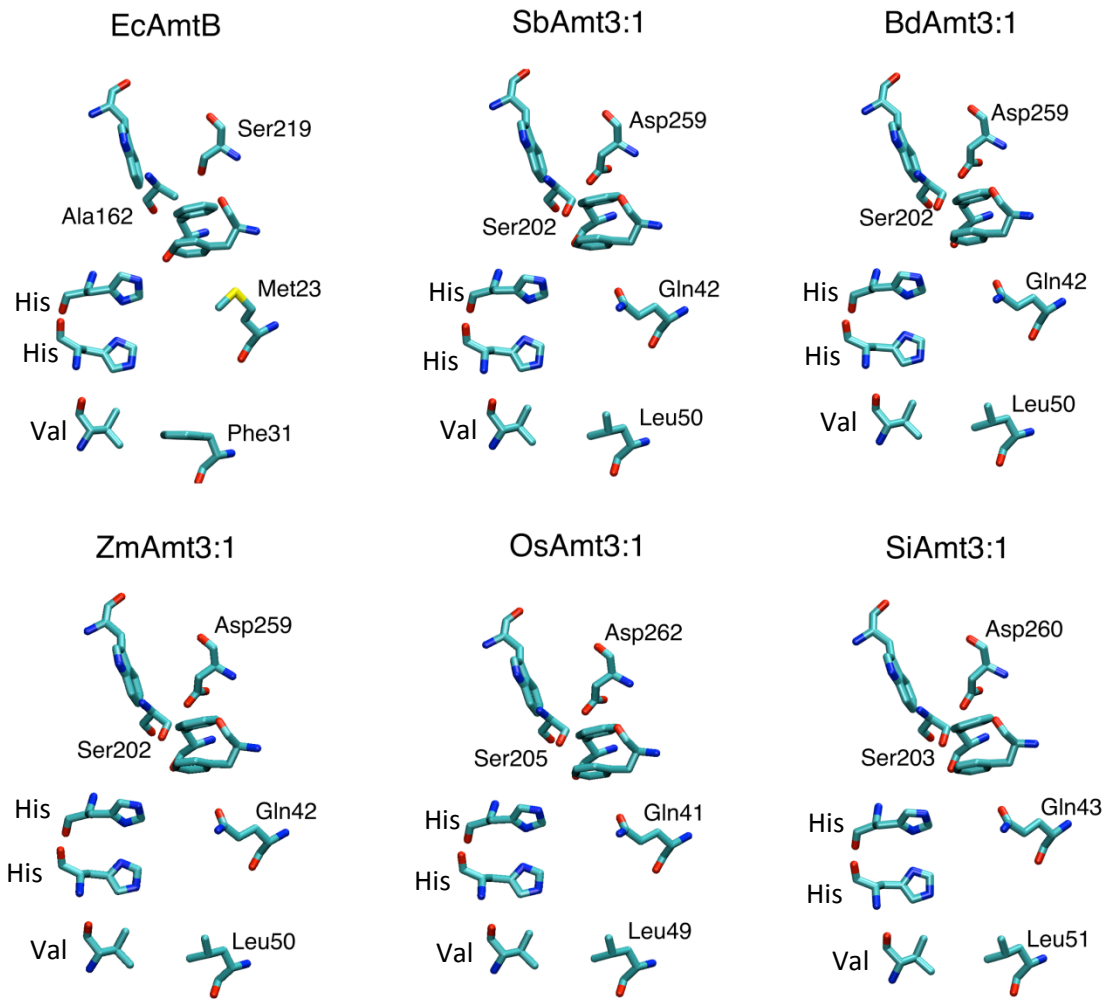


Figure 3.6: Homology models of the SbAMT3;1, OsAMT3;1, ZmAMT3;1, BdAMT3;1, and SiAMT3;1 transporters. Only key residues along the permeation pore are shown. The structure of the EcAmtB transporter, which served as template, is also illustrated. Abbreviations: rice, Os; maize, Zm; purple false brome, Bd; sorghum, Sb; foxtail millet, Si; Escherichia coli, Ec.

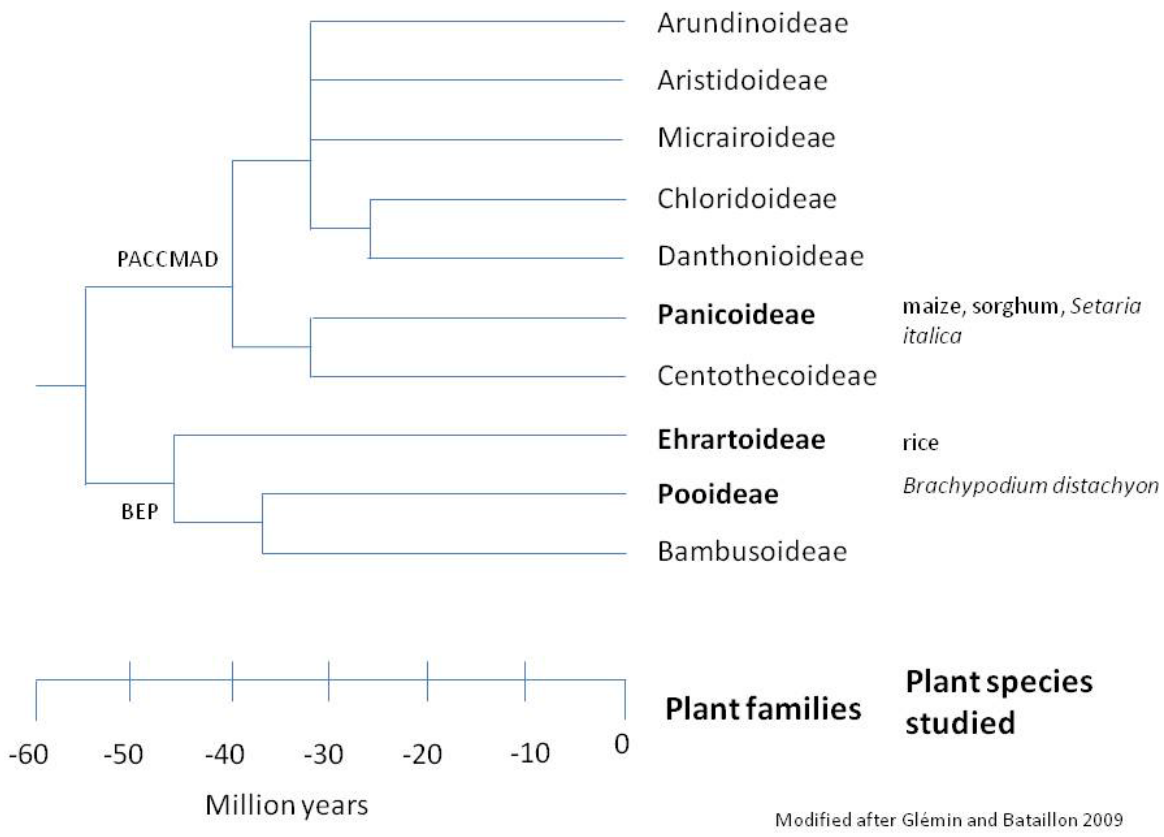


Figure. 3.7: Evolution of the Poaceae (Modified after Glémin & Bataillon 2009)

Table 3.1: Modifications made to the Hoagland solutions after Gabor & Wetter (1975) to obtain the treatments "-N", and "1xNH_4^+".

Hoagland solution after Gabor & Wetter (1975) Solution D	"-N"	"NH_4^+"
39.805 mMol $Ca(NO_3)_2 \cdot 4H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$
20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$
65.275 mMol KNO_3	75.70 mMol KCl	75.70 mMol KCl
10.433 mMol $NH_4H_2PO_4$	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4
		155.318 mMol NH_4Cl

Table 3.2: Characteristics of the AMT3;1 genes (a) and of the AMT4 genes (b) in *Sorghum bicolor, Zea mays, Oryza sativa, Brachypodium dystachion* and *Setaria italica*

a)

Model name	Gene name	Organism	Strand	Introns	CDS (bp)	Length of the AA sequence	Supporting EST	Location	Best blast hit with *AMTs* (%)	Sequence logo of AMT1 and AMT2 signal
Sb03g041140	SbAMT3;1	*Sorghum bicolor*	+	1	1467	488	+	3:68682524-68685235	*Zea mays* GRMZM2G335218_T01	DYSGGyvIhlsSGvaGFtaaYWvGpR
GRMZM2G335218	ZmAMT3;1	*Zea mays*	+	1	1467	488	+	8: 166105184 - 166106880	*Sorghum bicolor* Sb03g041140	DYSGGyvIhlsSGvaGFtaaYWvGpR
LOC_Os01g65000	OsAMT3;1	*Oryza sativa*	+	1	1497	498	+	1: 37735280 - 37737641	*Brachypodium distrachyon* Bradi2g56300.1	DYSGGyvIhlsSGvaGFtaaYWvGpR
Bradi2g56300	BdAMT3;1	*Brachybodium distrachyon*	+	0	1476	491	+	2: 54682765 - 54684240	*Oryza sativa* LOC_Os01g65000.1_GX7M	DYSGGyvIhlsSGvaGFtaaYWvGpR
Si004697m	SiAMT3;1	*Setaria italica*	+	1	1470	489	+	5: 42463676 - 42466199	*Zea mays* GRMZM2G335218_T01	DYSGGyvIhlsSGvaGFtaaYWvGpR

b)

Model name	Gene name	Organism	Strand	Introns	CDS (bp)	Length of the AA sequence	Supporting EST	Location	Best blast hit with *AMTs* (%)	Sequence logo of AMT1 and AMT2 signal
Sb01g008060	SbAMT4	*Sorghum bicolor*	-	1	1425	474	+	1: 6978013 - 6979495	*Oryza sativa* Os03g53780	DFAGGyvIhlsSGiaGFtaaYWvGpR
GRMZM2G473697	ZmAMT4	*Zea mays*	-	1	1395	464	+	5: 7260902 - 7262381	*Oryza sativa* Os03g53780	DFAGGyvIhlsSGiaGFtaaYWvGpR
Os03g53780	OsAMT4	*Oryza sativa*	+	2	1495	497	-	3: 30830441 - 30831935	*Brachypodium distrachyon* Bradi1g08587	DFAGGyvIhlsSGiaGFtaaYWvGpR
Bradi1g08587	BdAMT4	*Brachybodium distrachyon*	-	1	1434	477	+	1: 6068637 - 6070124	*Oryza sativa* Os03g53780	DFAGGyvIhlsSGiaGFtaaYWvGpR
Si039811m	SiAMT4	*Setaria italica*	-	1	1425	474	+	9: 5543610 - 5545092	*Oryza sativa* Os03g53780	DFAGGyvIhlsSGiaGFtaaYWvGpR

Table 3.3: Primers for RT-qPCR and full-length gene amplification.

	RT-qPCR Forward	RT-qPCR Reverse	Full-length Forward	Full-length Reverse
SbAMT3;1	GGCCTCGTCTGCATCACT	GGGTGTCGTCCACTTGCT	CCGCGAGGATGGCGAGCC	CGCCGTGCACGGCGTCGTCGCC
ZmAMT3;1	GTGGCTGTGCTGGGTCAT	TGGGATACAAGGGCGTGA	ATGTCGACCACCGCCTA	TCAGACGTTCTGCGTGT
OsAMT3;1	CATCACGCTCATCCTCCTC	CCTCTCCCTGTCCTTGGTC	ATGTCGGGGGACGCGTT	TCAGACGTTCTGCGTGACG
SiAMT3;1	GGCCTCGTCTGCATCACT	GGGTGTCGTCCACCTGCT	ATGAGCATCGACGACCCTTTG	TCAGACGTTCTGCGTGACGCC
BdAMT3;1	GCGACGGAGAGAAGTACGAC	GCTGAGTGGTCTCGATGTCA	ATGGCGACCGCCGATTTC	TTAGACGTTCTGCGTGACG
SbUbi	CAAGGAGTGCCCCAACAC	GGTAGGCGGGTAAAGCAAA		
ZmUbi	CCACTTGGTGCTGCGTCTTAG	CCTTC TGAATGTTGTAATCCGCA		
OsUbi	AATCAGCCAGTTTGGTGGAGCTG	ATGCAAATGAGCAAATTGAGCACA		
SiUbi	CAAGGAGTGCCCCAACAC	GGTAGGCGGGTAAAGCAAA		
BdUbi	GCCCAAGAAGCAAAAGCA	GCGTCGTCCACCTTGTAGA		

Table 3.4. Mean and Standard deviation of plant dry weight (g) colonized or not by *G. mosseae* in the different N treatments (-N and NH_4^+). Differences in dry weight between the treatments were performed with a one-way ANOVA (Scheffe's F test). Letters indicate a *p*-value < 0.05.

	-AM fungi		+AM fungi	
	-N	+N	-N	+N
Sorghum (*S. bicolor*)	1.37 +/- 0.18 a	2.65 +/- 0.42 b	1.00 +/- 0.14 a	2.96 +/- 0.09 b
Maize (*Z. mays*)	6.65 +/- 1.01 a	7.69 +/- 2.41 a	6.95 +/- 0.43 a	8.09 +/- 0.42 a
Rice (*O. sativa*)	1.38 +/- 0.08 a	2.77 +/- 0.20 c	1.19 +/- 0.02 a	2.33 +/- 0.09 b
B. distachyon	0.65 +/- 0.04 a	0.77 +/- 0.03 b	0.57 +/- 0.01 a	0.72 +/- 0.04 b
S. italica	1.44 +/- 0.25 a	2.97 +/- 0.33 b	1.29 +/- 0.21 a	3.32 +/- 0.11 b

Table 3.5. Mean and Standard deviation of N concentration in plants (mgN/gDW) colonized or not by *G. mosseae* in the different N treatments (-N and NH_4^+). Differences in dry weight between the treatments were performed with a one-way ANOVA (Scheffe's F test). Letters indicate a *p*-value < 0.05.

	-AMF		+AMF	
	-N	+N	-N	+N
Sorghum (*S. bicolor*)	4.18 +/- 0.08 a	5.89 +/- 0.46 b	4.48 +/- 0.23 a	5.42 +/- 0.27 b
Maize (*Z. mays*)	6.91 +/- 0.25 ab	8.03 +/- 0.91 bc	6.67 +/- 0.27 a	8.42 +/- 0.53 c
Rice (*O. sativa*)	5.02 +/- 0.47 ab	5.66 +/- 0.87 b	4.34 +/- 0.26 a	4.86 +/- 0.02 ab
B. distachyon	5.52+/- 0.52 a	7.24 +/- 0.40 b	5.04 +/- 0.46 a	6.89 +/- 0.54 b
S. italica	5.18 +/- 0.12 b	6.77 +/- 0.46 c	4.60 +/- 0.26 a	6.55 +/- 0.33 c

Table 3.6 Pairwise sequence identities

Sequence1	Sequence2	% Identity	Sequence1	Sequence2	% Identity
SbAmt3:1	ZmAmt3:1	92.2	EcAmtB	ZmAmt3:1	31.9
SbAmt3:1	OsAmt3:1	84.4	EcAmtB	OsAmt3:1	31.6
ZmAmt3:1	OsAmt3:1	86.37	EcAmtB	SbAmt3:1	31.73
SbAmt3:1	BdAmt3:1	84.7	EcAmtB	BdAmt3:1	32.3
SbAmt3:1	SiAmt3:1	91.2	EcAmtB	SiAmt3:1	32.93

Table 3.7 Residues forming the pores of EcAmtB, ZmAmt3:1, SbAmt3:1, OsAmt3:1, BdAmt3:1 and SiAmt3:1. Different residues with respect to the ones in EcAmtB are highlighted in light blue color. Abbreviations: rice, Os; maize, Zm; purple false brome, Bd; sorghum, Sb; foxtail millet, Si; Escherichia coli, Ec.

EcAmtB	ZmAmt3	SbAmt3	OsAmt3	BdAmt3	SiAmt3
His168	His208	His208	His211	His208	His209
His314	His362	His362	His365	His362	His363
Phe215	Phe255	Phe255	Phe258	Phe255	Phe256
Phe107	Phe147	Phe147	Phe150	Phe147	Phe148
Trp212	Trp252	Trp252	Trp255	Trp252	Trp253
Val314	Val358	Val358	Val361	Val358	Val359
Phe31	Leu50	Leu50	Leu49	Leu50	Leu51
Ile28	Leu47	Leu47	Leu46	Leu47	Leu48
Tyr32	Tyr51	Tyr51	Tyr50	Tyr51	Tyr52
Leu114	Leu154	Leu154	Leu157	Leu154	Leu155
Leu208	Leu248	Leu248	Leu251	Leu248	Leu249
Thr273	Thr313	Thr313	Thr316	Thr313	Thr314
Ile110	Ile150	Ile150	Ile153	Ile150	Ile151
Met23	Gln42	Gln42	Gln41	Gln42	Gln43
Val270	Val310	Val310	Val313	Val310	Val311
Val167	Ile207	Ile207	Ile207	Ile207	Ile208
Ala162	Ser202	Ser202	Ser205	Ser202	Ser203
Trp148	Trp188	Trp188	Trp191	Trp188	Trp189
Ser219	Asp259	Asp259	Asp262	Asp259	Asp260
Phe103	Phe143	Phe143	Phe146	Phe143	Phe144
Asp160	Asp200	Asp200	Asp203	Asp200	Asp201
Asn216	Asn256	Asn256	Asn259	Asn256	Asn257

4. Nitrogen source effect on the *Rhizophagus irregularis* – *Sorghum bicolor* symbiotic interaction

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4.1 Abstract

Arbuscular mycorrhizal (AM) can take up N in form of nitrate, ammonium or amino acid. In the extraradical mycelium of the fungi, arginine is synthesized and transported to the intraradical mycelium. There, arginine is broken down and ammonium is delivered to the plant. In this study, we assessed the effect of different N sources (without N, NO_3^-, NH_4^+, urea, glycine and arginine) on the expression of genes coding for enzymes of the N cycle and for N transporters in the AM fungus *Rhizophagus irregularis* and in sorghum (*Sorghum bicolor*) plants. Expression of the genes was significantly influenced by all the N sources and was different in the intraradical mycelium and in the extraradical mycelium. We also found that some plant genes were coregulated with fungal genes.

4.2 Introduction

Current data show that AM fungi can take up inorganic N as ammonium or nitrate (Govindarajulu *et al.*, 2005), as well as organic N in the form of amino acids (Leigh *et al.*, 2009). As a matter of fact, plant associated with AM fungi are able to take up several amino acid more efficiently than non-mycorrhized plants (Whiteside *et al.*, 2012). Once taken up by the extra-radical mycelium, N is translocated to the intraradical hyphae in form of amino acids (mainly arginine) (Govindarajulu *et al.*, 2005) and finally transferred to the plant as ammonium (Tian *et al.*, 2010). The question of the relevance of AM fungi in plant N nutrition was raised since N has a high mobility in the soil limiting the depletion zone around roots (Smith & Smith, 2011). However, some studies could show that significant amount (21 to 75%) of plant root N was derived from the fungal extraradical mycelium (ERM) (Toussaint *et al.*, 2004; Govindarajulu *et al.*, 2005; Jin *et al.*, 2005; Tanaka & Yano, 2005). Additionally, N supply can rescue the phenotype of a phosphate transporter mutant that was not able to do a functional AM symbiosis (Javot *et al.*, 2011), showing the importance of N transfer.

Interestingly, Tanaka & Yano (2005) found out that the amount of N delivered to the plant by the fungi depended on the N form supplied to the fungi. They shown that the rate of transfer was 10 times higher for NH_4^+ than for NO_3^- in maize plants mycorrhized by *Glomus aggregatum*. Another factor influencing N uptake by AM fungi is the C resource availability: an increase availability of C is related with an increased N uptake by the fungal ERM (Fellbaum *et al.*, 2012). These results indicate a possible "reward" mechanism between plant and fungi.

In fungi and in plants, enzymes involved in the assimilation of N into organic form are crucial for growth and transfer processes. Tian *et al.* (2010) described 11 fungal genes involved in the N primary metabolism and tested the expression of these genes 2h, 4h, 8h, 24h and 72h after the addition of 4 mM KNO_3 to the fungal ERM. The pattern of gene expression was different in the ERM compared to the intraradical mycelium (IRM). Indeed, a nitrate transporter (NT), two glutamine synthetase (GS1 and GS2), a carbamoyl-phosphate synthase (CPS), an arginosuccinate synthase (ASS), an arginosuccinate lyase (AL) and a glutamate synthase (GluS) were regulated in the ERM and not in the IRM after addition of KNO_3. In contrary, an arginase (ARG), two ornithine aminotransferase (OAT1 and OAT2), an ornithine decarboxylase (ODC) and an urease (URE) were regulated in the IRM and not in the ERM under the same conditions. These data indicate the production of arginine in the ERM, its transport and breakdown in the IRM to permit the transfer of NH_4^+ from the IRM to the plant.

Here, we studied the effect of different N sources on the expression of genes coding for enzymes of the N cycle and for N transporters in the AM fungus *Rhizophagus irregularis* (previously *Glomus intraradices*) and in sorghum. In *R. irregularis*, we analyzed the expression of 13 different genes coding for enzymes of the N metabolism and of four different genes coding for N transporters. In sorghum, we analyzed the expression of nine enzymes and eight transporters. Following N treatments were applied: without N, with NO_3^-, with NH_4^+, with urea, with glycine and with arginine. Our results shown that N treatment significantly influenced the expression of different enzymes and transporter genes of the N cycle and had an effect on plant growth and N content.

4.3 Material and Methods

Plant, arbuscular fungi and growth conditions

Experiments were performed with sorghum (*Sorghum bicolor* (L.) Moench), cv Pant-5. This cultivar is closely related to BTx623, the sorghum cultivar used for genome sequencing (Paterson et al., 2009). Seeds of cv Pant-5, kindly provided by sorghum breeders of I.G.F.R.I. (CCS Agriculture University of Hissar, Haryana, India) and G.B. Pant University of Agriculture and Technology (Pantanagar, Uttaranchal, India) were surface sterilized (10min in 2.5% KClO and then rinsed with sterile deionized water several times for one day) and soaked in sterile deionized water over night. Seeds were pre-germinated on autoclaved Terra Green (Oil Dri US-special, American aluminiumoxide, type III/R; Lobbe Umwelttechnik, Iserlohn, Germany) at 25°C for 24h and then grown in the dark at room temperature for 72h. The fungal strain *Rhizophagus irregularis* BEG-75 was propagated by trap cultures set up as previously described (Oehl *et al.*, 2003). To establish AM symbiosis, pre-germinated seeds were individually inoculated in 350ml pots containing a mixture of acid washed Terragreen, sand and loess soil (5:4:1 w/w/w). About 100 spores were added to the mixture. For the non-mycorrhized plants, the same amount of autoclaved inoculum was added to the mixture. To correct for possible differences in microbial communities, each pot received one ml of filtered washing of AM fungal inoculum (van der Heijden *et al.*, 1998). Plants were grown in a greenhouse with day-night temperatures of 28 and 15°C, respectively. Plants were watered twice a week during the whole experiment. From the first week on, 8ml of modified Hoagland solution was applied weekly. Five different Hoagland solutions modified after Gamborg & Wetter (1975) to obtain different N sources were prepared (Tab.4.1): "-N", "NO_3^-" , "NH_4^+", "Glycine", "Arginin e", "Urea". All treatments were independently repeated four times. A total of 40 pots were prepared.

Staining of AM fungi in plant roots and quantification of root colonization

From each analyzed plant, one subsample of 100mg of fresh roots was used to determine the degree of AM fungal colonization, as follows. Root subsamples were stained with trypan blue (0.005% w/v in lactic acid, glycerol, water, 1:1:1, w:w:w) at 60°C for 10 minutes in 15ml tubes in a water bath and destained 24h in glycerol: 1%HCl (w:w). Root colonization was quantified according to the grid intersection method as described by Brundrett *et al.* (1984). Total colonization comprised intersections containing hyphae, vesicles, spores or arbuscules. Differences between means of variables were analyzed by ANOVA ($p \leq 0.05$), using SPSS 18.0.

Samples, RNA isolation and quantitative reverse transcription-PCR

The pots were harvested 13 weeks after inoculation. Four soil subsamples of 50g were taken. Plant roots were carefully washed under tap water to remove root adherent substrate. Four subsample of 100mg of fresh roots were taken. RNA extraction from soil was done using Geneclean Turbo Kit (MP biomedicals, Santa Ana, CA, USA) according to manufacturer's instructions. RNA extraction from roots and cDNA synthesis from root and soil samples was performed as described by Courty *et al.* (2009), using the following conditions: mycorrhizal roots were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Full-length doubled-stranded cDNAs corresponding to mRNAs expressed in plant roots were obtained using the SMART–PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Quantification of AMT transcripts was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in real time quantitative PCR reactions with gene-specific primers from Tian et al. (2010) or designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Table 4.2). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C and a GC percentage > 50%. Primers used as controls or for analysis had an efficiency ranging between 90% and 110%. Target gene expression was normalized to the gene encoding the *G. intraradices* beta-tubulin (gene AY326321). Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. For data analysis, the geometric mean of the biological replicates (n = 4) was calculated.

C and N analysis

The remainder of the root samples and the shoot material were dried at 80°C for 72h and weighed. These samples were ground in 1.5 ml Eppendorf® tubes using 1.1 mm diam. tungsten carbide balls (Biospec Products, Inc., Bartlesville, Oklahoma, USA) in a Retch MM301 vortexer (Retch Gmbh and Co., Haan, Germany). Total N and C were measured using an on-line continuous flow CN analyzer coupled

with an isotope ratio mass spectrometer (ANCA-SL MS 20-20 system, Sercon Ltd, Crewe, UK).

Statistical analyses

An analysis of variance (ANOVA) was performed on the gene expression for each treatment separately, where the two latter parameters were arcsine-transformed to fit the assumption of normal distribution. The ANOVA was based on N treatments, ERM and IRM. Pairwise comparisons between the treatments were done with planned contrast analysis. Independent paired ttests were performed. A pr obability of $P \leq 0.05$ was considered to be significant.

Data were clustered by Java MultiExperiment View (MeV4.6.2) (Saeed *et al.*, 2006) with Pearson correlation and average linkage clustering.

4.4 Results and discussion

The actual pathway proposed for N uptake and transfer through AM symbiosis implicates the uptake of nitrate, ammonium or amino acid (2005; Leigh *et al.*, 2009; Whiteside *et al.*, 2012) and assimilation by the extraradical mycelium (ERM), followed by the synthesis of arginine and its transfer to the intraradical mycelium (IRM). In the IRM, enzymes for arginine breakdown are active and the resulting ammonium is transfer from the AM fungi to the plant (Tian *et al.*, 2010). This pathway is supported by labeling and enzymatic activity measurements (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005) as well as by molecular analyses. Indeed, Tian *et al.* (2010) studied the expression of genes coding for enzymes of the N cycle including two glutamine synthetase (GS1 and GS2), one carbamoyl – phosphate synthase (CPS), one argininosuccinate synthase (ASS), one arginosuccinate lyase (AL), one arginase (CAR), one ornithine decarboxylase (ODC), one ornithine aminotransferase 1 and 2 (OAT1 and OAT2), one urease (URE) and one glutamate synthase (GOGAT) and found different regulation levels of the enzymes in the ERM compared to the IRM. Here, we studied the effect of different N sources on the nutritional status of the plant as well as on the transcript regulation of genes coding for enzymes of the N cycle and genes coding for N transporters in the AM fungus *R. irregularis* and in sorghum plants. In addition to the AM fungal enzymes studied by Tian et al. (2010), we studied the expression of three nitrate reductases (NR), three ammonium transporters (AMT) and an asparagine synthtase (AspS). All fungal genes were studied in the ERM and in the IRM of the fungus. In the plant, we studied the expression of eight ammonium transporters (AMT-P) (Koegel *et al.*, 2013), three glutamate synthase (GOGAT-P), one nitrate reductase (NR-P), one aminomethyl transferase (gvcT-P), two asparagine synthase (AspS-P), two glutamate dehydrogenase (GDH-P) and one glutamine synthase (GS) (Fig.4.1).

Plant N nutrition and root length colonization: Root length colonization, dry weight and N content in *S.bicolor* plants were significantly lower in the treatment without N (-N) compared to the treatments with N (+N, NO_3^+, NH_4^-, urea, glycine and arginine) (Fig. 4.2, 4.3 and 4.4). Indeed, assimilation of N has effects on plant productivity, biomass and crop yield (Lam *et al.*, 1996).

Mycorrhized plants fed with glycine had a significantly higher N content and dry weight than mycorrhized plants fed with the other N sources (Fig. 4.3 and 4.4). These results show that the nutritional status of AM plants is influenced by the source of N added to the substrate similarly to non-mycorrhized plants were different sources of N affected growth rate, leaf sucrose phosphate synthase,

starch formation and photosynthesis (Kerr *et al.*, 1984; Raab & Terry, 1994). Interestingly, our results shown that AM plants feed with different organic N sources (Glycine or Arginine) differed significantly in their N content (Fig.4.4). This result could be explained by the fact that AM fungi do not take up different simple organic N sources with the same efficiency (Whiteside *et al.*, 2012).

Here, we did not find any differences in the N status of plants feed with different inorganic N sources (Fig. 4.4) in contrast to Ngwene *et al.* (2012) who shown that more ^{15}N was transferred to cowpea plants when the AM fungus *R. irregularis* had access to labeled nitrate compared to NH_4^+. In fact, the ^{15}N shoot/root ratio of the plants was clearly higher in the $^{15}NO_3^-$ treatments. NH_4^+ was preferentially kept in the fungal mycelium or in plant roots indicating a much lower rate of transfer in the case of NH_4^+. However, contrasting results were obtained by Tanaka & Yano (2005): they observed that the AM fungus *Glomus aggregatum* could rapidly deliver N to maize plants when it had access to NH_4^+ but not when it had access to NO_3^-. These differences in the results obtained might be due to the different AM fungi and host plant used. It raises the question of the right N source to use as fertilizer in sustainable agricultural systems.

Fungal and plant N cycle: The analysis in the IRM and in the ERM of 15 fungal genes coding for enzymes of the N cycle GS1 and GS2, CPS, ASS, AL, CAR, ODC, OAT1 and OAT2, URE, AspS, NR1, NR2, NR3, GOGAT and of genes coding for one nitrate (NT) and three ammonium (AMT1, AMT2, AMT3) transporters shown different expression pattern depending on the N source. Interestingly, the expression pattern of the analyzed genes was differing between the two organic N sources, glycine and arginine. This could be a reason for the differences in N nutritional status of the plants.

In the ERM, transcript level of OAT1 and 2, AL, CPS, AspS, NR3 and AMT1 and 2 was below the detection limit. Expression of GOGAT was not detected in the ERM and in the IRM.

Interestingly, relative expression of GS2-ERM was similar to GS2-IRM for the different N conditions (Fig. 4.5) and was coregulated with ASS-IRM and GOGAT3-P. Expression of NT was also similar in the ERM and the IRM for the different N conditions and was coregulated with CAR-ERM, CPS-ERM, OAT-ERM and NR-P (Fig. 4.5).

The analysis of ten plant genes coding for enzymes of the N cycle (GOGAT1-P, GOGAT2-P and GOGAT3-P, NR-P, gvcT-P, AspS1-P, AspS2-P, GDH1-P, GDH2-P, GS-P) and for ammonium transporters (Koegel *et*

al., 2013) revealed the down-regulation of most genes in the conditions +N (NO_3^-, NH_4^+, urea, glycine, arginine) compared to the condition –N (Fig. 4.1). Similarly, Sukanya *et al.* (1994) did not find accumulation of GS1 transcript in maize (*Zea mays*) under NH_4^+ condition. GS1-P and gvcT-P were coregulated with AMT1-1-P and AMT1-2-P and with AMT3-ERM, URE-ERM and ASS-ERM (Fig. 4.5).

In plants, AMTs were studied intensively and can be classified in two distinct subfamilies: AMT1 and AMT2 (Loque & von Wiren, 2004). The AMT1 subfamily forms a well defined group in monocots and dicots. The AMT2 subfamily can be further divided in three subclades (Suenaga *et al.*, 2003) and is more closely related to bacterial AMTs than to the AMT1 subfamily. Interestingly, AMT1-1-P and AMT1-2-P, belonging to the AMT1 subfamily, were distinctively regulated than the AMT transporters belonging to the AMT2 subfamily (Fig. 4.5).

As mentioned before, the pathway of N transport in the AM symbiosis (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005; Tian *et al.*, 2010) implies the expression of genes coding for N transport, assimilation and arginine synthesis in the ERM and the expression of genes coding for arginine and urea breakdown in the IRM. Indeed, we found a higher expression of AMT3 in the ERM and OAT1 and CPS were highly up-regulated in the IRM (Fig. 4.6). However, we also found a high up-regulation of GS1, NR1, NR2 and NR3 in the IRM compared to the ERM and URE was up-regulated in the ERM compared to the IRM (Fig.5.6). These data can be explained by the finding from Tian *et al.* (2010) that gene expression is varying strongly within some hours. Additionally, we could show that N source had an influence on gene expression. In ectomycorrhizal fungi, gene expression can also vary in the ERM compared to the Hartig net (Nehls *et al.*, 2001): in the poplar – *Amanita muscaria* symbiosis, a phenylalanine ammonia lyase gene was differentially expressed in both tissues in response to glucose treatment. Additionally, N treatment influence the gene expression of ectomycorrhizal fungi (Chen *et al.*, 2003).

AM fungi are key soil microorganisms contributing to crop productivity and ecosystem sustainability (Gianinazzi *et al.*, 2010): they do not only improve plant nutrition but also prevent erosion and increase stress resistance to drought and other factors (Smith & Read, 2008). For this reason, AM fungi are very important for sustainable agriculture. One point that agriculture has to face today is to achieve an increased production to feed the growing world population but at the same time to protect resources and to manage ecosystems in a more sustainable way. Here we could show an effect of different N

sources on the gene expression of sorghum plants and *R. irregularis* as well as on plant N status. In view of our results, the optimization of the N source could be of great interest for sustainable agriculture.

4.5 Acknowledgments

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Table 4.1: Modifications made to the Hoagland solutions after Gabor & Wetter (1975) to obtain the treatments "-N", "NO_3^-" , "NH_4^+", "Urea", "Glycine" and "Arginine".

Hoagland solution after Gabor & Wetter (1975) Solution D	"-N"	"NO_3^-"	"NH_4^+"	"Urea"	"Glycine"	"Arginine"
39.805 mMol $Ca(NO_3)_2 \cdot 4H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $Ca(NO_3)_2 \cdot 4H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$	39.805 mMol $CaCl_2 \cdot 2H_2O$
20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$	20.286 mMol $MgSO_4 \cdot 7H_2O$
65.275 mMol KNO_3	75.70 mMol KCl	65.275 mMol KNO_3	75.70 mMol KCl	75.70 mMol KCl	75.70 mMol KCl	75.70 mMol KCl
10.433 mMol $NH_4H_2PO_4$	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4	10.433 mMol KH_2PO_4
			155.318 mMol NH_4Cl	77.652 mMol CH_4N_2O	155.318 mMol $C_2H_5NO_2$	38.828 mMol $C_6H_{14}N_4O_2 \cdot HCl$

Table 4.2: Specific primers for RT-qPCR. White color indicate primer for *R. irregularis*. Grey color indicate primers for *S. bicolor.*

	Forward	Reverse	After
AMT1	TGTGTCAGCATTGTCTTCAGT	GGCAAGTGCGGGTGTAATAG	
AMT2	GTGCCAATGCCGCTAACA	GCCAGAACAGAATCCCAAAG	
AMT3	GGGCTTGACTTTGCTGGT	TTCGTCCCTTCCATGACC	
NR1	GCGCAGGAAATCGTCGTA	CGCAATGGCACACCTTTC	
NR2	GCTTCGGTACAGGTCCATGA	CGGTTGACAGGAAAACATCC	
NR3	GGAGAGGTGTTCCATTACGA	CCCGAGAATCATCCATTG	
AspS	GTTGAACGATGGATTCCAAGA	CATTAGCAACATTATCATAAGCAGA	
CAR	TGATGCGGTGAATCCTAAGAGA	GATCAAGTGCATCAACGTCAAAG	Tian et al. (2010)
ASS	GCATTGGTCGTATTGATATTGTTGA	TTGTTCCCCCAGGAGTTTCA	Tian et al. (2010)
CPS	GATCGCCGTCGTTGACTGT	CGCGCCGCGTTTAACTAA	Tian et al. (2010)
GS1	AGTGGCCTTCGTTCAAAGACTAG	CATCACCAGGTGCTTGATTAGTA	Tian et al. (2010)
GS2	CCAACATTGATCCTTATCGTGTC	CTCCTAAATTAGAGAAAGAAAAAAAGGG	Tian et al. (2010)
URE	CGCAAATGGGAGATGCAAAT	CAGCACTTGCATAATGACCAAAC	Tian et al. (2010)
GOGAT	TTCGCAGCCGGTGATTG	CGACCCTCATTTATACCCCATACA	Tian et al. (2010)
18S	TGTTAATAAAAATCGGTGCGTTGC	AAAACGCAAATGATCAACCGGAC	Tian et al. (2010)
OAT1	GGTTCGAGCGGATATTGTCATAC	AGGACTGCTGATATTGGGTAAACG	Tian et al. (2010)
OAT2	CGGGTAAGATGCTTTGTCAAGA	GCCTGAAAGTGCTTTACCAAGTATAAC	Tian et al. (2010)
ODC	TTGATTGCGTTACCAAAAATGG	TCGAAATACAACCAGTCACCAAGA	Tian et al. (2010)
AL	ACGGACTTGGCTGAATATTTGGT	GCCCCTGCAATATGATGAGTTT	Tian et al. (2010)
NT	GGCTTGCTGCGGTTCAGT	CGACTAATCCTGATATTGCACCAA	Tian et al. (2010)
AMT1-1	GCTGTGGTTCGGCTGGTA	GGACTTGAGGATGGTGGTGAA	Koegel et al. (2013)
AMT1-2	TCCATTGCTCCTCGTTGC	GGCTTTGCTCCCTCTTCC	Koegel et al. (2013)
AMT2-1	TCCCGCCCGCCTACAGCT	GTCACCATTCAGCTGTAG	Koegel et al. (2013)
AMT2-2	GCGGCTTCCTCTACCAGTG	CCTCTCCCTGTCGCTCTTC	Koegel et al. (2013)
AMT3-1	GGCCTCGTCTGCATCACT	GGGTGTCGTCCACTTGCT	Koegel et al. (2013)
AMT3-2	CCGCACGCACTCTATCTGTA	TCGCTGCTTATTGGGGTTAG	Koegel et al. (2013)
AMT3-3	CGTCATTGCCTGGAACATC	AGCATCATCCCCGATAAGC	Koegel et al. (2013)
AMT4	CGAACAACATTCTCCTGACG	CCCGAACACGAAGCAGTC	Koegel et al. (2013)
GOGAT1	CAGAACACGAACCGAGCA	CAGCAGGCAATCCACTCA	
GOGAT2	GCCCTCCCCTTCAGAGTC	CATCAGCCCTCCAATACGA	
GOGAT3	GAATGGGCTCCGTCCAG	CCAACCTCAGATGCGACA	
AspS1	CTGTTGCTTCACGGCACTTA	CTTCCTCCAAGGCATCTATCC	
AspS2	ACCCCCTCGGTTCCATAC	GAGAGCAGGACGCCAAAG	
GDH1	GCATCCGCTACCATCCTG	CGTCAACCGCTCCAACTC	
GDH2	TATGGCAACGAGGGAAGC	ATTGACGCCGCTGTTTTC	
gvcT	GCACATCACGCCAGTTGA	GCCTTCCGATTTCCTCCT	
NR	CTGCCGAGGAAATGGAAC	GTTGATGTGCGGGTGCTC	
Ubi	CAAGGAGTGCCCCAACAC	GGTAGGCGGGTAAAGCAAA	
GS	TGCCCGCTACATTCTCG	ACCCTGGATTGGCTTCG	

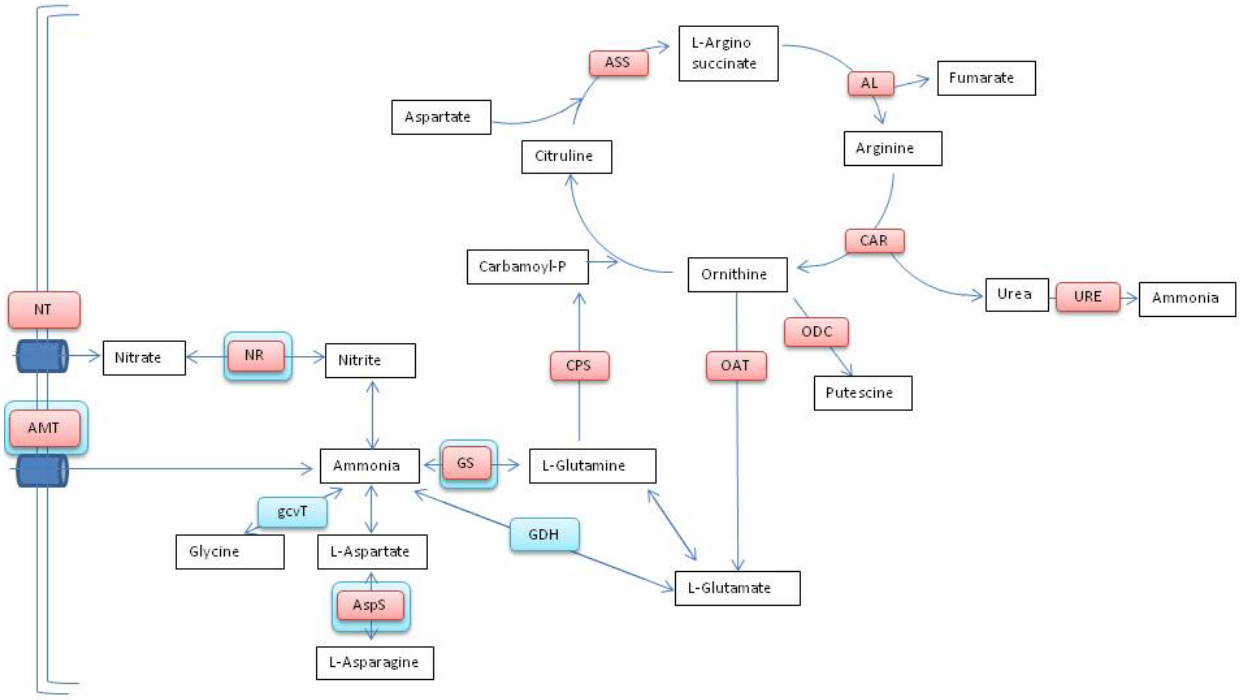

Figure 4.1. N pathway with enzymes and transporters studied in *R. irregularis* (red) and in *S. bicolor* (blue). Modified after www.genome.jp/kegg. NT, nitrate transporter; AMT, ammonium transporter; NR, nitrate reductase; GS, glutamine synthetase; GDH, glutamate dehydorgenase; AspS, asparagine synthetase; gcvT, aminomethyltransferase; CPS, carbamoyl-phosphate synthase; ASS, arginosuccinate synthase; AL, arginosuccinate lyase; CAR, arginase; URE, urease; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

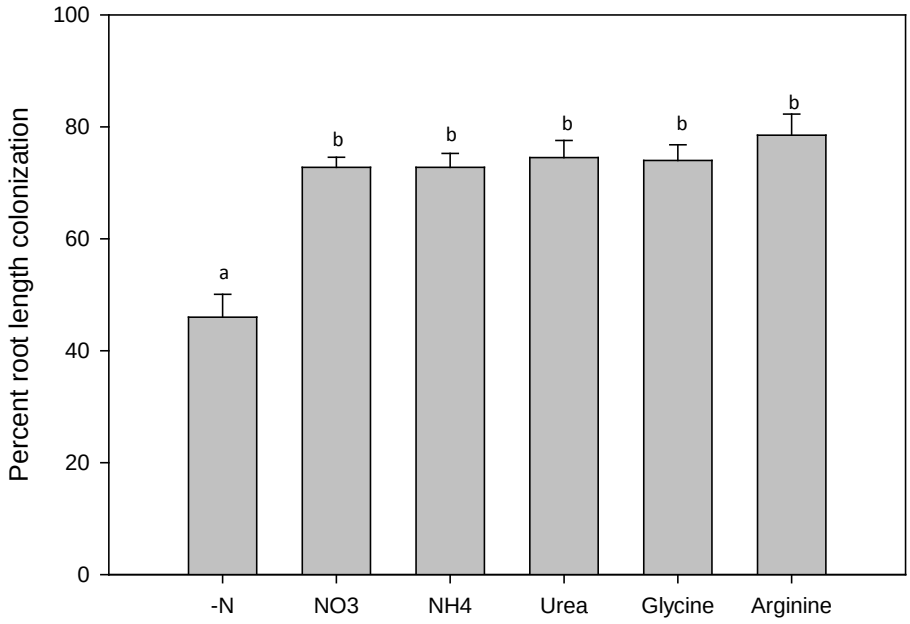

Figure 4.2. Percentage of root length colonized after 13 weeks post inoculation in plants colonized by *Rhizophagus irregularis* in the different N treatments (-N, NO_3^-, NH_4^+, urea, glycine and arginine). Values are mean of four replicates. Differences in N content between the treatments were performed with a one-way ANOVA (Scheffe's *F*-test). Letters indicate a *P*-value <0.05.

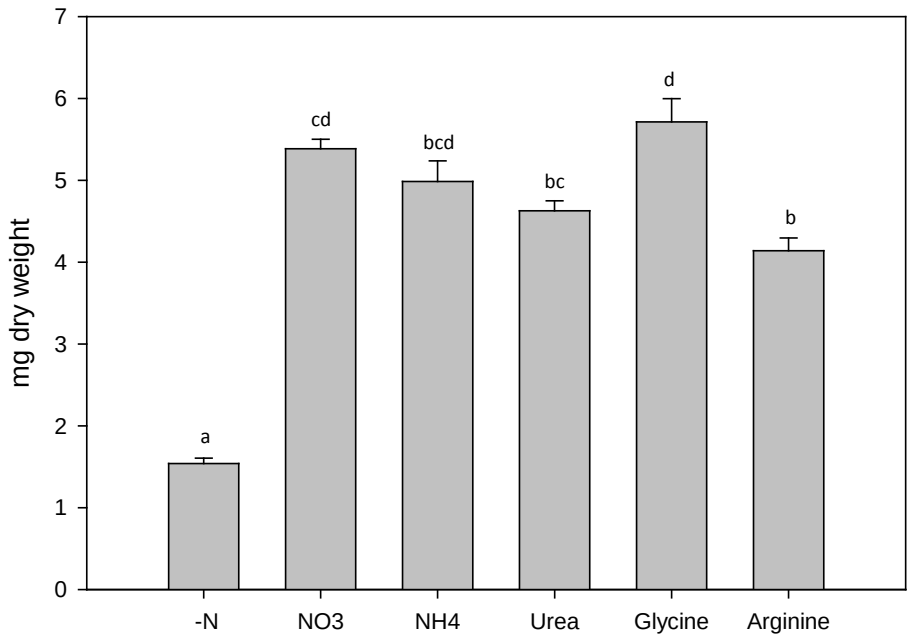

Figure 4.3: Dry weight (g) after 13 weeks post inoculation in plants colonized by *Rhizophagus irregularis* in the different N treatments (-N, NO_3^-, NH_4^+, urea, glycine and arginine). Values are mean of four replicates. Differences in N content between the treatments were performed with a one-way ANOVA (Scheffe's *F*-test). Letters indicate a *P*-value <0.05.

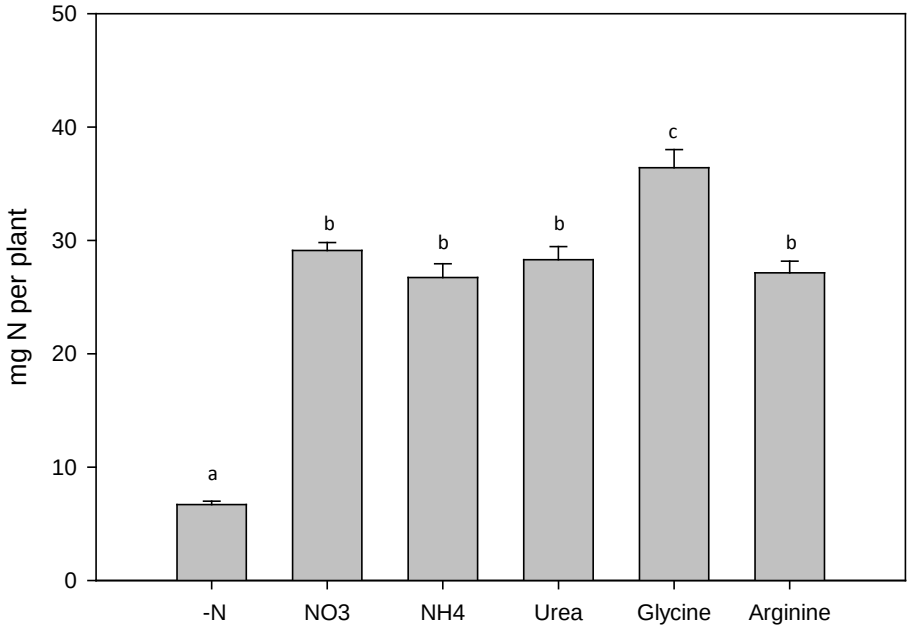

Figure 4.4: Total N content after 13 weeks post inoculation in plants colonized by *Rhizophagus irregularis* in the different N treatments (-N, NO_3^-, NH_4^+, urea, glycine and arginine). Values are mean of four replicates. Differences in N content between the treatments were performed with a one-way ANOVA (Scheffe's *F*-test). Letters indicate a *P*-value <0.05.

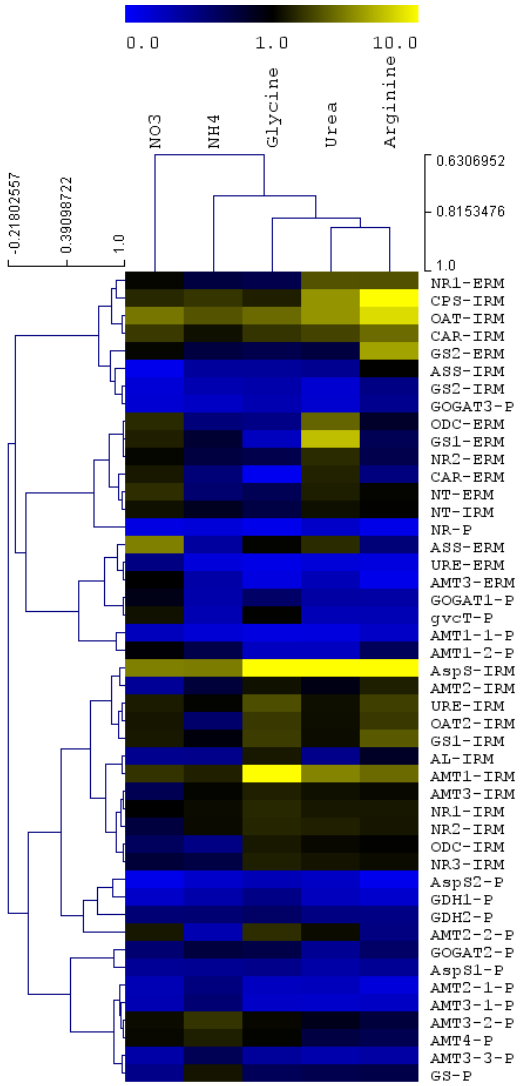

Figure 4.5. Hierarchical clustering showing the quantification by reverse transcript polymerase chain reaction (qRT-PCR) of the transcripts abundance of NT, AMT1, AMT2, AMT3,ASS, CPA, OAT1, OAT2, CAR, URE, GS1, GS2, GLO, AL, APS, UDC, NR1, NR2, NR3 in the IRM and in the ERM of *Rhizophagus irregularis* and GOGAT1, GOGAT2, GOGAT3, AspS1, AspS2, GDH1, GDH2, gvcT, NR, GS, AMT1-1, AMT1-2, AMT2-1, AMT2-2, AMT3-1, AMT3-2, AMT3-3, AMT4 in the plant (-P) in the different nitrogen (N) treatments (-N, NO_3^-, NH_4^+, urea, glycine arginine). The values are the means of four replicates. 18S was used as the reference transcript for the fungus. Ubiquitin was used as the reference transcript for the plant. Gene expression was normalized to the –N treatment.

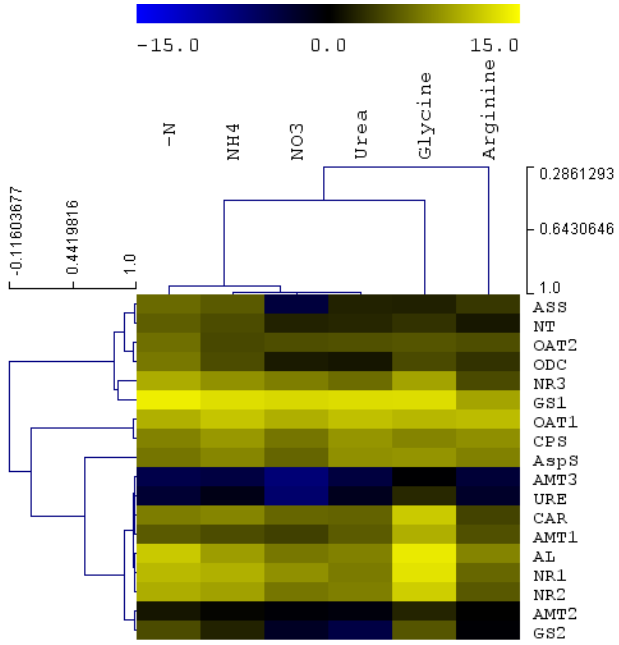

Figure 4.6. Hierarchical clustering showing ratio IRM/ERM of the quantification by reverse transcript polymerase chain reaction (qRT-PCR) of the transcript abundance of NT, AMT1, AMT2, AMT3,ASS, CPA, OAT1, OAT2, CAR, URE, GS1, GS2, GLO, AL, APS, UDC, NR1, NR2, NR3 in the different nitrogen (N) treatments (-N, NO_3^-, NH_4^+, urea, glycine and arginine). The values are the means of four replicates. Beta-tubulin was used as the reference transcript. Data are log2 transformed.

5. Rapid nitrogen transfer in the *Sorghum bicolor* – *Glomus mosseae* arbuscular mycorrhizal symbiosis

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5.1 Abstract

We have recently described two ammonium transporters in *Sorghum bicolor* roots that are induced in the symbiosis with arbuscular mycorrhizal fungi. To study the role of ammonium transport in this symbiosis, we studied the transfer of ^{15}N derived from ammonium in the soil to *Sorghum bicolor* plants via *Glomus mosseae* fungal mycelium, using compartmented microcosms. The hyphal compartment contained a source of $^{15}NH_4^+$, which was not accessible to the roots in the plant compartment. We found an unexpected velocity and efficiency of symbiotic N transfer: ^{15}N was present in plant roots and leaves already 48 hours after exposition of the arbuscular mycorrhizal fungi to $^{15}NH_4^+$. Our data support the idea that the arbuscular mycorrhizal symbiosis contributes in an important way to plant nitrogen nutrition.

5.2 Keywords

Nitrogen, mycelium, arbuscular mycorrhizal fungi, transfer, sorghum

5.3 Main text

We are interested in the arbuscular mycorrhizal symbiosis(Smith & Read, 2008; Smith & Smith, 2011), using sorghum (*Sorghum bicolor*) and *Glomus mosseae* as a model. Recently, we described the expanded family of ammonium transporters (AMTs) in sorghum (Koegel *et al.*, 2013). We shown that two AMTs, *SbAMT3;1* and *SbAMT4*, are up-regulated in mycorrhized plants and that *SbAMT3;1* is localized at the plant-fungus interface. We wondered about the biological significance of ammonium transport in the symbiosis.

Indeed, nitrate and ammonium, the two main sources of inorganic N present in the soil, have a high mobility, limiting the depletion zone around the roots (Smith & Smith, 2011). Nevertheless, different studies estimated that AM fungi delivered between 30% and 42% of the N taken up by the plant (Mader *et al.*, 2000; Govindarajulu *et al.*, 2005). In addition, AM fungi could play an important role in N nutrition under drought stress conditions or in marginal soil were nutrient supply is reduced, (Smith & Read, 2008). This may be particularly relevant for the symbiosis that we study, since sorghum can grow under more arid conditions than most other grain crops, and makes it an existential source of food, feed, or fiber for many farmers living in the semi-arid tropics of Africa, Asia and South America (Paterson *et al.*, 2009).

To determine the importance of AM fungi in the sorghum N nutrition, we set up an experiment using compartmented microcosms (Figure 1) where one plant and one hyphal compartment are connected, but separated by two 21µm nylon mesh unsheathing an air gap. The two compartments were filled with sterile (120°C, 20 min) growth substrate consisting of a mixture of Terragreen (American aluminum oxide, oil-dry U.S. special, type III R, 0.125 mm; Lobbe Umwelttechnik), sand (quartz sand from Alsace, 0.125–0.25mm; Kaltenhouse), and Loess from a local site (5:4:1, w/w/w). Sorghum (*Sorghum bicolor* (L.) Moench), cv Pant-5 seedlings were inoculated with a 2-g (approximately 100 spores) inoculum of *G. mosseae* ISCB13, or with 2 g of sterilized (120°C, 20 min) inocula as a non-mycorrhizal control. In the center of the hyphal compartment, a 21µm nylon mesh bag of 15ml was inserted and kept empty before labeling. Twelve weeks after inoculation, the nylon mesh bag was filled with 13g of sand including 10mg

of ^{15}N ammonium sulfate (Cambridge isotope Laboratory). Two ml of water was added to wet the labeled sand without inducing mass flow. The microcosms were watered with distilled water twice a week. In addition, every week, the compartments were amended with 8 mL of a Hoagland solution (Koegel *et al.*, 2013). Plants were grown under controlled conditions (16 h of light at 28°C and 8 h of dark at 15°C, constant relative aerial humidity of 65%).

A total of 40 microcosms were prepared. Four compartmented microcosms were harvested separately 0, 12, 24, 48 and 72 hours after labeling. From the plant compartments, roots, green parts of the leaves and wilted, yellow leaves were harvested separately. In the mycorrhized systems, the AM fungal mycelium was harvested both in the plant and in the hyphal compartment. Total amount of N and of ^{15}N were measured using a ThermoFinnigan FlashEAseries 1200 elemental analyzer. Liberated carbon dioxide and nitrogen were cleaned and dried through a series of chemical traps and separated by gas chromatography. Nitrogen isotopes were determined using a ThermoFinnigan DeltaV Advantage Continuous-Flow IRMS. Sample data was corrected versus internal standards EDTA (-1.1 per mil) and ammonium oxalate (+32.7 per mil), both of which had previously been calibrated against international standards.

No ^{15}N was transferred to the non-mycorrhized plants (Supplementary Tab.1). In contrary, we found ^{15}N already after 24 hours in the AM fungal mycelium from both the hyphal and from the plant compartment, indicating that the AM fungi were able to exploit the newly added ammonium very rapidly (Fig.2, Supplementary Tab.1). Mycorrhized plants received ^{15}N rapidly as well: the percentage of ^{15}N was significantly higher in the mycorrhized compared to the non-mycorrhized plant systems ($p=0.00$, $F=40.83$) after 48 hours (Supplementary Tab.1). The ^{15}N label was present both in roots and in green leaves, indicating a substantial transfer to the entire plant, except to the wilted yellow leaves, which did not display a ^{15}N signal (Fig. 2 and 3, Supplementary Tab. 1). ^{15}N concentration increased between 48 and 72 hours, indicating a continuous transfer (Fig. 2, Supplementary Tab. 1).

Interestingly, percentage of ^{15}N from total N was higher in fungal mycelium than in the plant. However, plant tissues (except the wilted yellow leaves) contained more μg ^{15}N than the fungal mycelium confirming the accumulation of ^{15}N in the plant tissues (Fig. 3).

Our data underline the effectiveness of AM fungi in the absorption of ammonium and in symbiotic N transfer. It remains to be seen what the role of the plants' AM-inducible ammonium transporters have in this process. AM fungi are able to take up N is form of nitrate, ammonium or amino acids from the soil, far away from the plant (Govindarajulu *et al.*, 2005; Leigh *et al.*, 2009), and they are believed to transfer it to the arbuscules, through the extraradical mycelium, in the form of arginine (Tian *et al.*, 2010). There, they may produce ammonium and deliver it to the plant, which is then taken up by the plants' AM-inducible ammonium transporters at the symbiotic interface.

5.4 Acknowledgments

This project was supported by the Swiss National Science Foundation (grant nos. 130794 to A.W. and PZ00P3_136651 to P-E.C).

Figure 5.1: Compartmented microcosms. One plant and one hyphal compartment are separated by two 21µm nylon meshes and an air gap to prevent mass flow. A 21µm nylon mesh bag of 15ml was inserted in the center of the hyphal compartment and filled with 13g of sand including 10mg of ^{15}N ammonium sulfate twelve weeks after inoculation.

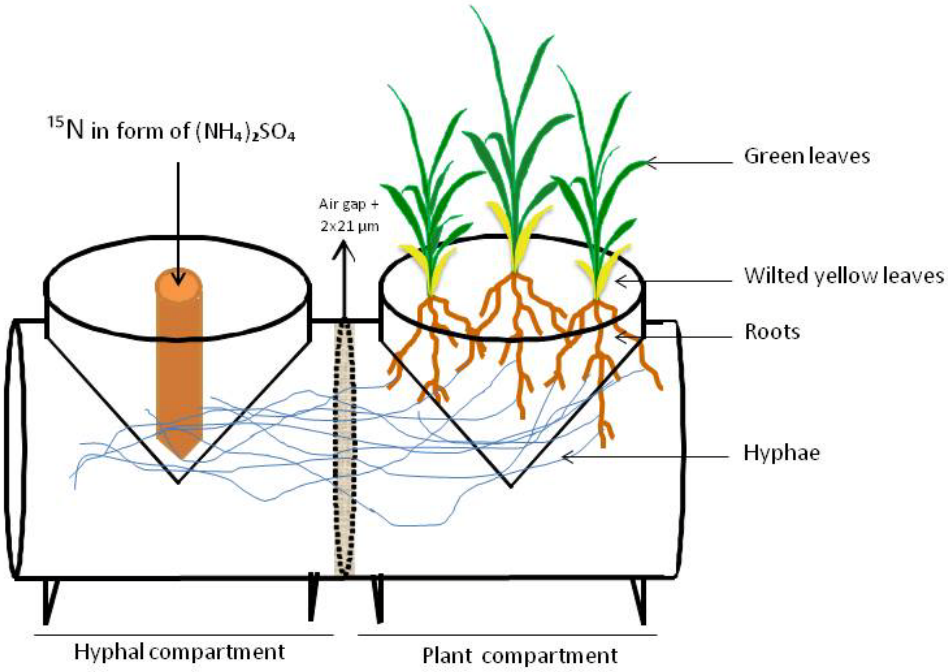


Figure 5.2: Time course experiment on the transfer of $(^{15}NH_4)_2SO_4$ as N source from the soil to *Sorghum bicolor* plants via *Glomus mosseae* ISCB13 mycelium.

^{15}N was measured in different tissues after 0, 12, 24, 48, and 72 hours of labeling: roots, green leaves and wilted, yellow leaves of plants as well as in the AM fungal mycelium from the plant and from the hyphal compartment. Values are the means of four replicates.

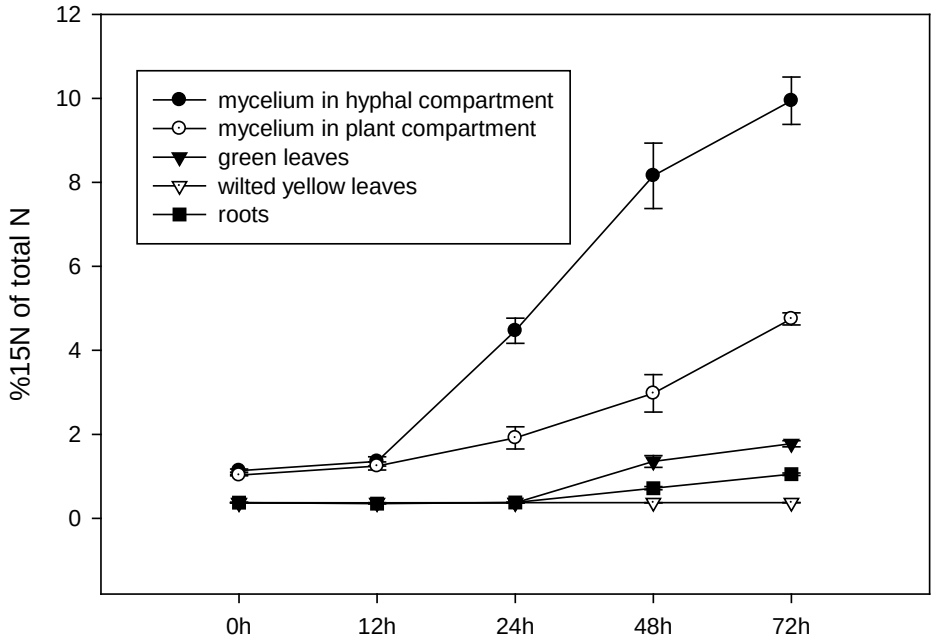
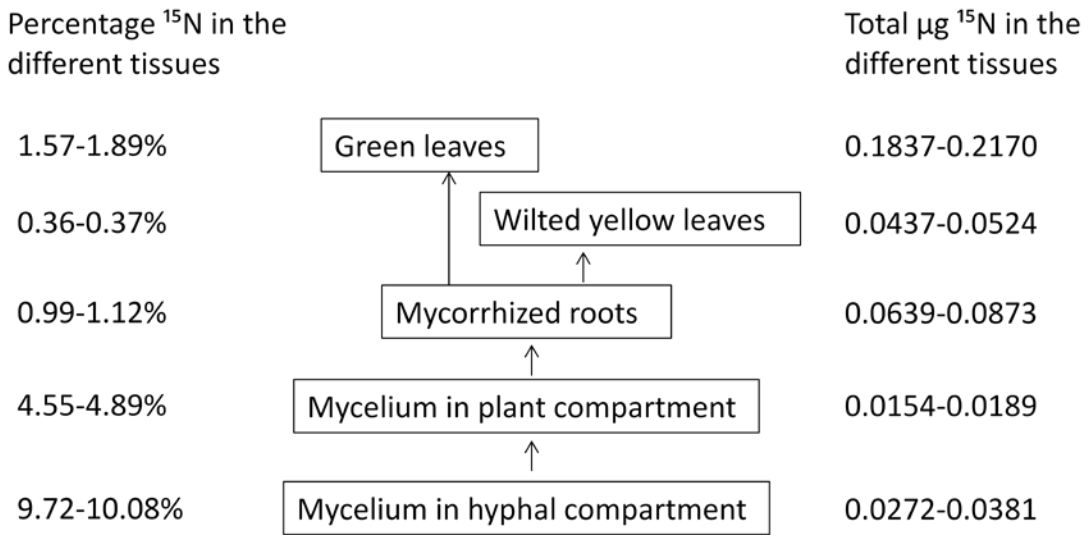


Figure 5.3: Percentage of ^{15}N and total ^{15}N (µg) in the different tissues of mycorrhized sorghum plants harvested in the compartmented microcosms after 72h of labeling.

Values are range of four replicates.



	Hyphae		Roots		Green leaves		Wilted yellow leaves	
	Hyphal compartment	Plant compartment	-Myc	+Myc	-Myc	+Myc	-Myc	+Myc
0h	1.13 (a)	1.03 (a)	0.36 (a)	0.37 (a)	0.36 (a)	0.36 (a)	0.37 (a)	0.37 (a)
12h	1.35 (a)	1.24 (a)	0.37 (a)	0.35 (a)	0.36 (a)	0.36 (a)	0.37 (a)	0.37 (a)
24h	4.46 (b)	1.91 (ab)	0.36 (a)	0.37 (a)	0.36 (a)	0.36 (a)	0.37 (a)	0.37 (a)
48h	8.15 (c)	2.97 (b)	0.37 (a)	0.71 (b)	0.36 (a)	1.39 (b)	0.37 (a)	0.37 (a)
72h	9.94 (c)	4.75 (c)	0.36 (a)	1.05 (c)	0.36 (a)	1.77 (c)	0.37 (a)	0.37 (a)

Supplementary table 5.1: Percentage of ^{15}N of total N in plant and AM fungal mycelium.

Values are the means of four replicates. Differences in relative gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F test). Different letters indicate significant differences between the harvesting time points within one treatment.

6. General discussion

6.1 Ammonium uptake mediated by AM fungi in agroecosystems

N is a key element for plants and microorganisms' growth but its mineral forms (ammonium and nitrate) are often limited in ecosystems. For this reason, microorganisms such as AM-fungi developed the capability to rapidly absorb N. However, in agro-ecosystems, microorganisms have a lower activity and biomass due to the absence of available C and cannot trap all the N added to the substrate (Kuzyakov & Xu, 2013). In this context farmers also often overfertilize field to improve plant growth. Reduced microorganisms activity and overfertilization result in N leakage which has negative effects on the environment such as groundwater pollution or soil degradation (Tilman *et al.*, 2002). Here, the use of AM fungi could reduce the problem by making N present in the soil more rapidly available for the plant, thus reducing the need of N fertilizers. Interestingly, in **chapter 4**, we shown that different N sources had an impact on the gene expression of the fungi and on the dry weight of the plants. These data raise the question of the useful N source for a sustainable agriculture system to fully exploit AM fungi uptake and transfer capabilities improving plant growth under low-input systems. Indeed, AM fungi can take up different forms of N like nitrate, ammonium or amino acids (Govindarajulu *et al.*, 2005; Whiteside *et al.*, 2012) and recent studies have shown that the assimilated N was transformed to arginine in the ERM of the fungi to be transferred from the ERM to the IRM. In the IRM, arginine was brokendown to allow the transfer of ammonium from the fungi to the plants (Tian *et al.*, 2010). The presence of AM-inducible AMTs localized at the plant – fungus interface (**chapter 2**) confirm these findings. However, the transfer of N in form of nitrate between AM fungi and plants is not excluded. Ngwene *et al.* (2012) hypothesized two different N pathways for N transfer from the AM fungi to the plants: one for ammonium and one for nitrate. The authors suggested that nitrate, in contrast to ammonium, might not be assimilated to organically bound N but rather directly transferred to the plant. This way of N transfer would be much faster and more efficient. To confirm this hypothesis, regulation of nitrate transporters in plants under different N treatments should be assessed. Additionally, the form of N present in the ERM, in the IRM and in mycorrhized roots should also be analyzed under different N treatments. These data could help to manage the use of N fertilizer in agriculture. In view of the potential benefits of AM fungi, efforts

have to be made to lower the negative impact of intensive agricultural practices and plant selections on these fungi (Oehl *et al.*, 2003; Kiers *et al.*, 2007).

6.2 Evolution of AM-inducible transporters: Comparison of AMTs and Phts

Beside N, a main nutrient taken up and transferred by AM fungi to their plant partner is phosphorus (P) (Smith & Read, 2008). Interestingly, evolutionary characteristics of AM-inducible AMTs and Phts are very similar: PT11 type proteins of the PHT1 family form a monophyletic group within mycorrhizal angiosperms indicating functional conservation across the monocots and the dicots (Yang *et al.*, 2012). The mycorrhizal function of PT11 might have evolved later or was alternatively lost in some lineages as plants like *Physcomitrella patens* have a PT11 protein but are not capable to form mycorrhizal symbiosis (Yang *et al.*, 2012). Similarly, AMT3;1 type protein of the AMT3 cluster also forms a monophyletic group within mycorrhizal angiosperms (Fig. 1.5). The branch point between the five monocots AMT3;1 and poplar and soybean AMT3;1 demonstrates that these transporters share an ancient common ancestor. These findings attest from the evolutionary antiquity of proteins involved in symbiotic nutrient transfer. For the AMT3;1, the presence of mycorrhizal function in a common ancestor and its later lost in poplar appear as a credible hypothesis.

More recently, the Poaceae acquired a second AM-inducible Pht1, PT13. It was found to be up-regulated upon colonization by AM fungi in rice, *B. distachyon* and sorghum (Hong *et al.*, 2012; Yang *et al.*, 2012; Walder *et al.*, in Preparation). For this reason, a similarity in its function with PT11 can be assumed. Similarly to PT13 proteins found in the Poaceae, the AMT4 proteins are clustering together and their expression is induced by AM fungi showing the presence of this protein in a common ancestor of these plants. A related function of AMT4 proteins in the different Poaceae species can also be assumed.

The question of the redundancy of AM-inducible Phts has been raised since mutants lacking one AM-inducible Pht in tomato did not show any AM-related phenotype (Nagy *et al.*, 2005). However, Yang *et al*. (2012) shown that the two AM-inducible Phts in rice had no redundant function. For AM-inducible AMTs, to my knowledge, no similar studies have been done. However, our results on the expression of AMT3;1 and AMT4 in different plant tissues and our characterization of the transporters in yeast (**chapter 2**) shown that both transporters have different expression pattern and different affinities for

ammonium transport, an indication for non-redundant function in the plant. By testing amiRNA mutants of OsAMT3;1 and OsAMT4, the two AM-inducible AMTs in rice (**appendix III**), we will map the precise function of both transporters and answer the question of their redundancy.

6.3 The role of reciprocal nutrient exchange for a functioning AM symbiosis

The main function of AM symbiosis is the reciprocal exchange of nutrients between plants and their fungal partners. AM fungi are obligate symbionts and receive all the C they need from their plant partners (Smith & Read, 2008).

In the other direction, plants can take up nutrients directly with their roots and do not obligatorily need AM fungi. However, AM fungi are able to search a much bigger surface for nutrients and their very thin hyphae also permit them to enter smaller soil pores than plant roots (Smith & Read, 2008). For this reason, it is of advantage for a plant to associate with AM fungi.

The question of the mechanism regulating AM symbiosis and of the importance of reciprocity in resource exchange has been raised. On one hand, the nutrient exchange doesn't have to be reciprocal to allow functional AM symbiosis (Walder *et al.*, 2012). On the other hand, a minimal delivery of P seems to be required for regular AM colonization. Indeed, rice plants lacking PT11, the Pht responsible for symbiotical P transfer in rice, shown a very reduced colonization and arbuscule level (Yang *et al.*, 2012) similarly to *M. truncatula* mutants lacking the AM-inducible Pht, MtPT4 (Harrison *et al.*, 2002b; Javot *et al.*, 2007). Reciprocal rewards also seems to stabilize AM symbiosis: plants and AM fungi appear to detect and react to changes in nutrient transfer (Kiers *et al.*, 2011).

Our results shown that AM inducible AMTs evolved in cereal plants at about the same time than AM inducible Phts. Their functionality was confirmed by yeast characterization and computer simulation (**Chapter 2 and 3**). However, nothing is known about their role for the establishment of AM symbiosis. Thus, like AM-inducible Phts, AM-inducible AMTs might be crucial for arbuscule development and maintain inside plant cortical cells. We hypothesize that mutants lacking one AM-inducible AMT will have an impaired AM fungal colonization leading to a reduced or an absence of AM fungal mediated nutrient uptake. This hypothesis is supported by the fact that N depletion can recover the phenotype of the *M. truncatula* mutant *mtpt4*: arbuscule lifespan is similar in the mutant compared to the wild type in condition of low N supply (Javot *et al.*, 2011). In contrast, in non-mutant cereal plants, we could show

that N treatment had no effect on root length colonization and on arbuscule level after 13 weeks. The expression of AM-inducible AMTs was also not modified. However, the combined effect of low N and low P on the expression of AM-inducible AMTs and its impact on the establishment of AM symbiosis would be of great interest here. Actually, it is well known that the deficiency of one mineral nutrient can influence the uptake of other nutrients (Schachtman & Shin, 2007). As shown by Wu *et al.* (2003), P starvation reduced photosynthetic activity, which affected C reduction and N assimilation. Thus, after 24h of P starvation, expression of genes for nitrate reductase or glutamine synthetase was repressed (Wu *et al.*, 2003). Similarly, P starvation of the plant combined with its N status could have an effect on the expression of AM-inducible AMTs.

6.4 Conclusion and outlook

In this work we focused on N transfer during AM symbiosis (**chapter 5**) and on the regulation of AM-inducible AMTs (**chapter 2 and 3**). Here, micro RNA naturally present in plants could play an important role in the regulation of nutrient transfer during AM symbiosis as well as in the regulation of AM-inducible transporters. For this reason, it would be interesting to assess all the micro RNA present in mycorrhized and non-mycorrhized plants under different N conditions. A special focus could be put on the role of miR399 and miR530, two micro RNA implicated in nutrition pathways (Cai *et al.*, 2012).

Furthermore, our study revealed two AM-inducible AMTs in sorghum, maize, rice, *S. italica* and *B. distachyon*. These transporters might be important for symbiotic N transfer. To test this hypothesis, the capability of the mutants lacking one AM-inducible AMT to transport N from a labeled source to the plant should be studied. A kinetic of the transport in the mutant compared to the wild-type could also bring interesting inputs. In overall, these data would provide very helpful information on the function of the different AM-inducible AMTs and on the importance of such transporters for the AM symbiosis.

The main aspect of this work was the symbiosis between plants belonging to the Poaceae and AM fungi belonging to the Glomeromycota. Many plants belonging to the Poaceae are of agricultural interest and build the principal source of food for people around the world. However, intensive agricultural practices have negative impact on the environment. To meet the increasing global food demand, achieve ecological goals and face the global warming, it is important to find sustainable strategies maintaining

high plant yield under low fertilizer input. AM fungi are one possibility to achieve this goal: their capability to rapidly absorb N from the soil and to link different plants together (Smith & Read, 2008), thus promoting plant facilitation is of great interest. Indeed, it was shown that legumes fixing N in symbiosis with rhizobia can provide N to other plants *via* a common mycorrhizal network (Martensson *et al.*, 1998). Particularly, the association between legumes fixing N and grasses, a mixed-culture model often used in traditional agriculture, could be a solution to the overfertilization and should be more intensively studied. Here we focused on the transfer of N *via* AM fungi and mapped important aspect of this transfer reveling the potential of AM fungi for plant N nutrition. Transfer of N is only one aspect of the mycorrhizal symbiosis. However this aspect was long underestimated and might play a very important role in the view of a more sustainable agriculture.

Appendix I : Paper on sorghum phosphate transporters

Expression of phosphate transporter genes in *Sorghum*: Developmental aspects and regulation in a common mycorrhizal network

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Abstract

- Arbuscular mycorrhizal (AM) fungi are important plant symbionts, trading mineral nutrients beyond the reach of roots, in particular inorganic phosphate (Pi), in exchange to their hosts' photosynthetic products.
- In a mixed-culture between flax (*Linum usitatissiumum*) and sorghum *(Sorghum bicolor*), flax took up much more of the Pi delivered by the common mycorrhizal network (CMN) than sorghum, although sorghum invested much more carbon into the CMN than flax (Walder *et al.*, 2012). Is this difference in Pi uptake due to differential regulation of Pi transporters in the two host plants?
- A baseline was first established by identifying all eleven and nine members of the Pi transporter family Pht1 in sorghum and flax, respectively. Two Pht1 sorghum (*SbPT10* and *SbPT11*) and four flax (*LuPT2*, *LupT5* and *LuPT8*/*LuPT9*) transporters were expressed in the roots in the presence of AM fungi. When expression of the mycorrhiza-inducible Pi transporters was studied in mono-cultures or mixed-cultures of flax and sorghum, it turned out that the expression of AM-inducible *Pht1* genes was only weakly related to mycorrhizal Pi uptake, but that it was differentially regulated depending on the fungal partner and the co-cultured plant.
- We conclude that in both sorghum and flax, expression of AM-inducible Pht1 transporters is initiated by arbuscule formation, but that the differential Pi delivery by the CMN is not directly dependent on differential Pht1 gene expression in the two host plants.

Introduction

Phosphorus (P) is an essential macronutrient playing a central role in developmental and metabolic processes in plants, and one of the most important growth-limiting factors in many natural and agro-ecosystems (Marschner, 1995). The primary source of P for plants is inorganic orthophosphate (Pi). Pi concentrations in the soil solution are very low (1-10 µM) and rapidly drop to sub-micromolar levels at the root-soil interface, where a narrow depletion zone is generated because of the highly efficient Pi uptake systems of the roots, combined with the extremely slow diffusion of Pi in the soil solution (Hinsinger *et al.*, 2005; Richardson *et al.*, 2009). To overcome this limitation, Pi fertilizers are used extensively in agriculture. However, readily available P may be exhausted at the end of this century; therefore, it is a major challenge for plant research to increase P acquisition from the soil in a sustainable way (Vance *et al.*, 2003; Cordell *et al.*, 2009).

Since cellular Pi concentrations are in the millimolar range, direct Pi uptake from the soil solution requires an energy dependent transport system; in the plant root, this function is fulfilled by the Pi/H^+ symporters belonging to the *Pht1* gene family, which are fueled by the protein gradient established at the plasma membrane (Rausch & Bucher, 2002). Indeed, many *Pht1* genes are strongly expressed in the rhizodermal cells, including root hairs and in cortical root cells, indicating a role in direct Pi uptake from soil (Daram *et al.*, 1998; Chiou *et al.*, 2001; Ai *et al.*, 2009). But expression has also been observed in leaves and inflorescences, indicating additional roles of Pht1 transporters such as remobilization of Pi from leaves (Rae *et al.*, 2003), or Pi uptake in the elongating pollen tube (Mudge *et al.*, 2002). *Pht1* genes were often over-expressed when plants were P limited (Bucher, 2007).

In natural ecosystems, plants use a series of adaptations to increase the acquisition of poorly available P, of which the symbiosis with arbuscular mycorrhizal fungi (AMF) is one of the most important. AMF are soil fungi of the phylum Glomeromycota forming symbiotic association with about 80% of terrestrial plants (Parniske, 2008). AMF are not host specific; as a consequence, they may form common mycorrhizal networks (CMNs), colonizing simultaneously several plants from the same or different species (Bever *et al.*, 2010). For mycorrhizal plants, AMF play a crucial role in Pi acquisition, accounting

for up to 90% of their P requirements (van der Heijden *et al.*, 2008). AMF, with their extraradical hyphae, act as functional extensions of the root system and can access nutrients such as Pi much beyond the root-determined depletion zone (Richardson *et al.*, 2009). In CMNs, some plants may increase their access to Pi by exploiting the CMN more efficiently than the interconnected plants (Zabinski *et al.*, 2002; Walder *et al.*, 2012).

Plants engaged in a the arbuscular-mycorrhizal (AM) symbiosis can absorb Pi through two different pathways; (1) directly *via* the rhizodermis, including the root hairs, through high-affinity plant Pht1 transporters, as described above, and (2) indirectly *via* the AM symbiosis. The indirect mycorrhizal pathway begins with Pi uptake by high-affinity fungal Pht1 transporters expressed in the extraradical hyphae (Smith & Smith, 2011). Pi is then translocated towards the roots and released from the arbuscule, by unknown mechanisms, to the periarbuscular space. There, it is taken up across the plant's periarbuscular membrane, most likely by AMF-inducible Pht1 transporters of the plant (Smith & Smith, 2011). Such AMF-inducible Pi transporters have been identified in many plant species of monocots and dicots (Javot *et al.*, 2007), including perennial trees (Loth-Pereda *et al.*, 2011). They all belong to the family of Pht1 transporters but cluster in two different subgroups, named subfamily I and III (Bucher, 2007). Most members of subfamily I are only expressed in arbuscule-containing cortical cells during AM symbiosis, as revealed by immunolocalization (Harrison *et al.*, 2002a; Javot *et al.*, 2007; Tamura *et al.*, 2012). AM-induced *Pht1* genes of subfamily III were found to be expressed more generally in plant roots, but specifically induced in the cortical cells during AM symbiosis (Rausch *et al.*, 2001; Maeda *et al.*, 2006). Studies of mutants with reduced expression of these two types of AMF-inducible *Pht1* genes revealed that both were important for symbiotic Pi uptake (Maeda *et al.*, 2006; Javot *et al.,* 2007). Recent investigations on the promoter region of AM-inducible *Pht1* genes revealed that at least two cis-regulatory elements, the mycorrhiza transcription factor binding sequence (MYCS) and P1BS, mediate the transcriptional activation of these Pi transporters (Chen *et al.*, 2010).

The study presented here is based on our recent finding that flax (*Linum usitatissimum*) and sorghum *(Sorghum bicolor*), connected by a CMN, had rather different terms of trade with their AMF partners

(Walder *et al.*, 2012). In particular, flax took up more than ten times more of the Pi delivered by the CMN than sorghum, although sorghum invested much more carbon into the CMN than flax. Was this difference in Pi uptake due to differential regulation of Pi transporters in the two host plants?

To study this question, we first established a comprehensive genome-wide inventory of Pi transporters of the *Pht1* gene family in sorghum, based on its the genome sequence (Paterson *et al.*, 2009), and identified eleven *Pht1* transporter genes and their corresponding transcripts. We also assessed the genome organization and the evolutionary history of sorghum *Pht1* genes, along with their expression patterns in various tissues. We then considered the expression of the transporters in the presence or absence of mycorrhizal fungi, and low or high concentrations of available Pi. Additionally, we identified nine Pht1 transporters of flax based on its the genome sequence (Wang, Z *et al.*, 2012), two of which were AM-inducible. With these tools at hand, we examined the expression of the Pht1 transporters of flax and sorghum when they were engaged in a CMN with a nonspecific plant, as a model for a mono-culture, or a heterospecific plant, as a model for a mixed-culture (Walder *et al.*, 2012). The strong asymmetries in Pi partitioning between interconnected flax and sorghum plants, which were also influenced by the identity of the fungus forming the CMN (either *Glomus intraradices* or *G. mosseae*) were not reflected by correspondingly different expression levels of the Pht1 transporters, implying that posttranscriptional regulatory phenomena must be involved in the different terms of trade between the symbiotic partners.

Materials and methods

Plant growth conditions and experimental set-up

Seeds from *Sorghum bicolor* (cultivar Plant Chari 5) were surface sterilized (10min in 2.5% KClO) and then rinsed with sterile deionized water several times during one day) and soaked in sterile deionized water over night. Seeds were pre-germinated on autoclaved sand (Quartz sand of Alsace, 0.125 – 0.25 mm, Kaltenhouse, France) for 72h in the dark at room temperature. To establish AM symbiosis, pre-germinated seedlings were planted individually in 350ml pots containing a mixture of sand, acid washed Terragreen (American aluminum oxide, oil dry US special, type III R, 0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany) and Loess from a local site (4:1:1 [wt/wt/wt]) and were inoculated at the same time with 2 g AMF inoculum containing about 100 spores. Inocula of the two fungal strains, *Glomus intraradices* "TERI commercial" (Mathimaran *et al.*, 2008) and *G. mosseae* ISCB 22 (Botanical Institute, University of Basel, Switzerland), were prepared as previously described (Oehl *et al.*, 2003). For the non-mycorrhizal plants, the same amount of autoclaved (120°, 20 min) inoculum was added. To correct for possible differences in microbial communities, each pot received 5 ml of filtered washing of AMF inoculum (Koide & Elliott, 1989). The pots were watered weekly with deionized water. Plants were grown in a greenhouse. Night temperature was set to 18°C and day temperatures varied between 23 and 30°C depending on weather conditions. Additional lighting was provided with high-pressure mercury vapor lamps (Philips HPL-N, 400 W) to a day length of 16 h per day.

To study the effect of phosphorus on gene expression, 8ml of full or P-free Hoagland Solution (with or without the component $NH_4H_2PO_4$) was applied weekly (Zabinski *et al.*, 2002) from the first week. The experiments were set up in a randomized block design with four replicates yielding in a total of 24 pots.

For the studies on the gene expression in mono-culture or mixed-culture, the plant material was taken from the microcosms described previously, in which sorghum and flax plants were raised in separate compartments, connected by a common hyphal compartment (Walder *et al*., 2012).

Staining of AMF in plant roots

Trypan Blue staining was used to identify mycorrhizal structures inside the root (Phillips & Hayman,

1970). The percentage of root length colonized by hyphae, arbuscules and vesicles was estimated for each sub-sample by a modified line intersection method (McGonigle *et al.*, 1990). A minimum of 50 line-intersections per root sample was scored for AMF structures.

DNA isolation from field samples and fungal diversity analyses

Diversity of AMF species associated to sorghum roots harvested in a field site (northeastern France; 47°62'N, 7°52'E; September 2011) was assessed. For each of the three plants, two subsamples (about 100mg) of fresh roots were snap frozen and stored at -80°C. DNA was extracted and analyzed as described earlier (Courty *et al*., 2011). Briefly, after isolation of DNA with the NucleoSpin tissue KS kit (Macherey-Nagel), the ITS region of nuclear ribosomal DNA was amplified on a T3 thermocycler (Biometra), and the amplified fragments were then sub-cloned using the TOPO-TA cloning kit (Invitrogen). Sequences were manually corrected using Sequencher 4.2 (Gene Codes). To identify fungal species, BLASTN searches were carried out against the sequence databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Computational identification and characterization of sorghum and flax *Pht1* genes

Genes encoding Pi transporters of the Pht1 family were identified *in silico* using five approaches, based on the full genome sequence of *S. bicolor* (Paterson *et al*., 2009) available at the JGI interface and *L. usitatissimum* (Wang *et al*., 2012), available at the Phytozome interface website (http://www.phytozome.net): (1) known Pht1 protein sequences from *Arabidopsis* and other plants (*e.g., Medicago truncatula, Oryza sativa, Lycopersicon esculentum*, *Populus trichocarpa*) were used to query flax and sorghum gene catalog using TBLASTN, (2) these plant *Pht1* gene sequences were also used in TBLASTN query against the JAZZ sequence assembly for sorghum (Paterson *et al*., 2009) and against the WGS sequence assembly for flax (Wang *et al*., 2012), (3) gene models with a predicted Pi permease domain (IPR004738) were identified using InterProScan and ScanProsite (http://www.expasy.org/tools/scanprosite/), (4) these putative Pht1 transporters were selected on the basis of a Pht1 conserved specific amino acid signature (Karandashov & Bucher, 2005), (5) confirmation

of these Pht1s models was based on expressed sequence tag (EST) support. Throughout the paper, JGI protein identification (ID) was used to identify these sorghum gene models. The final set of Pht1 proteins comprised the following; SbPT1 (JGI Protein ID: 5050188), SbPT2 (JGI Protein ID: 5056998), SbPT3 (JGI Protein ID: 5030542), SbPT4 (JGI Protein ID: 5048813), SbPT5 (JGI Protein ID: 5050189), SbPT6 (JGI Protein ID: 5058886), SbPT7 (JGI Protein ID: 5048812), SbPT8 (JGI Protein ID: 5033142), SbPT9 (JGI Protein ID: 5041365), SbPT10 (JGI Protein ID: 5041362) and SbPT11 (JGI Protein ID: 5036358). For flax, transcript names at the Phytozome interface website were used to identify these flax gene models. The final set of Pht1 proteins comprised the following; LuPT1 (Lus10022547), LuPT2 (Lus10016635), LuPT3 (Lus10011826), LuPT4 (Lus10021191), LuPT5 (Lus10014754), LuPT6 (Lus10033886), LuPT7 (Lus10003560), LuPT8 (Lus10012860) and LuPT9 (Lus10030506). Prediction of putative transmembrane (TM) segments for Pht1 proteins was performed using the programs TMHMM, TM pred and Top Pred (http://www.expasy.ch/proteomics). For sorghum, putative cis-acting elements were searched 2 kb upstream the start codon of the 11 Pht1 genes by DNA-pattern matching analysis.

For phylogenetic analysis, the Pht1 amino acid sequences were aligned with ClustalX using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbor joining trees were constructed using the Jones-Taylor-Thornton (JTT) substitution rate matrix for distance computation in MEGA5. Bootstrap analysis was carried out with 500 replicates. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Gene models used for phylogenetic analysis are given in the Supplemental Materials and Methods.

Samples, RNA isolation and quantitative reverse transcription-PCR

The procedure used for RNA extraction and cDNA synthesis was as described by Courty *et al*. (2009) and in Supplemental Materials and Methods S1. Primers used as controls or for analysis had efficiency ranged between 90% and 110%. Target gene expression was normalized (Muller *et al.*, 2002) to the gene encoding ubiquitin in sorghum (JGI Protein ID: 5060159) and in flax (Huis *et al.*, 2010). Considering the high sequence similarity (>95%) of LuPT3 and LuPT4, LuPT6 and LuPT7, and LuPT8 and LuPT9, it was not possible to design specific PCR primers for the amplification of corresponding cDNAs (Supplemental

Table S5). All used primers are listed in Supplemental Table S1.

From each of the three *S. bicolor* plants harvested in the field site described above, three subsamples (about 100mg) of roots, shoots, stem, pistils and stamina were snap frozen and stored at -80°C for further gene expression analysis.

Concerning the effect of P nutrition in the single pot experiment and of common mycorrhizal networks in mono-culture or mixed-culture, plant roots were carefully washed under tap water to remove root adherent substrate. Three subsamples of 100mg of fresh roots were snap frozen and stored at -80°C for further gene expression analysis.

Carbon, nitrogen and P analysis

Shoots and root subsamples were dried 72h at 65°C and dry weights were estimated. Dried shoots and roots were homogenized and ground at 30 Hz in a ball mixer mill (MM2224, Retsch, Haan, Germany). Aliquots of 2 mg were weighed for elemental analyses. Contents of nitrogen and carbon were determined using an ANCA elemental analyzer/mass spectrometer (Europa Scientific Ltd., Crewe, UK). P content of shoots and roots was measured photometrically, using the molybdate blue method after acid digestion (Murphy & Riley, 1962).

Statistical analyses

An analysis of variance (ANOVA) was performed on the total biomass, on the C and N content, and on the total and arbuscular colonization for each treatment separately, where the two latter parameters were arcsine-transformed to fit the assumption of normal distribution. The ANOVA was based on N treatments and AMF treatments. Pairwise comparisons between the treatments were done with planned contrast analysis. Independent paired-tests were performed. A probability of $P \leq 0.05$ was considered as representing a significant difference.

Results

Annotation and distribution of sorghum *Pht1* genes

Fourteen different gene models coding for putative Pht1 transporters were identified in the predicted gene catalog resulting from the automated annotation of *S. bicolor* genome assembly (Version 6.0.176; http://genome.jgi-psf.org/Sorbi1/Sorbi1.home.html). Examination of these gene models revealed two truncated gene models (Protein ID: 5033055 and 5036201), and one gene model (Protein ID: 4801120) missing the Pht1 specific signature (Karandashov & Bucher, 2005). The remaining set of eleven putative *Pht1* genes (for *S. bicolor* Pi transporter family 1) were named according to the Commission on Plant Gene Nomenclature as *SORbi;Pht1;1* through *SORbi;Pht1;11*; for simplification, they will be called *SbPT1* through *SbPT11* in this article. Except for SbPT3, ESTs have been identified for these 11 Pht1 transporters, confirming that they are expressed (Supplemental Table S12). In the *S. bicolor* genome assembly, these 11 putative *Pht1* genes were located on chromosomes 1, 2, 3, 6 and 7 (Supplemental Fig. S1). Interestingly *SbPT1* and *SbPT5*, as well as *SbPT4* and *SbPT7*, were present as pairs on chromosome 1, forming a sort of inverted repeats with an intervening putative promoter sequence of only 0.89 kb and 2.77 kb, respectively. However, the sequence similarity within these pairs was not particularly high (73.6 % and 75%, respectively; Supplemental Table S4), indicating that they have evolved independently. *SbPT3, SbPT8, SbPT11* and *SbPT6* are located on chromosome 1, 2, 3 and 7, respectively. *SbPT2, SbPT9* and *SbPT10* were all located on chromosome 6, but none of them appears to be tandem repeat sequences (Supplemental Fig. S1).

All putative Pht1 proteins of sorghum belonged to the "major facilitator superfamily" (Pao *et al.*, 1998) with 12 predicted trans membrane (TM) segments, separated into two blocks of six TM segments by a long hydrophilic loop, and hydrophilic N and C terminus (Supplemental Table S2). The Pht1 specific signature (GGDYPLSATIMSE) was conserved and identical for all Pht1 proteins. Eight out of 11 *Pht1* genes are intron-less in sorghum (Supplemental Table S2). The 2kb up-stream region of the Pht1 genes harbor potential regulatory elements involved in P and AM response, from one in *SbPT5* to 17 in *SbPT4* (Supplemental Fig. S2). The most prominent element, P1BS (GNATATNC), exhibiting a crucial role in Pi starvation response, one copy of P1BS is present in the promoter region of *SbPT1, SbPT3, SbPT4, SbPT6,*

SbPT7 and *SbPT8,* and five copies in the promoter region of *SbPT11*. A root motif box (ATATT or AATAT) is present in the promoter region of all 11 *Pht1* genes. Some of the promoter regions harbor two motifs-OSEROOTNODULE (AAAGAT) and NODCON2GM (CTCTT), which are conserved elements associated with AM and nodule induced leghaemoglobin gene regulation. Additionally, w-boxes (TTGACY) and "unknown elements" (TCTTGTT) were discovered.

Identification of *Pht1* genes of flax

Nine different gene models coding for putative Pht1 transporters were identified in the predicted gene catalog resulting from the *de novo* prediction of flax (*L. usitatissimum*) genome assembly available at Phytozome interface. Commission on Plant Gene Nomenclature as *LINus;Pht1;1* through *LINus;Pht1;9*; for simplification, they will be called *LuPT1* through *LuPT9* in this article. Except for LuPT4 and LuPT5, ESTs have been identified for six Pht1 transporters, confirming that they are expressed (Supplemental Table S3). Actually, some limitations on long-range accuracy of the flax genome restricted a precise localization of these nine putative *Pht1* genes. All putative Pht1 proteins of flax belonged to the "major facilitator superfamily" (Pao *et al*., 1998) with 12 predicted TM segments (Supplemental Table S3) and contained the Pht1 specific signature (GGDYPLSATIxSE), slightly modified for LuPT8 and LuPT9 (GGDYPLSAVIMSE). Two out of nine *Pht1* genes are intron-less in flax (Supplemental Table S3).

Phylogenetic analysis of the Pht1 transporters

A neighbor-joining tree of Pht1 transporters was constructed by multiple sequence alignment, comparing the relevant protein sequences from sorghum and flax and with sequences from other plants and fungi (Fig. 1). As has been described for other monocotyledon plant species (Nagy *et al*., 2006), Pht1 proteins of sorghum clustered into four subfamilies (Fig. 1). SbPT11 is the only transporter belonging to the subfamily I and appears to be closely related to AM-inducible Pht1 transporters ZmPT6 of *Zea mays* (Glassop *et al.*, 2005), OsPT11 of *Oryza sativa* (Paszkowski *et al*., 2002) and BdPT7 from *Brachipodium distachyon* (Hong *et al.*, 2012). Sorghum also harbors two non-orthologous Pi transporters SbPT9 and SbPT10, belonging to Pht1 subfamily IV, which also contains OsPT13, another AM-inducible Pi

transporter of *O. sativa* (Güimil *et al.*, 2005). SbPT9 is closely related to the Pht1 transporters ZmPT5 of *Z. mais* (Nagy *et al.*, 2006). SbPT8 clusters in a small subfamily harboring three other cereal Pht1 transporters of *B. distachyon* (BdPT3)*, Triticum aestivum* (TaPTmyc) and *Hordeum vulgare* (HvPT8); TaPTmyc and HvPT8 are known to be AM-inducible (Glassop *et al*., 2005). All Pht1 transporters of sorghum except SbPT11 belong to Pht1 subfamilies present only in monocotyledons.

The nine Pht1 proteins of flax were distributed in two distinct subfamilies (Fig. 1). LuPT8 and LuPT9 clustered in subfamily I harboring AM-inducible Pht1 transporters and was closely related to GmPT7 and PtPT8, two AM-inducible Pi transporter of *Glycine max* (Tamura *et al*., 2012) and *Populus trichocarpa* (Loth-Pereda *et al*. 2011), respectively. The six other Pht1 proteins clustered in subfamily III, specific for dicotyledonous plant species (Fig. 1). Except *LuPT5*, all genes encoding Pht1 were resulting from a recent duplication event (5-9 MYA, Wang *et al.*, 2012).

Gene expression of *Pht1* transporters in different sorghum tissues

Expression of Pht1 transporters in different tissues of sorghum was examined in plants from a grain field. The roots of these plants were colonized by AMF (degree of colonization 42 % ± 3 SEM, data not shown), primarily by *G. mosseae*, as revealed by sequence analysis of fungal ITS sequences (data not shown). In sorghum, ten out of 11 *Pht1* genes were expressed in roots; only *SbPt3* was not detectable in any of the tissues investigated (Fig. 2). Highest expression levels were measured for *SbPT7* in all tissues and for *SbPT11* in roots. *SbPT2, SbPT6* and *SbPT7* were constitutively expressed in all the tissues. *SbPT1* and *SbPT10* were only detectable in mycorrhizal roots. *SbPT4* was only expressed in vegetative tissues. *SbPT9* was detected in mycorrhizal roots and leaves. Besides in mycorrhizal roots, *SbPT11* was expressed in both female and male inflorescence, whilst *SbPT5* and *SbPT8* were only expressed in stamina.

Influence of Pi availability and AM colonization on *Pht1* gene expression in sorghum roots

The expression level of *Pht1* genes studied in sorghum roots under conditions of low Pi supply, either in the absence of AMF or in the presence of *G. intraradices* or *G. mosseae* (Fig. 3). Expression of most *Pht1* genes was only minimally affected by mycorrhiza formation. However, expression levels of *SbPT10 and SbPT11* were at least 1000 fold higher in mycorrhizal roots than in non-mycorrhizal ones. The expression of *SbPT9* was significantly enhanced in roots colonized by *G. mosseae*, but not in those colonized by *G. intraradices* but not in (Fig. 3). *SbPT11* was only detectable in mycorrhizal roots. In contrast, expression of *SbPT1, SbPT2* and *SbPT6* was significantly repressed in mycorrhizal roots. *SbPT4, SbPT5* and *SbPT7* transcripts were not modified upon AM colonization.

Additional Pi fertilizer only weakly affected sorghum plant growth performance, but P content of plants was significantly higher with additional Pi supply (Supplemental Fig. S3). All mycorrhizal plants exhibit an arbuscular colonization of 46 % ± 3 SEM without significant differences among P treatment and AMF species (p-value = 0.91). Plants growing in Pi-poor conditions were compared with plants growing with additional Pi fertilization in non-mycorrhizal roots and in roots colonized by *G. intraradices* or *G. mosseae*. Genes exhibiting a modification in expression of at least the factor two are considered to be up- or down-regulated. In non-mycorrhizal roots, Pi supply significantly repressed the expression of *SbPT1* and *SbPT8*, but significantly enhanced *SbPT6* (Supplemental Fig. S4). In contrast, in mycorrhizal roots, neither *SbPT1* nor *SbPT8* were down-regulated upon additional Pi fertilization. *SbPT6* was also significantly over-expressed in mycorrhizal roots (Supplemental Fig. S4). Expression of the *SbPT2, SbPT4, SbPT5, SbPT7, SbPT9, SbPT10* and *SbPT11* was not or marginally modified by Pi availability. Remarkably, expression of *SbPT5* was repressed with additional Pi supply in non-mycorrhizal roots and roots colonized by *G. intraradices*, but rather enhanced in roots colonized with *G. mosseae*.

***Pht 1* gene expression in sorghum and flax sharing a mycorrhizal network**

As shown in Figure 4, *Pht1* genes of sorghum exhibited similar expression patterns upon mycorrhization in mono-culture as in single pot culture described above (Fig. 3). Again, *SbPT8*, *SbPT9*, *SbPT10* and

SbPT11 were strongly induced in association with AMF; moreover, *SbPT10* and *SbPT11* were even only detected in mycorrhizal roots (Fig. 4). The expression of *SbPT1* was repressed by the factor 100 in AM symbiosis. *SbPT2* and *SbPT5* were significantly up-regulated in association with *G. intraradices,* which contrasts to the repression measured in single pots (Fig. 3). *SbPT6* was significantly repressed in roots colonized by *G. mosseae* and in roots colonized by *G. intraradices* in mixed-cultures. Similarly, expression of *SbPT10* was more enhanced when colonized by *G. mosseae* than by *G. intraradices* (Fig. 4). However, different culture systems modified the expression of *Pht1* genes in sorghum only marginally compared to the effect of AMF colonization. In normalized expression, only the expression level of *SbPT6* was significantly reduced in mixed-cultures, as compared to mono-cultures, when associated with *G. intraradices*, and the expression level of *SbPT10* was significantly increased in a CMN with flax formed by *G. mosseae* (Fig. 4).

The expression level of *LuPT1* was constant in mono- and mixed-culture, except in *G. intraradices* mixed-cultures where it was down-regulated. In mono-culture, expression of *LuPT3*/*LuPT4* was repressed in mycorrhizal roots, whilst the expression level of the AM-inducible Pht1 *LuPT2*, *LuPT5* and *LuPT8*/*LuPT9* shown increased expression levels (Fig. 5). In flax, and in association with *G. intraradices*, *LuPT6*/*LuPT7* and *LuPT5* were significantly up-regulated in mixed-culture compared to mono-culture, and *LuPT2* was significantly down-regulated. In association with *G. mosseae*, *LuPT8*/*LuPT9* were significantly less expressed in mixed-culture than in mono-culture and were showing similar expression levels as in non-mycorrhizal roots (Fig. 5). In mixed-culture, *LuPT2* was the only Pht1 significantly over-expressed in association with *G. mosseae* compared to *G. intraradices*.

The differences in expression of AM-inducible *Pht1* genes between mono- and mixed-culture are more precisely displayed in comparing their relative expression (Table 1). In sorghum roots associated with *G. mosseae*, *SbPT9* and *SbPT10* were more expressed in mixed-culture than in mono-culture, whereas in association with *G. intraradices* no difference was observed. In contrast, the AM-induced *Pht1* genes of flax, *LuPT8*/*LuPT9* and *LuPT2* were repressed in mixed-culture when associated with *G. mosseae* and *G. intraradices*, respectively while *LuPT5* was up-regulated when associated with *G. intraradices*.

Discussion

Phylogenetic relation of Pht1 transporters

The phylogenetic analysis revealed the relation of the 11 sorghum transporters (Fig. 1) with members of previously described Pht1 subfamilies (Karandashov & Bucher, 2005). Subfamily I harbors the AM-inducible Pi transporters from both monocotyledonous and dicotyledonous plant species, indicating their evolution before the separation of the two plant groups. In sorghum SbPT11, the protein encoded by one of the AM-induced *Pht1* genes, fall into this group. The three other AM-inducible Pi transporter of sorghum, SbPT8, SbPT9 and SbPT10, fall into divergent subfamilies harboring transporters of monocotyledonous plant species, which are partially AM-inducible. These subfamilies seems to be evolutionary younger than the subfamily I, because they comprise proteins solely from monocotyledonous species. This indicates a separate evolutionary gain of AM-inducibility of genes involved in P uptake, both in monocotyledonous and dicotyledonous species (Karandashov *et al*., 2004; Nagy *et al*., 2006). In addition, the Pht1 transporters SbPT1 to SbPT7 fall also in the monocot-specific subfamily IV. Genes coding for SbPT1 to SbPT8 have no introns, suggesting that they arise from duplication events from a primordial gene (Lynch & Conery, 2000). The tandem repeat sequences of *SbPT1*/*SbPT5* and *SbPT4*/*SbPT7* are likely to be most recent duplications. Concerning linum transporters, LuPT3 and LuPT4, LuPT6 and LuPT7, and LuPT8 and LuPT9 had an identity higher than 95 % and were resulting from a recent duplication event (5-9 MYA, Wang *et al.*, 2012). Two out of the nine Pi transporters (LuPT8 and LuPT9) fall into the the AM-inducible Pi transporters subfamily I. The six other Pi transporters belong to subfamily III, specific from dicotyledonous species.

Expression of *Pht1* genes in different sorghum tissues

Pht1 genes exhibit substantial differences in their expression in the various organs examined. *SbPT2, SbPT6* and *SbPT7* were constitutively expressed in sorghum and thus may be involved in P homeostasis (Jia *et al.*, 2011). The transcripts coding for *SbPT5*, *SbPT8* and *SbPT11* were expressed in roots, but in addition, they were also strongly expressed in inflorescence, mainly in stamina, similar to their homologs from *Arabidopsis* and maize, which were suggested to fulfill Pi uptake in the elongating pollen tube (Mudge *et al.*, 2002; Nagy *et al.*, 2006). The specific expression of *SbPT9* in leaves in addition to

roots indicates a role in Pi mobilization in leaves, as suggested for a homologous transporter in poplar (Loth-Pereda *et al.*, 2011). *SbPT1* was only detected in the roots and down-regulated upon P fertilization in non-mycorrhizal roots (Fig. 2 and 3), indicating a role in direct Pi uptake by the roots (Karandashov & Bucher, 2005; Ai *et al.*, 2009). With respect to root expression, most interestingly, *SbPT10* and *SbPT11* were detected in AMF-colonized roots (Fig. 3), and therefore probably involved in symbiotic Pi uptake (Smith *et al.*, 2011). *SbPT3* was not detected in any of the examined tissue types under the present experimental conditions (Fig. 1, 2, 4 and Supplemental Fig. S4). Thus, this *Pht1* gene could be expressed under specific conditions, which were not included in our experimental set up, or, alternatively, its expression levels were too low to be detected.

Physiological measurements have shown that P starved plants rapidly increase their capacity in Pi uptake by transcriptional up-regulation of *Pht1* genes (Smith *et al.*, 2003). One of the best-characterized regulatory elements involved in P regulation is the transcription factor PHR1, which activates a subset of P starvation induced genes by binding on cis-acting P1BS elements (Rubio *et al.*, 2001; Nilsson *et al.*, 2007). In the promoter region of 7 of the 11 *Pht1* genes of sorghum, we found at least one P1BS element (Supplemental Fig. S2), including *SbPT1, SbPT6* and *SbPT8*. Interestingly, *SbPT1* and *SbPT8* were strongly down-regulated in non-mycorrhizal roots in response to P fertilization, while *SbPT6* was clearly up-regulated under the same conditions (Supplemental Fig. S4), indicating that similar regulatory elements may have opposite functions in gene expression.

In the roots, two genes were practically exclusively expressed in the presence of mycorrhiza, namely *SbPT10* and *SbPT11* (Fig. 3). *SbPT11* belongs to subfamily I of Pht1 genes, comprising mycorrhiza-inducible Pht1 genes of both in monocots and dicots. It is also expressed in flower organs. *SbPT10* is a member of the monocot-specific subfamily IV;. some members of the latter group have previously been shown to be AM-inducible (Fig. 1). Several plant species, e.g. *O. sativa, L. esculentum, S. tuberosum* possess multiple AM-induced *Pht1* genes (Güimil *et al.*, 2005; Nagy *et al.*, 2005), resulting in a functional redundancy of symbiotic Pi transport (Nagy *et al.*, 2005), whereas other plant species harbor a single AM-induced *Pht1* gene which appears to be crucial for the symbiotic P acquisition (Javot *et al.*, 2007).

AM-dependent induction of Pi transporters is often accompanied by the down-regulation of other *Pht1* genes; in particular those suggested being involved in the direct P uptake (Rausch *et al.*, 2001; Paszkowski *et al*., 2002; Glassop *et al*., 2005). Such an AM-dependent down-regulation was apparent in the lowered transcript level of *SbPT1* in mycorrhizal roots. Transcription of mycorrhiza-inducible genes has been suggested to be regulated by two cis-acting regulatory elements, P1BS and MYCS, in solanaceous species (Chen *et al.*, 2011); P1BS, in particular, might have a double role as a regulatory element in response to Pi availability as well as to AM symbiosis (Smith *et al.*, 2011). However, P1BS is only present in the AM-inducible *Pht1* genes *SbPT8* and *SbPT11*, whilst *SbPT10* does not harbor a P1BS element in their promoter region (Supplemental Fig. S2). The transcriptional regulation of AM-inducible genes is still poorly understood; the discovery of more AM-specific regulatory elements, active in abroad range of mycorrhizal plants might shed new light on AM-inducibility.

Role of transcriptional regulation of *Pht1* transporters in Pi acquisition *via* a CMN

In experimental monocultures, two individual sorghum plants connected to a CMN received an equal share of the Pi accessible only to the AMF, but *G. mosseae* was twice as effective to deliver Pi when compared to *G. intraradices* (Walder *et al.*, 2012) This might be related to the stronger induction of the AM-inducible *Pht1* genes (Fig. 3 and Supplemental Fig. 4S) by *G. mosseae*, as compared to *G. intraradices.* Interestingly, the sorghum roots harvested in the field were almost exclusively colonized by *G. mosseae*, indicating a preference under field conditions (Helgason *et al.*, 2002; Davison *et al.*, 2011). Our experiment thus reflected the interdependence of preference and compatibility in the sorghum - *G. mosseae* association.

In mixed-culture sharing the mycorrhizal network with associated flax, sorghum acquired 6% of Pi *via* the CMN built by *G. intraradices* and 52% when built by *G. mosseae* (Walder *et al.*, 2012). Expression of the two main AM-induced *Pht1* genes of sorghum, *SbPT10* and *SbPT11*, was very strongly induced even in the presence of *G. intraradices*, under conditions where sorghum received virtually no CMN-mediated Pi, and thus exhibited an inactive mycorrhizal pathway (Fig. 3 and 4). It is worth noting, however, that the expression of *SbPT9* and *SbPT10* was enhanced in mixed-culture with flax when the CMN was

formed by *G. mosseae,* i.e. under conditions where sorghum obtained more Pi through the mycorrhizal pathway (Table 1). In mixed-cultures, flax acquired the double amount of Pi compared to the flax mono-culture *via* both CMNs (Walder *et al.*, 2012), Nevertheless, when the CMN was formed by *G. mosseae*, the AM-induced *Pht1* genes from flax were less expressed in mixed-cultures compared to mono-cultures and dropped down even to the non-mycorrhizal level (Fig. 5 and Table 1).

Our data show, in accordance with current literature (Smith & Smith, 2011), that transcriptional regulation of specific members of the Pht1 transporter family occurs, both in sorghum and in flax, in response to mycorrhiza formation. However, in the situation of mixed-cultures between sorghum and flax engaged in a CMN, transcriptional regulation of these transporters is not related to the differential gain of P by the interconnected plants. Besides the expression level of the transporter genes, post-transcriptional regulation and the rates of protein turnover influence the number and functioning of transporter in the plasma membrane (Smith & Smith, 2011). Transcriptional regulation appears to be crucial for the induction of Pht1 transporters during initialization of the symbiotic P exchange at periarbuscular membrane (Harrison *et al*., 2002), whereas fine-tuned regulation of the transport system probably may be more dependent on posttranscriptional regulation and protein turnover.

Even if the expression of AM-inducible *Pht1* genes of sorghum and flax were only marginally related to Pi supply via mycorrhizal network, the relative expression elucidated differences between the culture systems (Table 1). In mixed-culture and *G. mosseae* as fungal partner, *SbPT9* and *SbPT10* were up-regulated, and *LuPt8*/*LuPT9,* and *LuPT2* and *LuPT5* were repressed and not regulated, respectively. In mixed-culture and *G. intraradices* as fungal partner, *LuPt6*/*LuPT7* and *LuPT5* genes were up-regulated whilst *LuPT2* was down-regulated. Therefore, it appears that expression of AM-inducible *Pht1* genes is affected not only by the fungal partner, but also by the interconnected plant species. To explain these data, jumping in applied ecology could help. A genotype has a traditional phenotype exhibited in association with individuals of the same species. However, in interacting with individuals of other species, the traditional phenotype is extended to another level, called community or ecosystem phenotype(Whitham *et al.*, 2006). In other words, here, flax and sorghum plants interconnected *via* the

CMN are forming a unique "community phenotype" (Whitham *et al.*, 2006), in which expression of the AM-inducible *Pht1* genes could be mediated by complex interactions between the three partners (sorghum, flax, AMF species).

Acknowledgments

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Tables and Figures

Table 1. Relative expression of AM-induced *Pht1* genes of sorghum and flax

AMF species	Plant species			
	Sorghum		Flax	
	Pht1 gene	Rel. Expression	*Pht1* gene	Rel. Expression
G. intraradices	*SbPT1*	1.18 ± 0.40	*LuPT2*	0.5 ± 0.16
	SbPT8	1.08 ± 0.16	*LuPT5*	3.3 ± 0.54
	SbPT9	1.09 ± 0.34	*LuPT8/LuPT9*	0.9 ± 0.18
	SbPT10	0.98 ± 0.47		
	SbPT11	1.43 ± 0.41		
G. mosseae	*SbPT1*	1.23 ± 0.41	*LuPT2*	1.8 ± 0.4
	SbPT8	1.33 ± 0.24	*LuPT5*	1.5 ± 0.36
	SbPT9	4.27 ± 1.32	*LuPT8/LuPT9*	0.6 ± 0.08
	SbPT10	2.99 ± 0.39		
	SbPT11	1.00 ± 0.26		

Quantification by RT-qPCR analysis of the transcript levels of AM-induced *Pht1* genes in sorghum and flax roots as affected by mono- and mixed-culture. Roots were colonized by G*lomus intraradices* and *G. mosseae*. Genes were considered to be biologically regulated, if modified by more than the factor two. Transcript levels in roots grown in mono-culture were used as control values for relative expression. Transcripts were normalized against ubiquitin. Values are means (± SEM) of four replicates. CMN-mediated Pi uptake was estimated by measuring ^{33}P uptake only accessible for AMF hyphae. In comparison of the mono-cultures and the mixed-culture, sorghum received 0.1 and 0.6 times less CMN-mediated Pi in association to *G. intraradices* and to *G. mosseae*, respectively. In contrast, flax received 2.1 and 1.8 times more CMN-mediated Pi in association to *G. intraradices* and to *G. mosseae*, respectively.

Supplemental data

1. **Supplemental methods**
2. **Supplemental Figure S1**. Gene distribution of the 11 *Pht1* genes on the 10 chromosomes (Ch) of *Sorghum bicolor* according to (Paterson et al., 2009).
3. **Supplemental Figure S2.** Putative cis-regulatory elements involved in Pi and AM response in the promoter region of the 11 *Pht1* genes.
4. **Supplemental Figure S3.** P content of plants as affected by Pi availability in non-mycorrhizal roots and roots colonized by *Glomus intraradices* or *G. mosseae*.
5. **Supplemental Figure S4.** Quantification by RT-qPCR analysis of the transcript levels of the 11 sorghum *Pht1* genes in sorghum roots in response to Pi availability.
6. **Supplemental Table S1.** Primers for qRT PCR analysis of Pht1 transcripts of sorghum and flax.
7. **Supplemental Table S3.** Characteristics of the *Sorghum bicolor Pht1* gene family.
8. **Supplemental Table S3.** Characteristics of the *Linum usitatissimum Pht1* gene family.
9. **Supplemental Table S4.** Similarity/identity matrix of *Sorghum bicolor* Pht1 protein sequences.
10. **Supplemental Table S5.** Similarity matrix of *Linum usitatissimum* Pht1 protein sequences.

Figure 1. Neighbor-joining tree for Pht1 transporters based on aligned sequences (727 amino acids). Sequence names consist of species code (first letter of genus and first letter of species name) and gene name (see Supplementary Material for details). Fungal Pht1 transporters and chloroplastic Pht2 transporters serve as outgroups. Roman numbers indicate four different plant Pht1 subfamilies thought to different in evolutionary age. The 11 sorghum and the 9 flax Pht1 transporters are highlighted in bold black. Asterisks indicate Pht1 transporters known to be induced in response to mycorrhiza formation.

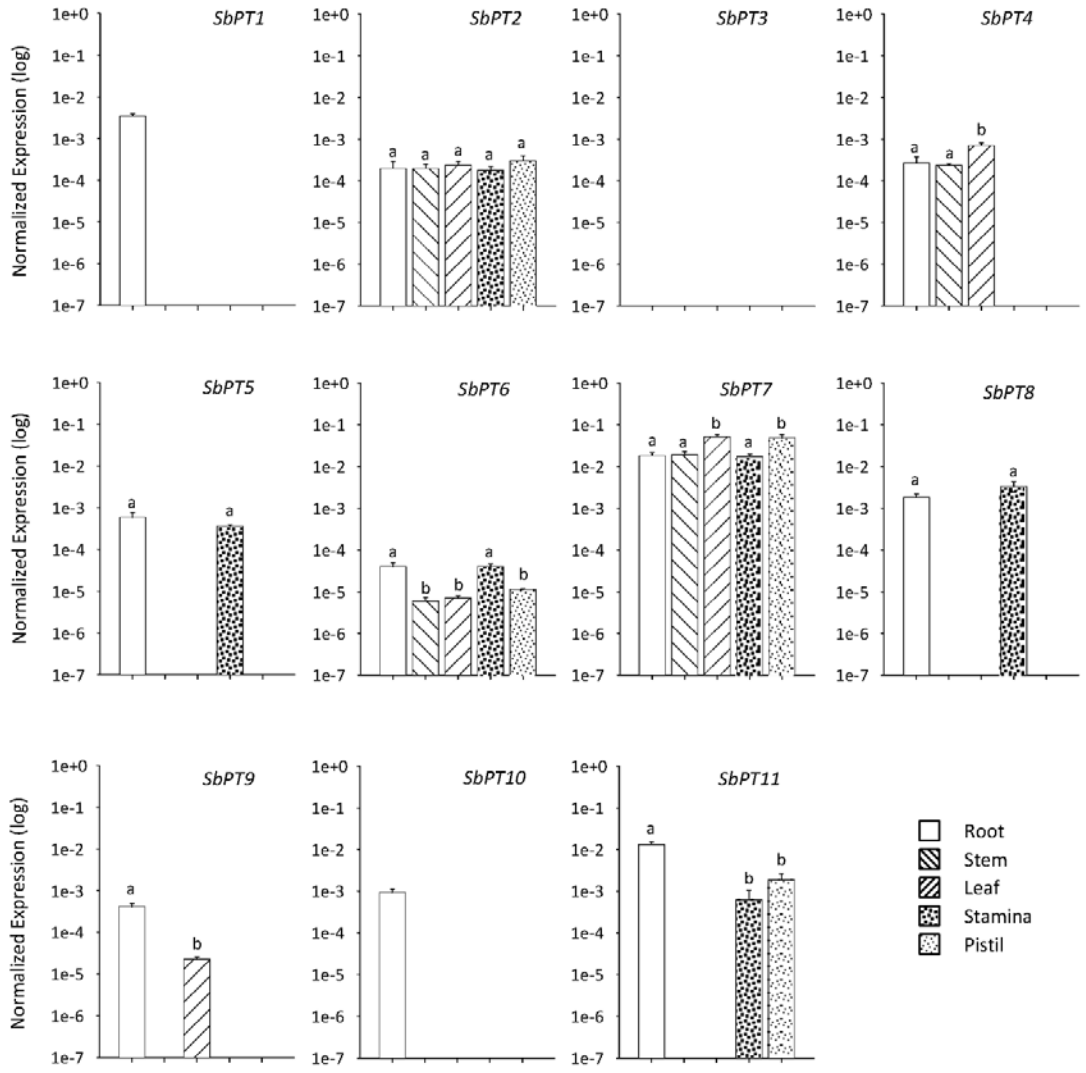


Figure 2. Quantification by qRT-PCR analysis of transcript levels of the 11 sorghum *Pht1* genes in different tissue types. Sorghum plants were harvested in a farmer's field; it was found that roots were primarily colonized by *G. mosseae* isolates (42 % ± 3). Transcript levels were normalized against ubiquitin. Values are means of three replicates; error bars represent SEM. For each gene, different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.

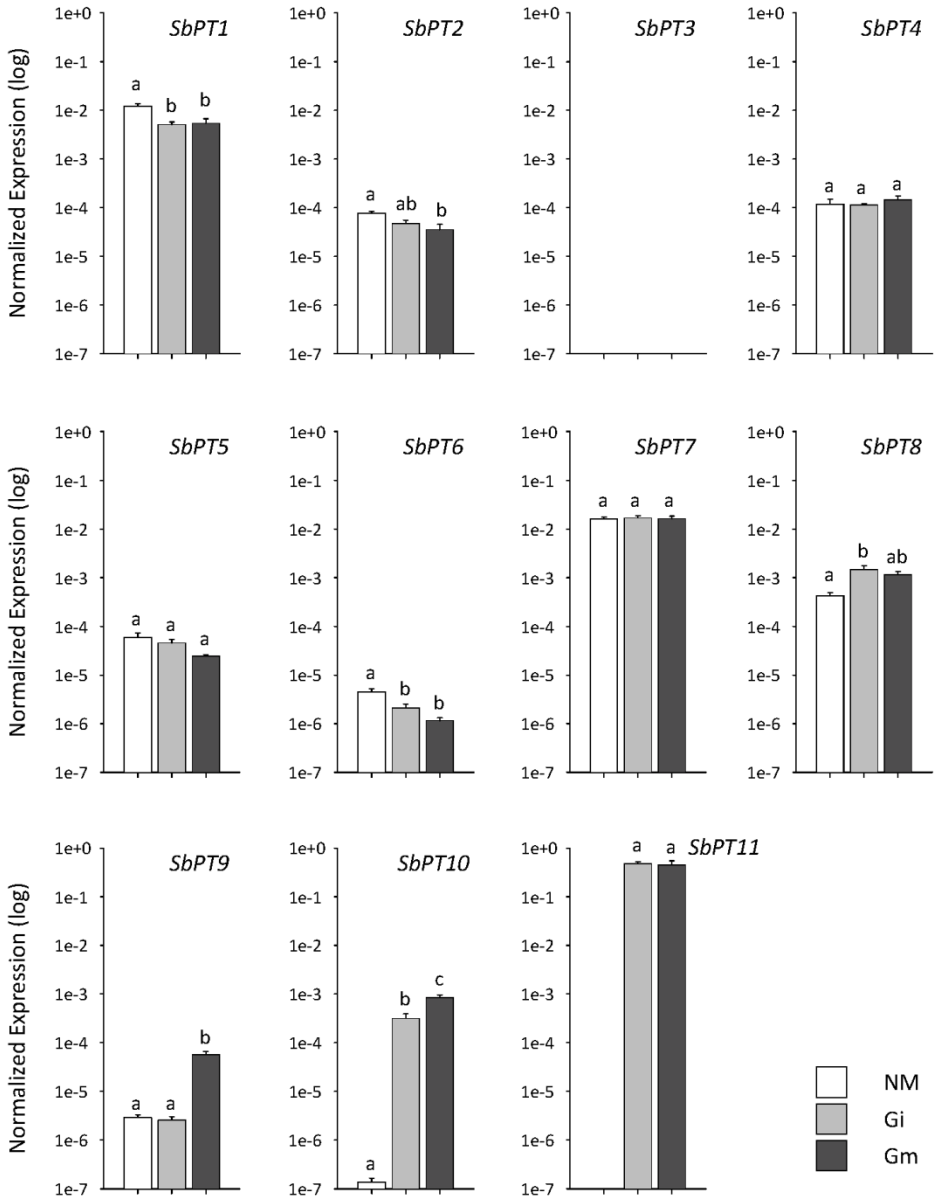


Figure 3. Quantification by qRT-PCR of the transcript levels of the 11 sorghum *Pht1* genes in non-mycorrhizal roots (NM) and roots colonized by *G. intraradices* (Gi) or *G. mosseae* (Gm). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. For each gene, different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.

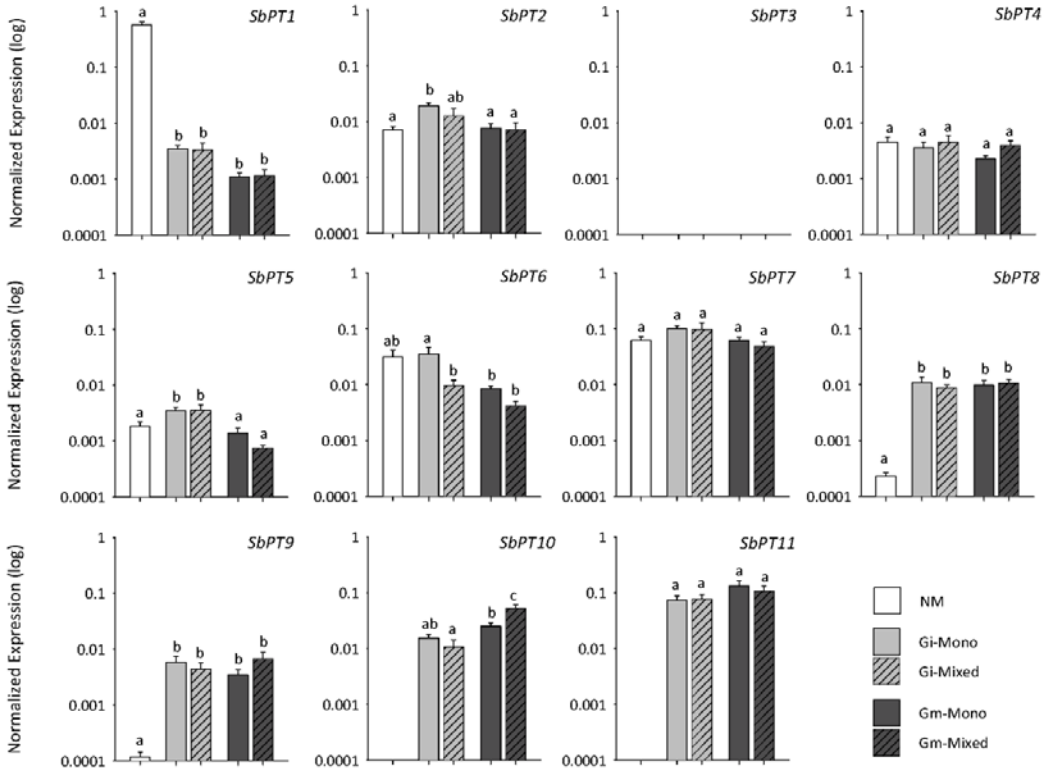


Figure 4. Quantification by qRT-PCR analysis of transcript levels of the 11 sorghum *Pht1* genes in non-mycorrhizal roots (NM) or in roots connected to a mycorrhizal network built by G*lomus intraradices* (Gi) or *G. mosseae* (Gm) shared with a co-cultured sorghum (Mono) or with a co-cultured flax plant (Mixed). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.

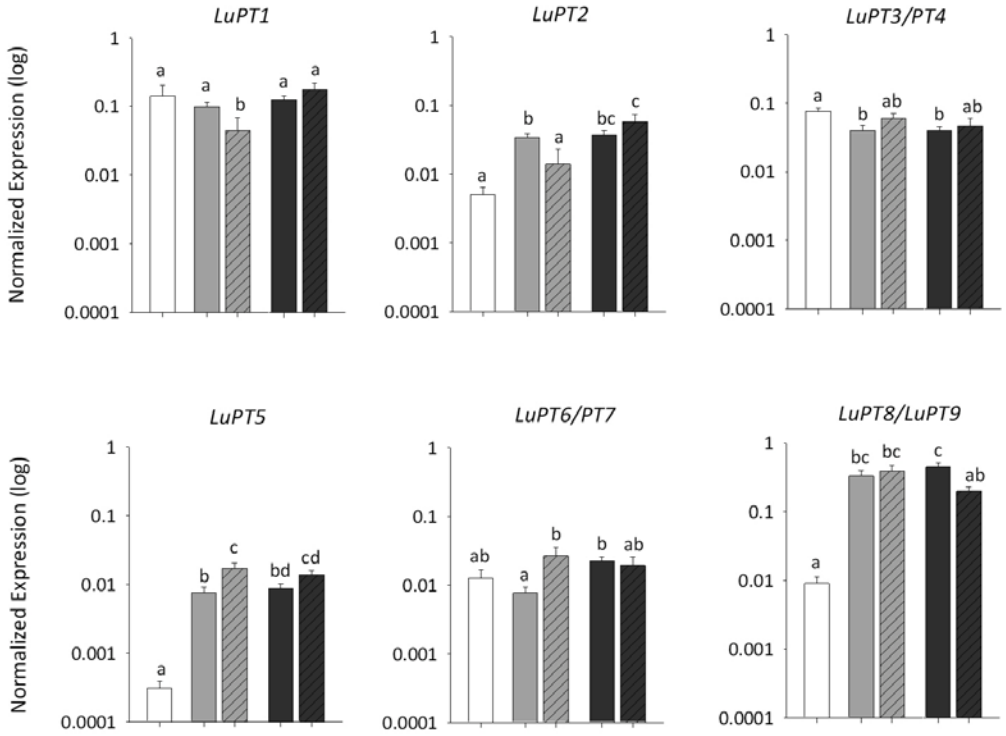


Figure 5. Quantification by qRT-PCR analysis of transcript levels of the nine flax *Pht1* genes in non-mycorrhizal roots (NM) or in roots connected to a mycorrhizal network build by *Glomus intraradices* (Gi) or *G. mosseae* (Gm) shared with a co-cultured flax (Mono) or with a co-cultured sorghum plant (Mixed). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.

Supplemental data

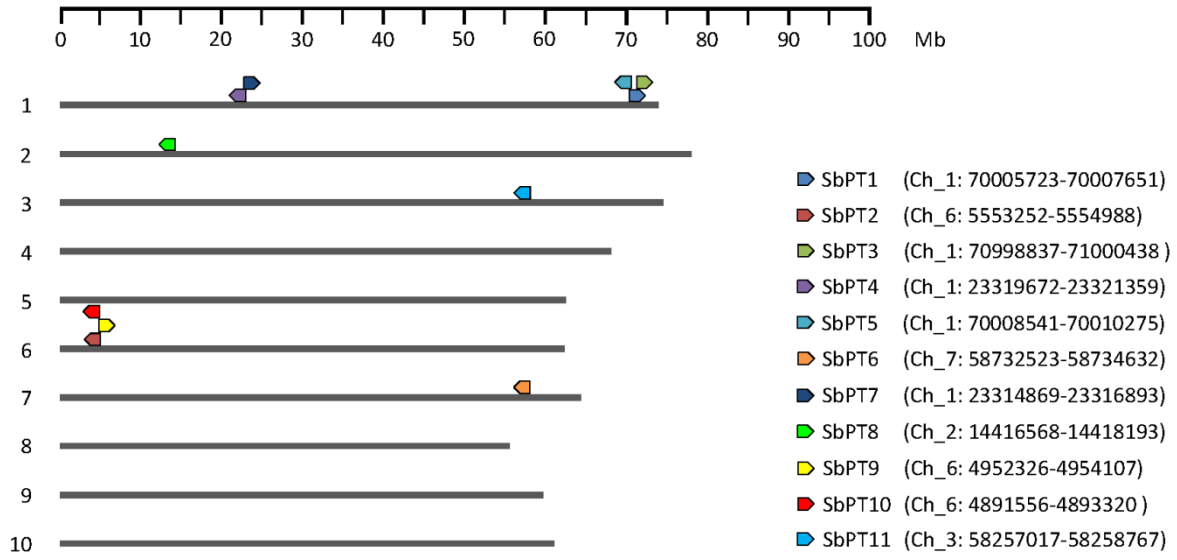
The phylogenetic analysis presented in Fig. 1 is based on the putative amino acid sequences of Pi transporters based on DNA sequences, as archived in the UniProt data base. Sequences retrieved from NCBI GenBank were: *Arabidopsis thaliana;* AtPT1 (Q8VYM2), AtPT2 (Q96243), AtPT3 (O48639), AtPT4 (Q96303), AtPT5 (Q8GYF4), AtPT6 (Q9ZWT3), AtPT8 (AEE79268.1), AtPT9 (Q9S735); *Medicago truncatula*: MtPT1 (AAB81346), MtPT2 (AAB81347), MtPT4 (AAM76743), MtPT2;1 (XP_003628943); *Glycine max*: GmPT1 (ACP19339), GmPT2 (ACN80147), GmPT3 (ACP19343), GmPT4 (ACP19342), GmPT5 (ACP19340), GmPT6 (ACP19341), GmPT7 (ACP19345), GmPT8 (ACP19338), GmPT9 (ACP19347), GmPT10 (ACP19346), GmPT11 (ACP19344); *Lotus japonicus*: LjPT1 (BAE93354), LjPT2 (BAE93355), LjPT3 (BAE93353); *Lupinus albus*: LaPT1 (AAK01938), LaPT2 (AAK38197); *Lycopersicon esculentum*: LePT1 (AAB82146), LePT2 (AAB82147), LePT3 (AAV97729), LePT4 (AAX85193); *Ricinus communis*: RcPT1 (XM_002531486), RcPT2 (XM_002524576), RcPT3 (XM_002517998); *Lycopersicon esculentum*: LePT1 (AAB82146), LePT2 (NP_001234043), LePT4 (NP_001234674), LePT5 (AAX85194); *Solanum tuberosum*: StPT1 (CAA67395), StPT2 (CAA67396), StPT3 (AAV97729), StPT4 (AAW51149), StPT5 (AY885654), StPT2;1 (AAT35816); *Solanum melongena*: SmPT1 (ABK63959), SmPT3 (ABK63963), SmPT5 (ABK63969) *Nicotiana tabacum*: NtPT1 (AAF74025), NtPT2 (BAB21545), NtPT3 (ABK63964), NtPT4 (ABK63967), NtPT5 (ABK63970); *Populus trichocarpa*: PtPT1 (XP_002315705), PtPT2 (XP_002332753), PtPT3 (XP_002332751), PtPT4 (XP_002306844), PtPT5 (XP_002302047), PtPT6 (XP_002306623), PtPT7 (XP_002306845), PtPT8 (XP_002329198), PtPT9 (XP_002300626), PtPT10 (XP_002331845), PtPT11 (XP_002307816), PtPT12 (XP_002300153), PtPT2;1 (XP_002314598), PtPT2;2 (XP_002311765); *Oryza sativa*: OsPT1 (AAN39042), OsPT2 (AAN39043), OsPT4 (AAN39045), OsPT5 (AAN39046), OsPT6 (AAN39047), OsPT7 (AAN39048), OsPT8 (AAN39049), OsPT9 (AAN39050), OsPT10 (AAN39051), OsPT11 (AAN39052), OsPT12 (AAN39053), OsPT13 (AAN39054); *Hordeum vulgare*: HvPT1 (AAN37900), HvPT2 (AY187020), HvPT4 (AY187025), HvPT5 (AAO72435), HvPT6 (AAN37901), HvPT7 (AAO72436), HvPT8 (AAO72440); *Triticum aestivum*: TaPT1 (CAC69857), TaPT2 (CAC69855), TaPT8 (AAP49822), TaPT*myc* (AH25730); *Zea mays*: ZmPT1 (NP_001105269), ZmPT2 (NP_001105816), ZmPT3 (AAY42387), ZmPT4 (AAY42388), ZmPT5 (AAY42389), ZmPT6 (NP_001105776); *Brachypodium distachyon*: BdPT1 (XP_003558115), BdPT2 (XP_003560773), BdPT3 (XP_003557302), BdPT4 (XP_003558800), BdPT5 (XP_003562075), BdPT6 (XP_003573982), BdPT7 (XP_003569484), BdPT8 (XP_003573220), BdPT9 (XP_003573982), BdPT10 (XP_003581012), BdPT11 (XP_003581010), BdPT12 (XP_003581013), BdPT13 (XP_003581014); *Laccaria bicolor*: LbPT1 (XP_001889013), LbPT2 (XP_001889026), LbPT3 (XP_001889028), LbPT4 (XP_001880970), LbPT5 (XP_001888254); *Glomus intraradices*: GiPT (AAL37552); *Glomus mosseae*: GmosPT (AAZ22389); *Glomus versiforme*: GvPT (AAC49132); *Saccharomyces cerevisiae* S88c: ScPHO84 (NP_013583).

RNA isolation and quantitative reverse transcription-PCR

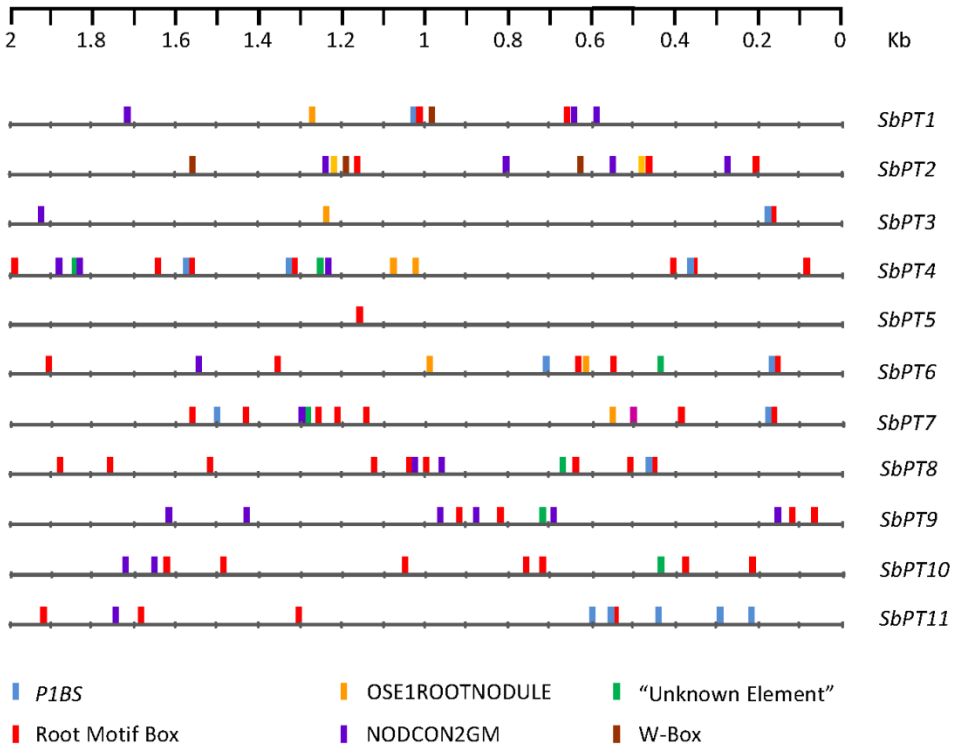
Mycorrhizal and non-mycorrhizal roots, as well as shoots, stems, stamina and pistils were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Full-length doubled-stranded cDNAs corresponding to mRNAs expressed in plant roots were obtained using the SMART–PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Quantification of AMT transcripts was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in real time quantitative PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Supplementary Data, Table 1). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C and a GC percentage > 50%. Target gene expression was normalized to the gene encoding the *S. bicolor* ubiquitin (Protein 5060159). Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. For data analysis, the geometric mean of the biological replicates (n = 4) was calculated. The primer efficiency ranged between 90% and 110%.

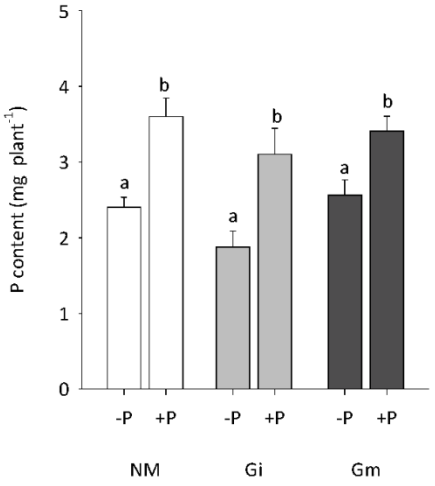
Supplementary Figures



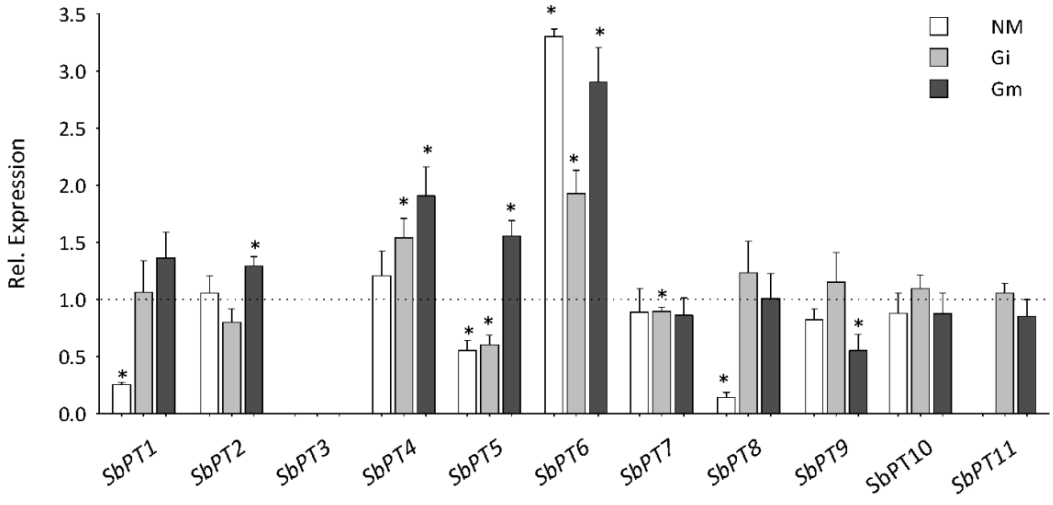
Supplemental Figure S1. Gene distribution of the 11 *Pht1* genes on the 10 chromosomes (Ch) of *Sorghum bicolor* according to (Paterson et al., 2009).



Supplemental Figure S2. Putative cis-regulatory elements involved in Pi and AM response in the promoter region of the 11 *Pht1* genes. The putative cis-regulatory elements were screened with DNA-pattern matching analysis within 2 kb upstream region of the start codon. P1BS, GNATATNC; root motif box, ATATT or AATAT; OSE1ROOTNODULE, AAAGAT; NODCON2GM, CTCTT; W-box, TTGACY and an unknown element, TCTTGTT.



Supplemental Figure S3. P content of plants as affected by Pi availability (low Pi availability (-P), high Pi availability with Pi fertilizer (+P)) in non-mycorrhizal roots (NM) and roots colonized by *Glomus intraradices* (Gi) or *G. mosseae* (Gm). Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.



Supplemental Figure S4. Quantification by RT-qPCR analysis of the transcript levels of the 11 sorghum *Pht1* genes in sorghum roots in response to Pi availability. Roots were non-mycorrhizal (NM), or colonized by G*lomus intraradices* (Gi) and *G. mosseae* (Gm). Genes were considered to be biologically regulated if affected by more than the factor two. Transcript levels in roots without any Pi fertilization were used as control values for relative expression. Transcripts were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Asterisk above bars indicate a significant difference ($P \leq 0.05$) according to Student's t-test.

Supplemental Table S1. Primers for qRT PCR analysis of Pht1 transcripts of sorghum and flax.

Name	Sequence (5'-3')
SbPT1a	GGCCAAGGTGCTCAAGAAG
SbPT1b	GGAGGAACTGCACCGAGAAG
SbPT2a	ACTAAGCAGCAGCCTCCGTA
SbPT2b	AAGCCACAAGGAAACCATTG
SbPT3a	TACTCGCGTATGAACATGCC
SbPT3b	TCCTCCTTATTGCCGATGTC
SbPT4a	GGCGCCGTCGTACCAGGACAA
SbPT4b	GAGCGCCGCCGGGATGGT
SbPT5a	GAGAATCTGGACGAGATCAC
SbPT5b	CAGGTTCTGGCTGTAGTAGG
SbPT6a	CAAGCTCGGCCGTAAGAAGG
SbPT6b	GCCAGAAGCGGAAGAAGCAC
SbPT7a	GGACACCAGCAAGGACAAC
SbPT7b	CGCGATGGAGCAGATGAC
SbPT8a	GCAGCGAGGCCAATGAGACT
SbPT8b	TTGGCTCCGGTAGGAAGCAG
SbPT9a	GAGGACGAGCCGTTCAAGAG
SbPT9b	CGCGACGGAGAAGAAGTACC
SbPT10a	CACCATGTGCTGGTTACTTC
SbPT10b	GATAATCGCCTGAGTACGTG
SbPT11a	CGTGGTTCCTTCTGGACATA
SbPT11b	TCTCGAACACCTCCTTGAGT
SbUBIa	CAAGGAGTGCCCCAACAC
SbUBIb	TGGTAGGCGGGTAAAGCAAA
LuPT1a	CGCCTGCGAAGACGATGAAC
LuPT1b	GCCGATGTGGTCGATTAGCG
LuPT2a	ATGGCTACCGCCGGCGAA
LuPT2b	GATTAGCGCGACCGTGAACCAG
LuPT3/4a	CGGGATGGTGTTTACGTTGT
LuPT3/4b	GGGCAAGTTGATTGAGTGAGT
LuPT5a	TTGCGATCGTGGTGTCGACT
LuPT5b	CGCCATACGTAGTCAGCTTGC
LuPT6/7a	GTTTTCAAAATCGCCAGAGC
LuPT6/7b	CCCTTGTGAGTCCAGTGGTT
LuPT8/9a	GATTCGAACGGGATCAAGAA
LuPT8/9b	GACGGCAACCACTTTCTCAT
LuUBIa	CTCCGTGGAGGTATGCAGAT*
LuUBIb	TTCCTTGTCCTGGATCTTCG*

*LuUBIa and LuUBI were published (Huis et al., 2010).

Supplemental Table 2. Characteristics of the *Sorghum bicolor Pht1* gene family.

Gene name	JGI protein ID	Chromo-some	Strand	Length of genomic sequence (bp)	Length of genomic sequence without UTR (bp)	Length of cDNA sequence (bp)	Length of amino acids sequence (AA)	Number and length of introns (bp)	Trans-membran domains	Suppo rting ESTs	Best blast hit with *Phts* (% ID)	Pht1 signature GGDYPLSA TIxSE
SbPT1	5050188	1	+	1929	1569	1569	523	-	12	+	ORYsa;Pht1;2 (80)	GGDYPLSA TIMSE
SbPT2	5056998	6	-	1653	1566	1566	522	-	12	+	ORYsa;Pht1;4 (82)	GGDYPLSA TIMSE
SbPT3	5030542	1	+	1602	1602	1602	534	-	12	-	ORYsa;Pht1;8 (78)	GGDYPLSA TIMSE
SbPT4	5048813	1	-	1688	1569	1569	523	-	12	+	ORYsa;Pht1;3 (88)	GGDYPLSA TIMSE
SbPT5	5050189	1	-	1735	1608	1608	536	-	12	+	ORYsa;Pht1;12 (85)	GGDYPLSA TIMSE
SbPT6	5058886	7	-	2110	1626	1626	542	-	12	+	ZEAma;Pht1;3 (87)	GGDYPLSA TIMSE
SbPT7	5048812	1	+	2025	1626	1626	542	-	12	+	ZEAma;Pht1;1 (95)	GGDYPLSA TIMSE
SbPT8	5033142	2	-	1626	1626	1626	542	-	12	+	HORvu;Pht1;8 (78)	GGDYPLSA TIMSE
SbPT9	5041365	6	+	1533	1533	1284	416	1 (249)	12	+	ORYsa;Pht1 (70)	GGDYPLSA TIMSE
SbPT10	5041362	6	-	1638	1638	1512	504	1 (126)	12	+	TRImo;Pht1 (67)	GGDYPLSA TIMSE
SbPT11	5036358	3	-	1665	1665	1578	526	1 (87)	12	+	ZEAma;Pht1;6 (87)	GGDYPLSA TIMSE

Supplemental Table S3. Characteristics of the *Linum usitatissimum Pht1* gene family.

Gene	Gene name	Scaffold	Position	Strand	Length of genomic sequence (bp)	Length of cDNA sequence (bp)	Length of Amino Acids sequence (AA)	Number and length of introns (bp)	Transmembran domains	Supporting ESTs	Pht1 signature GGDYPLSAxKSE
LnPT1	Lus10022547	465	503730-505353	-	1623	1623	541	524	12	+	GGDYPLSATMSE
LnPT2	Lus10016635	915	37088-37692	-	1605	1605	534	1539	12	+	GGDYPLSATMSE
LnPT3	Lus10011826	610	397068-398687	-	1620	1620	539	1557	12	+	GGDYPLSATSME
LnPT4	Lus10021191	11	355866-357506	-	1641	1641	546	1614	12	-	GGDYPLSATSME
LnPT5	Lus10014754	584	518975-520585	-	1611	1611	536	1551	12	-	GGDYPLSATMSE
LnPT6	Lus10033886	222	942722-944490	+	1769	1617	538	1584	12	+	GGDYPLSATMSE
LnPT7	Lus10003560	669	111348-113104	-	1757	1620	539	1569	12	+	GGDYPLSATMSE
LnPT8	Lus10012860	1313	225511-227157	+	1647	1647	548	1554	12	+	GGDYPLSAVMSE
LnPT9	Lus10030506	917	487461-489113	+	1653	1653	550	1623	12	+	GGDYPLSAVMSE

Supplemental Table S4. Similarity/identity matrix of *Sorghum bicolor* Pht1 protein sequences.

	% of Identity										
	SbPT1	SbPT2	SbPT3	SbPT4	SbPT5	SbPT6	SbPT7	SbPT8	SbPT9	SbPT10	SbPT11
SbPT1		70.2	68.2	83.6	73.6	72.7	73.9	57.7	53.7	53.1	56.4
SbPT2	79.2		68.4	71.8	71.6	73.9	73.7	61.5	54.6	53.3	52.9
SbPT3	82	80.7		68.5	68.4	74.8	69.1	56.8	51.7	50.9	51.5
SbPT4	91.4	83.1	82.7		75	73.4	75	59.4	54.6	53.3	57
SbPT5	83.6	83.4	81.5	85		74.5	80.7	60	55	52.5	55.7
SbPT6	82.6	84.3	86.3	84.1	84.3		78.4	61.2	55.6	53.1	55.7
SbPT7	84.1	86	82.8	86.3	88.4	87.8		60.5	55.9	54.3	58.2
SbPT8	73.8	75.8	74.5	75.2	76.5	77.1	77.6		49	47.5	49.4
SbPT9	70.5	69.8	69.2	71.1	69.9	70.4	70.6	64.9		55.6	46.7
SbPT10	69.5	71.8	69.2	69.5	69	70.5	71.2	67.3	71.7		47.5
SbPT11	70.9	70.4	69.5	70.4	70.9	71.1	73.3	67.9	62.3	66.4	
	% of Similarity										

Supplemental Table S5. Similarity matrix of *Linum usitatissimum* Pht1 protein sequences.

	LuPT1	**LuPT2**	**LuPT3**	**LuPT4**	**LuPT5**	**LuPT6**	**LuPT7**	**LuPT8**	**LuPT9**
LuPT1	-	80.2	63.1	62.1	60.4	61.7	62.4	46.4	45.4
LuPT2		-	79.5	78.2	76.9	75.8	76.7	55.7	54.8
LuPT3			-	**96.5**	73.5	74.5	74.3	57.7	57.3
LuPT4				-	74.5	73.5	73.9	56.5	56.1
LuPT5					-	79.6	79.7	56.2	55.8
LuPT6						-	**97.9**	56.9	56.9
LuPT7							-	56.5	56.2
LuPT8								-	**97.8**
LuPT9									-

Appendix II: Special techniques

I. Laser microdissection

C. Arnould[1], S. Koegel[2], L. Geay[1], M. Kälin[2], B. Valot[1], C. Henri[1], D. Wipf[1], E. Dumas-Gaudot[1], P-E. Courty[2]

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II. Artificial microRNA

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I. Studying gene expression using microdissection technology

Plant organs are made of different tissues with different cell types and functions. The processes happening in these tissues are distributed non-uniformly among cells. However, due to technical constraints, most studies are done on whole organs (Schnable *et al.*, 2004) and information about the contribution of individual cells to the biology of the organism is not provided (Day *et al.*, 2005). A good example is AM plants: to study AM processes, most of the approaches are made on whole plant or whole root RNA extracts (Hohnjec *et al.*, 2005; Hildebrandt *et al.*, 2007). However, only parts of the root cells contain mycorrhizal structures. Laser microdissection technology (LM) is a reliable method to study differences between cell populations (Nelson *et al.*, 2006). This technology was developed firstly to isolate selected human cell populations from heterogeneous tissues (Emmert-Buck *et al.*, 1996) and is now routinely used in pathology or in cancer biology research (Fend & Raffeld, 2000; Gillespie *et al.*, 2001). A big advantage of LM is that it does not require cell-specific markers or genetic lines and tissues can be viewed by conventional microscopy. In plant research, LM protocols for subsequent gene analyses have been developed for several plant species, including rice, maize and Arabidopsis (Asano *et al.*, 2002; Nakazono *et al.*, 2003; Casson *et al.*, 2005). Additionally, LM protocol for subsequent protein analyses has been developed for Arabidopsis (Schad *et al.*, 2005): after isolation of the cells and protein extraction, liquid chromatography combined with tandem mass spectrometry was adapted to study tissue specific protein repartition. LM was applied for the first time to study gene regulation in AM symbiosis by Balestrini *et al.* (2007): they analyzed gene expression in tomato roots colonized by *G.mosseae*. Then, Gomez & Harrison (2009) developed a method to collect cortical cells from *M. truncatula* colonized by *G. versiforme*. These studies confirmed the suitability of LM to study gene expression during AM symbiosis (Gomez *et al.*, 2009).

We developed high efficiency LM protocols for sorghum, medicago and poplar mycorrhized and non-mycorrhized by AM fungi. Selected and collected cells are of high quantity and quality for RNA or protein analyses. Indeed, RNA concentrations reached 3240 pg/µl for sorghum, 1728 pg/µl for medicago and 1282 pg/µl for poplar. RNA integrity numbers (RIN) upper 7 were obtained for all plants revealing a high quality of RNA extracts.

Additionally, we could analyze by tandem mass spectrometry more than 200 proteins isolated from mycorrhized and non-mycorrhized medicago plants. A poster from Arnould *et al.* 2012 showing these results is presented below.

LA MICRODISSECTION LASER

UNE TECHNIQUE D'ANALYSE DU PROFIL D'EXPRESSION GÉNIQUE ET PROTÉIQUE DES TYPES CELLULAIRES SPÉCIFIQUES DE LA SYMBIOSE MYCORHIZIENNE À ARBUSCULES

C. Arnould[1], S. Koegel[2], L. Geay[1], M. Kälin[2], B, Valot[3], C. Henri[3], D. Wipf[1], E. Dumas-Gaudot[1], P-E. Courty[2]

[1]UMR Agroecologie, Dijon, France; [2] Université de Bale, Suisse ; [3] PAPPSO, Jouy en Josas, France

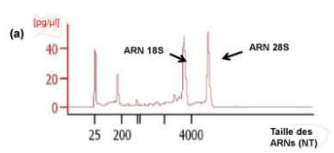
Introduction

La capture de cellules par microdissection laser (MDL) permet de récolter une population homogène de cellules provenant d'un même tissu. Dans l'étude de la symbiose mycorhizienne à arbuscule, cette technique permet de comparer différentes populations cellulaires: cellules contenant des arbuscules, cellules encore non-colonisées ou cellules de l'ectoderme.

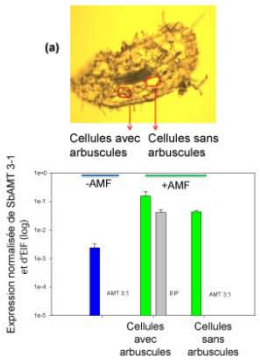
Notre étude porte sur la mise en place d'un protocole de routine sur 3 plantes modèles utilisées dans l'étude de la symbiose mycorhizienne à arbuscules : une plante pérenne (Populustrichocarpa, le peuplier), une plante fourragère (Medicago truncatula, la luzerne) et une céréale (Sorghumbicolor, le sorgho), et ce pour une plateforme de MDL de type Arcturus. Toutes ces plantes sont associées avec le même champignon, Rhizophagusirregularis.

Matériel et Méthodes

Analyses des ARN après MDL



	Qualité des ARNs (RIN*)	Concentration pg/µl
Luzerne	7,4	1728
Peuplier	7,8	1282
Sorgho	7,7	3240



Qualité et quantité des ARNs obtenus (BioAnalyseur Agilent)

(a) Electrophérogrammme indiquant la quantité des ARNs en fonction de leur taille

(b) Tableau récapitulatif (n=3).

*RIN (RNA integrity number): nombre compris entre 0 et 10. Lorsque RIN >7: haute qualité

Expression du transporteur d'ammonium SbAMT3; 1 induit par la mycorhization

(a) Coupe de racine de Sorgho mycorhizée

(b) Expression normalisée de SbAMT3.1. Gène contrôle: ubiquitine. EIF: facteur d'élongation de R. irregularis utilisé pour confirmer l'absence d'AMF (Arbuscule Mycorhization Fungus) dans les cellules sans arbuscules.

Analyses des protéines après MDL

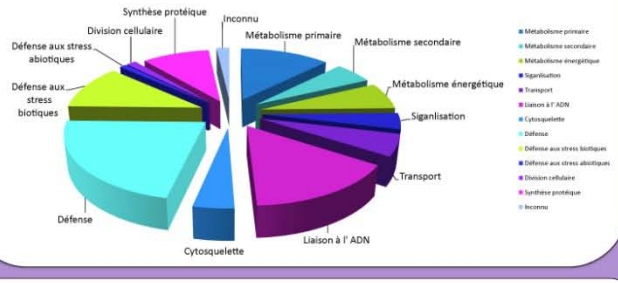
Les analyses ont été faites à partir d'échantillons microdisséqués de luzerne mycorhizée

Résultats quantitatifs (BioAnalyseur Agilent) : Rendements 80ng/µl

Résultats qualitatifs obtenus après spectrométrie de masse nanoLC MS/MS - ETD (QExactive)

Paramètres d'interprétation :
Espèce: M. truncatula, Version: 3,5v3 (47529 entrées)
Web site : http://www.jcvi.org/cgi-bin/medicago/annotation.cgi
Logiciel : Xtandem (Release 2011.12.01.1)
Filtrage : Protéines : log (evalue) < -4; minimun 2-3 peptides avec evalue < 0.03 pour valider une protéine ou isoforme

Les premiers résultats, portant sur la microdissection de 19 939 320 µm2 de cellules contenant des arbuscules de racines de M. truncatula, ont permis d'identifier 232 protéines qui ont été classées par catégories fonctionnelles représentées sur la figure suivante :



Conclusions et Perspectives

- La méthode développée pour les ARNs est utilisée en routine et permet d 'étudier finement l'expression de gènes par approche ciblée (qRT-PCR) ou globale (Puces génome entier).
- La méthode développée pour les protéines est prometteuse mais nécessite encore des ajustements pour extraire et solubiliser davantage les protéines membranaires et faire le différentiel entre les divers types cellulaires (cellules corticales avec ou sans arbuscules de racines colonisées et cellules corticales de racines non colonisées).

Remerciements

PEC remercie l'European Science Foundation (ESF) et le projet « Nitrogen in Europe:Assessment of current problems and futur solutions » pour son soutien financier . Les auteurs remercient également le projet Franco-Suisse Germaines de Stael. PEC et SK sont soutenu par le SNF(projet Ambizione PZ00P3_136651 et le projet SNF 130794). DW, EDG, LG et CA sont soutenus par le Conseil Régional de Bourgogne et l'Agence Nationale pour la Recherche, Le Centre de Microscopie CM/uB est remercié pour la mise à disposition du microscope à dissection laser.

II. Studying gene function using amiRNA technology

Pathogen-derived resistance was observed in plants expressing virus derived genes (Wilson, 1993). This mechanism could be explained by the process of post-transcriptional gene silencing reported in transgenic plants containing sense and antisense transgenes. Indeed, plants expressing a virus-derived sense or antisense RNA had virus resistance induced by post-transcriptional gene silencing (Baulcombe, 1996; Waterhouse *et al.*, 1998). This finding has lead to the production of virus-resistant potato plants for commercial use (Baulcombe, 1996). The mechanism of post-transcriptional gene silencing was then used to study the function of specific genes by silencing them. Recently, artificial microRNA (amiRNA) technology has been developed to silence precisely single or multiple genes in plants. It exploits endogenous microRNA (miRNA) precursors to generate *in vivo* a single specific miRNA (Niu *et al.*, 2006; Small, 2007; Tang *et al.*, 2007; Ossowski *et al.*, 2008). This miRNA targets messenger RNA (mRNA) and cleaves it at position 10 and 11 (Llave *et al.*, 2002) silencing the gene. The amiRNA must not be perfectly complementary to the target site to cleave it meaning that one amiRNA can be designed to target one or several genes (Alvarez *et al.*, 2006; Schwab *et al.*, 2006). Additionally, the effect of one amiRNA can be augmented by using a strong promoter. amiRNA were efficiently used in the dicotyledonous plants Arabidopsis, tomato or tobacco (Alvarez *et al.*, 2006; Schwab *et al.*, 2006; Qu *et al.*, 2007) but also in monocotyledonous rice plants (Warthmann *et al.*, 2008). amiRNA targeting rice genes can be designed at the WMD3 interface (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The importance of specific fungal genes for AM symbiosis was assessed by host-induced gene silencing: Helber et *al.* (2011) produced *M. truncatula* plants silencing a monosaccharide transporter (MST2) from *Glomus* sp DAOM 197198. When colonizing the transformed plants, expression of MST2 was significantly reduced and AM fungal growth arrest was observed.

Appendix III: Post transcriptional gene silencing of AMT3;1 and AMT4 in rice using amiRNA technology

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Methods and Preliminary results

Abstract

Arbuscular mycorrhizal (AM) fungi are important in plant Nitrogen (N) acquisition. Recent studies shown the transport of N in form of ammonium during AM symbiosis. Here, we hypothesize that induction of specific ammonium transporters (AMT) genes in Rice (*Oryza sativa*) during AM colonization might play a key role in functionality of the symbiosis. Rice is an important crop forming a symbiosis with arbuscular mycorrhizal (AM) fungi. We identified ten ammonium transporters (AMTs) in rice. Transcript profiling using quantitative real time PCR revealed that two AMTs (AMT3;1 and AMT4) are induced when rice roots are colonized by the AM fungi *Glomus mosseae* and *Glomus intraradices* ("AM-inducible AMTs**"**). Yeast complementation with a yeast mutant that lacks high-affinity ammonium transporters (see chapter 3) shown that AMT3;1 has high affinity transport activity. By knocking down AMT3;1 and AMT4 through amiRNA technology, we want to assess the physiological role of these AM-inducible AMTs in the functionality of AM symbiosis. Our working hypothesis is that the transformants will show a reduction of ammonium uptake via AM fungi and growth retardation.

Introduction

Rice (*Oryza sativa)* is the second world-leading crop (http://faostat3.fao.org/home/index.html). It can grow in wet environments where no other crops are able to grow. More than one billion people over the world depend on rice cultivation for their livelihood. In nature, many AM fungal species colonize rice roots (Solaiman & Hirata, 1995). AM fungi help the plant acquiring key mineral nutrients as Phosphorus (P) or Nitrogen (N) (Smith & Read, 2008). For this reason, AM fungi are of great interest for sustainable agriculture and have been the focus of many studies in the last decades (Abbott & Robson, 1982; Mosse, 1986; Werner *et al.*, 2005; Sawers *et al.*, 2008).

The role of AM fungi in plant N nutrition has been studied. AM fungi were shown to take up inorganic N as ammonium or nitrate (Govindarajulu *et al.*, 2005), as well as organic N in the form of amino acids (Leigh *et al.*, 2009). Once internalized, N is assimilated and translocated to the intraradical hyphae in form of amino acids, mainly in the form of arginine (Govindarajulu *et al.*, 2005) and finally transferred to the plant as ammonium (Tian *et al.*, 2010). In a root cell with an arbuscule, this transfer is expected to proceed by way of secretion of ammonium through unknown transporters present in the fungal plasma membrane into the periarbuscular space, followed by uptake through plant ammonium transporters (AMT) in the periarbuscular membrane. The periarbuscular membrane is derived from the plasma membrane and is the place where the plants takes up nutrients delivered by the fungi (Harrison *et al.*, 2002b; Kobae & Hata, 2010).

Bioinformatic analyses of the rice genome revealed 10 genes coding for AMTs. The transcript level of these transporters was measured in rice in the presence or absence of AM fungi under different regimes of N nutrition and in different plant tissues as root, shoot, stem, stamina and pistils. Two AMTs, OsAMT3;1 and OsAMT4, were expressed predominantly in roots and were induced in the presence of AM fungi independently of the N treatment. OsAMT3;1 was functionally characterized in a yeast mutant lacking high-affinity ammonium transporters. A computer simulation of the pore also confirmed its faculty to transport ammonium (see chapter 3).

Mutation of AM-induced transporters can bring new information about the role of these transporters in AM symbiosis. Indeed, rice plants lacking PT11, the PHT responsible for symbiotical P transfer in rice, shown a very reduced colonization and arbuscule level (Yang *et al.*, 2012). Similarly, *M. truncatula*

mutants lacking MtPT4, a PHT located at the periarbuscular membrane and essential for symbiotic P transport (Harrison *et al.*, 2002b), have arbuscule degenerating prematurely (Javot *et al.*, 2007).

Recently, artificial microRNA (amiRNA) technology has been developed to silence precisely single or multiple genes in plants. Before, posttranscriptional gene silencing was reported in transgenic plants containing sense and antisense transgenes. Indeed, plants expressing a virus-derived sense or antisense RNA had virus resistance induced by posttranscriptional gene silencing (Baulcombe, 1996; Waterhouse *et al.*, 1998). These findings lead to the use of posttranscriptional gene silencing to study the function of specific genes by silencing them. amiRNA technology exploits endogenous microRNA (miRNA) precursors to generate *in vivo* a single specific miRNA (Niu *et al.*, 2006; Small, 2007; Tang *et al.*, 2007; Ossowski *et al.*, 2008). This miRNA targets messenger RNA (mRNA) and cleaves it at position 10 and 11 (Llave *et al.*, 2002) silencing the gene. amiRNA targeting rice genes can be designed at the WMD3 interface (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Here, we tried to understand the mechanism regulating the mutualistic symbiosis between plants and AM fungi. By collaborating with REFUGE, an international hosting platform located in Montpellier, France we tried to elucidate gene function through functional genomics strategies using artificial miRNA technology. We transformed rice plants (*Oryza sativa*) to obtain plants silencing AMT3;1 and AMT4. We hypothesize that rice plant lacking an AM-inducible AMT will have a reduced N transfer through AM fungi. Additionally, changes in the arbuscule architecture and abundance are expected. We expect that our finding will help to understand more precisely the cross-talk between plant and fungi, and the way the mutualistic AM symbiosis stayed stable over the past 450 million years.

Material and Methods

Identification and characterization of rice AMT genes

Sequences from the *O. sativa* genome database were: OsAMT1;1 (Q7XQ12), OsAMT1;2 (Q6K9G1), OsAMT1;3 (Q6K9G3), OsAMT2;1 (Q84KJ7), OsAMT2;2 (Q8S230), OsAMT2;3 (Q8S233), OsAMT3;1 (Q84KJ6), OsAMT3;2 (Q851M9), OsAMT3;3 (Q69T29), OsAMT4;1 (Q10CV4).

For phylogenetic analysis, the AMTs amino acid sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbor joining trees were constructed using Poisson correction model for distance computation in MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was carried out with 1000 replicates. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Gene accession numbers of amino acids sequences are *Populus trichocarpa*: PtrAMT1;1 (B9HSW3), PtrAMT1;2 (B9IPE2), PtrAMT1;3 (B9HKW8), PtrAMT1;4 (B9GRB5), PtrAMT1;5 (B9GRB4), PtrAMT1;6 (B9HP47), PtrAMT2;1, (B9HCZ0), PtrAMT2;2 (B9IGE2), PtrAMT3;1 (B9GHA5), PtrAMT4;1 (B9GS88), PtrAMT4;2 (B9IKS2), PtrAMT4;3 (B9H8E7), PtrAMT4;4 (B9I5F0), PtrAMT4;5 (B9MX92). *Arabidopsis thaliana* (at NCBI): AtAMT1;1 (At4g13510), AtAMT1;2 (At1g64780), AtAMT1;3 (At3g24300), AtAMT1;4 (At4g28700), AtAMT1;5 (At3g24290), AtAMT2;1 (At2g38290), *Sorghum bicolor*: SbAMT1;1 (Sb06g022230), SbAMT1;2 (Sb04g026290), SbAMT2;1 (Sb09g023030), SbAMT2;2 (Sb03g038840), SbAMT3;1 (Sb03g041140), SbAMT3;2 (Sb01g001970), SbAMT3;3 (Sb04g022390), SbAMT4 (Sb01g008060), *Nitrosomona europeae:* NeAMT/Rh1 (Q82X47), *Escherichia coli:* EcAMTB (P69681). The accession numbers of the Phytozome 6.0 database were used for soybean (*Glycine max*) as follows: GmAMT1.1 (Glyma20g21030.1), GmAMT1;2 (Glyma10g26690.1), GmAMT1;3 (Glyma10g31080.1), GmAMT1;4 (Glyma10g31110.1), GmAMT1;5 (Glyma1031130.1), GmAMT1;6 (Glyma20g36390.1), GmAMT2;1 (Glyma07g18670.1), GmAMT2;2 (Glyma18g43540.1), GmAMT2;3 (Glyma01g30920.1), GmAMT3;1 (Glyma05g33010.1), GmAMT4;1 (Glyma09g41810.1), GmAMT4;2 (Glyma20g00680.1), GmAMT4;3 (Glyma19g43380.1), GmAMT4;4 (Glyma02g04960.1), GmAMT4;5 (Glyma02g16200), GmAMT4;6 (Glyma10g03600.1).

Plant growth conditions for tissue analysis and to test functionality of the constructs

Seeds from *O. sativa* (cultivar Baviva) were surface sterilized (10min in 2.5% KClO) and then rinsed with sterile deionized water several times during one day and soaked in sterile deionized water over night. Seeds were pre-germinated on autoclaved Terra Green (Oil Dri US-special, american Aluminiumoxide, type III/R; Lobbe Umwelttechnik, Iserlohn, Germany) at 25°C for 24h and then grown in the dark at room temperature for 72h. *G. mosseae* ISCB13 (Botanical Institute, Basel, Switzerland) fungal strain was propagated by trap cultures set up as previously described in detail (Oehl *et al.*, 2004). To establish AM symbiosis, pre-germinated seeds were individually inoculated at the same time in pots containing a mixture of acid washed Terragreen, sand and loess soil (5:4:1 w/w/w). About 100 spores were added to the mixture. For the controls (non-mycorrhizal plants), the same amount of autoclaved inoculum was added to the mixture. To correct for possible differences in microbial communities, each pot received one ml of filtered washing of AM fungal inoculum (van der Heijden *et al.*, 1998). Plants were grown in a greenhouse with day-night temperatures of ca. 28°C and ca. 15°C, respectively, photoperiod of 12h and humidity of 80%.

Plants were watered twice a week during experiments. From the first week on, 8 ml of modified Hoagland solutions, after Gamborg & Wetter (1975), were applied weekly. Two different Hoagland solutions were applied: "+N" ($Ca(NO_3)_2 \cdot 4H_2O$, KNO_3 and $NH_4H_2PO_4$ from the original solution were replaced by $CaCl_2 \cdot 2H_2O$, KCl, NH_4Cl and KH_2PO_4) and "-N" ($Ca(NO_3)_2 \cdot 4H_2O$, KNO_3 and $NH_4H_2PO_4$ from the original solution were replaced by $CaCl_2 \cdot 2H_2O$, KCl and KH_2PO_4). Additionally, for all the solutions, $(NH_4)_2MoO_4$ was replaced by Na_2MoO_4. Shoots and roots were harvested separately 13 weeks after inoculation.

Staining of AM fungi in plant roots and quantification of root colonization

From each analyzed plant, one subsample of 100mg of fresh roots was used to determine AM fungal colonization. Root subsamples were stained with trypan blue (0.005% w/v in lactic acid, glycerol, water, 1:1:1, w:w:w) at 60°C for 10 minutes in 15ml tubes in a water bath and destained 24h in glycerol: 1%HCl (w:w). Root colonization was quantified according to the grid intersection method as described by Brundrett *et al.* (1984). Total colonization comprised intersections containing hyphae, vesicles, spores or arbuscules. Differences between means of variables were analyzed by ANOVA ($p \leq 0.05$), using SPSS 18.0.

Samples, RNA isolation and quantitative reverse transcription-PCR

Mycorrhizal and non-mycorrhizal roots as well as leaves stem, stamina and pistils were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Full-length doubled-stranded cDNAs corresponding to mRNAs expressed in plant roots were obtained using the SMART–PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Quantification of AMT transcripts was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in real time quantitative PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Tab.1). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C and a GC percentage > 50%. Target gene expression was normalized to the gene encoding the *O. sativa* ubiquitin. Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. For data analysis, the geometric mean of the biological replicates (n = 4) was calculated. The primer efficiency ranged between 90% and 110%.

Designing amiRNA

Rice gene sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) and from TIGR5 for WMD (http://www.tigr.org/tdb/e2k1/osa1). Sequence homology of miRNA precursors to available rice cDNAs was determined by BLAST and multiple alignments were performed using Mega4 (Tamura *et al.*, 2007). The possible amiRNA candidate sequences were generated using WMD (Schwab *et al.*, 2006; Ossowski *et al.*, 2008). WMD3 (http://wmd3.weigelworld.org/) supports different plant species including *Oryza sativa* (TIGR v5) and designs 21bp sequences directed against one or several genes. The program suggests suitable amiRNA candidates after a two-step selection process based on empirically established

criteria for efficiency and specificity while minimizing possible off-target effects to other genes in the rice genome. The hybridization energy was chosen between -35 and -38 kCal.

amiRNA Constructs

According to (Warthmann *et al.*, 2008), two (for AMT4) and three (for AMT3;1) 21 bp amiRNAs were selected to target different sites in the target mRNA. Additionally, appropriate amiRNA* sequences that would in pairing to the amiRNA exactly mimic the foldback structure of the endogenous OsAMT3;1 and OsAMT4 were designed. Each primary amiRNA construct was engineered from the vector pNW55, replacing the 21 bp of the natural osa-MIR528 miRNA from pNW55 as well as the partially complementary region of the miRNA* by modification PCRs in a similar way as described earlier (Schwab *et al.*, 2006), following the PCR scheme in Figure 4.1. In detail, for each miRNA construct three modification PCRs were performed with primers G-4368+II, I+IV and III+G-4369 on pNW55 as template, yielding fragments of 256, 87 and 259 bp lengths, respectively. Primer I contains the amiRNA in sense orientation, primer II its reverse complement, primer III the amiRNA* sequence in sense and primer IV the amiRNA* sequence in antisense orientation. The primers G-4368 and G-4369 are vector primers and were the same for all amiRNA constructs. amiRNA primers were designed using the primer design function of WMD3. A list of all primers can be seen in Table 1. The three resulting fragments were gel purified with Zymoclean Gel DNA Recovery Kit (Zymo Research) and then fused by one PCR with the two flanking primers G-4368 and G-4369 on a mixture of 1 µl from each previous PCR as template. All PCRs were performed with Phusion DNA Polymerase (Biolabs) in a volume of 50 µl according to the manufacturer's recommendation: The fusion product of 554 bp was again gel purified (Zymo Research), cloned using the BP clonase step from Gateway Cloning Technology (Invitrogen) in a pC2300overExp vector (from Emmanuel Guiderdoni, CIRAD, France). The sequence was verified by excised with HindIII/Acc65I and sequencing. All five amiRNA plant expression vectors were transformed into *Agrobacterium tumefaciens*.

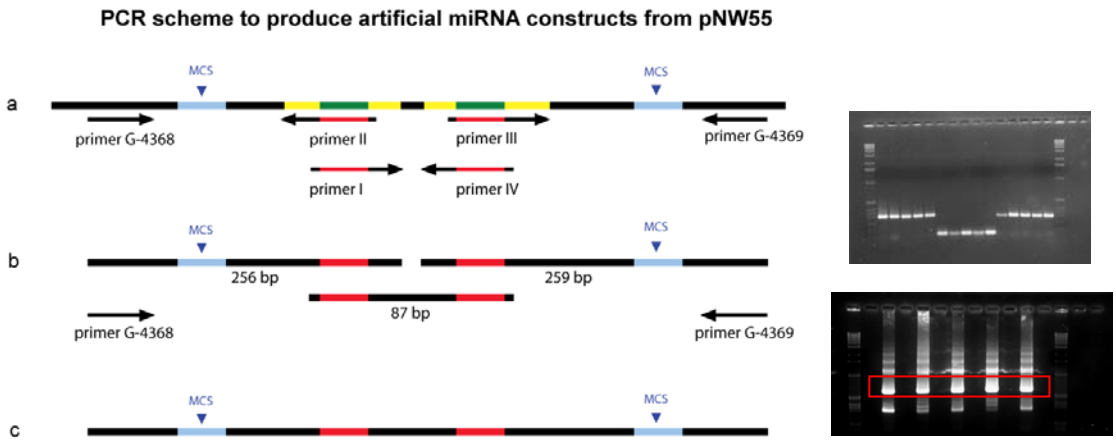

Figure 4.1: PCR scheme to produce artificial miRNA constructs from pNW55. (a) The original miRNA 528 and miRNA* sequences of pNW55 (green) are replaced by the amiRNA sequences (red) designed for OsAMT3;1 and OsAMT4 during the first PCRs. Sequences in pNW55 complementary to the primers are in yellow and multiple cloning sites in blue. (b) The three PCRs on pNW55 as template (G-4368 + II, I + IV, III + G-4369) result in 3 DNA fragments. (c) Fusion PCR of the 3 PCR products from (b) with the primers G-4368 + G-4369 results in one DNA fragment for subsequent cloning. After Warthmann *et al.* (2008)

Rice transformation and culture

Rice seeds from the variety Nipponbare (japonica) were transformed with the transgenes according to modified protocols from Hiei *et al*. (1994) developed by the CIRAD Montpellier and selected on hygromycin (Fig. 4.2). Control plants were obtained by transformation with the empty binary vector pC2300overExp. All regenerated T_0 transgenic plants were genotyped for the presence and the number of transgene copies. Plants with one or two copies were selected for seed production. Plants were grown in a greenhouse at the CIRAD Montpellier with 12h photoperiod at 28 °C (day), 23 °C (night) and 80-90% humidity.

Table 4.1: Primer sequences for direct mutagenesis of pNW55 and for quantitative real time PCR

OsAMT3;1	SK_AI	agTTAGAACTGCGTCGTTTCCGGcaggagattcagtttga
	SK_AII	tgCCGGAAACGACGCAGTTCTAActgctgctgctacagcc
	SK_AIII	ctCCGGATACGTCGCAGTTCTAAttcctgctgctaggctg
	SK_AIV	aaTTAGAACTGCGACGTATCCGGagagaggcaaaagtgaa
	SK_BI	agTACGACATGTTGTAGCGCCCGcaggagattcagtttga
	SK_BII	tgCGGGCGCTACAACATGTCGTActgctgctgctacagcc
	SK_BIII	ctCGGGCCCTAGAACATGTCGTAttcctgctgctaggctg
	SK_BIV	aaTACGACATGTTCTAGGGCCCGagagaggcaaaagtgaa
	SK_CI	agTCATCCACAGTATGGCGCCGCcaggagattcagtttga
	SK_CII	tgGCGGCGCCATACTGTGGATGActgctgctgctacagcc
	SK_CIII	ctGCGGCCCCAAACTGTGGATGAttcctgctgctaggctg
	SK_CIV	aaTCATCCACAGTTTGGGGCCGCagagaggcaaaagtgaa
OsAMT4	SK_DI	agTTATCCATATCATGTCGTCGAcaggagattcagtttga
	SK_DII	tgTCGACGACATGATATGGATAActgctgctgctacagcc
	SK_DIII	ctTCGACCACAAGATATGGATAAttcctgctgctaggctg
	SK_DIV	aaTTATCCATATCTTGTGGTCGAagagaggcaaaagtgaa
	SK_EI	agTACGTTGTTCGGAAGGGACGCcaggagattcagtttga
	SK_EII	tgGCGTCCCTTCCGAACAACGTActgctgctgctacagcc
	SK_EIII	ctGCGTCGCTTGCGAACAACGTAttcctgctgctaggctg
	SK_EIV	aaTACGTTGTTCGCAAGCGACGCagagaggcaaaagtgaa
pNW55	attB1-G-4368	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGCAAGGCGATTAAGTTGGGTAAC
	attB2-G-4369	GGGGACCACTTTGTACAAGAAAGCTGGGTGCGGATAACAATTTCACACAGGAAACAG
qPCR primers	OsAMT3;1Fw	CATCACGCTCATCCTCCTC
	OsAMT3;1R	CCTCTCCCTGTCCTTGGTC
	OsAMT4Fw	GTGATCCTGTACGGCGACTG
	OsAMT4R	AACGCCTCCCTGTCCTTCTC

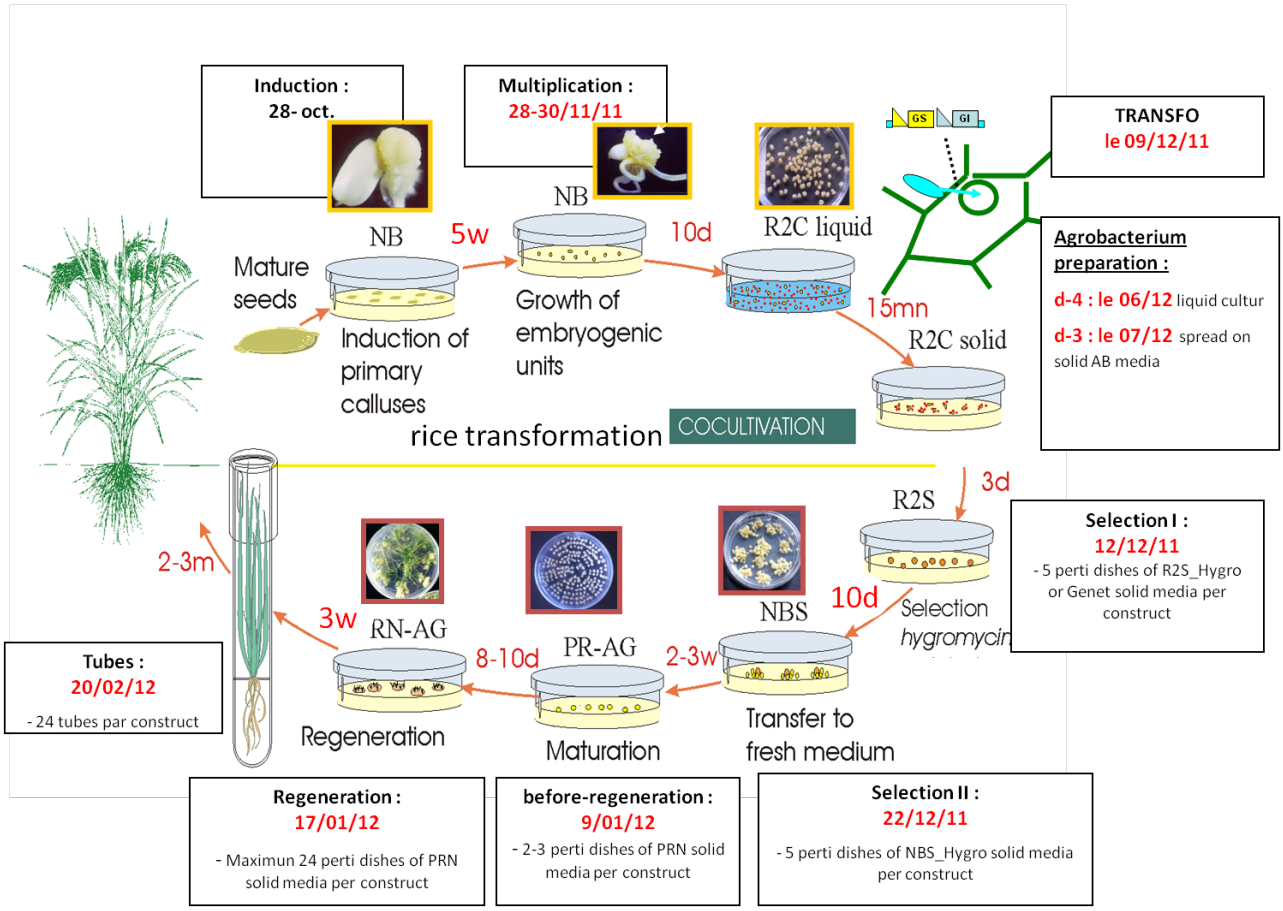

Figure 4.2: Transformation cycle developed by the CIRAD Montpellier after Hiei et al. (1994). The date show the time at which the different steps were executed for the transformation with the amiRNA targeting OsAMT3;1 and OsAMT4.

Preliminary results

AM-inducible AMTs in rice

Two rice AMTs, OsAMT3;1 and OsAMT4 were induced upon colonization by *G. mosseae* (Fig. 4.3). Both also shown a higher expression in mycorrhized roots compared to other tissues (Fig. 4.4). OsAMT3;1 was detected in all the tissues studied in contrary to OsAMT4 that was not detected in the stem and in the pistils.

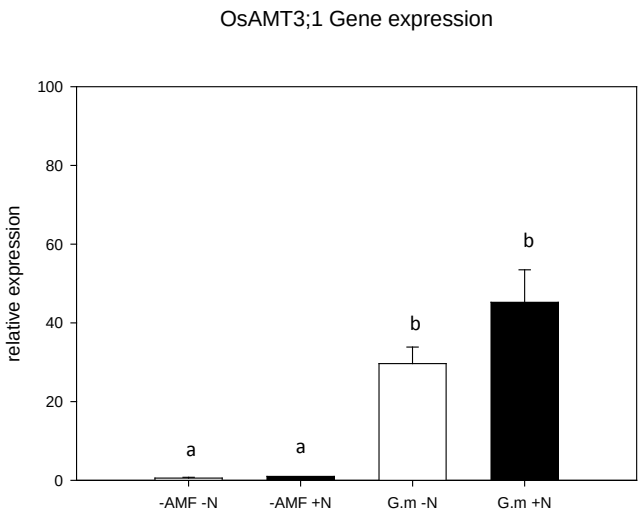

Figure 4.3. Quantification by qRT-PCR of the transcript levels of *OsAMT3;1* and *OsAMT4* in *Oryza sativa* roots either non-colonized or colonized by arbuscular mycorrhizal fungi (Gm; *Glomus mosseae*) 9 weeks post-inoculation in the different N treatments (-N and +NO_3^-).

The values are the means of four replicates. Ubiquitin was used as the reference transcript. Gene expression was normalized to the "-AMF, 1×NO_3^-" treatment. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F test). Letters indicate a *p*-value < 0.05.

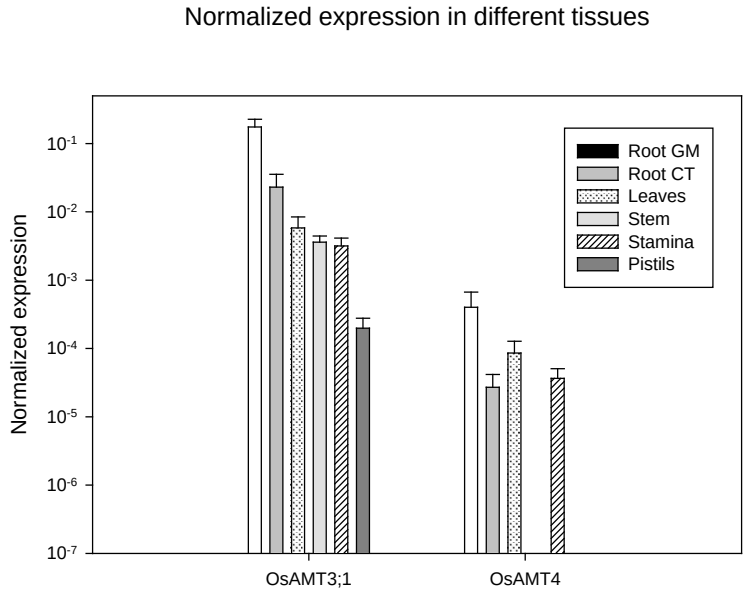

Figure 4.4. Quantification by qRT-PCR of the transcript levels of the two *Oryza sativa* AM-inducible AMT genes (AMT3; and AMT4) in different tissues. The values are the means of three replicates. Ubiquitin was used as the reference transcript. GM: *G. mosseae*, CT: control.

amiRNA constructs

Three amiRNA constructs were prepared for OsAMT3;1 and two amiRNA constructs were prepared for OsAMT4 (Tab.4.2). The amiRNA were selected to target different sites in the target mRNA (Tab.4.1).

Regeneration and selection of amiRNA

After transformation with Agrobacterium, selection with hygromycin was performed. Selected cali were regenerated and placed in tubes for 3 month. After this time, the regenerated plants were transferred to the greenhouse for seed production. Loss of plant material happened at every step and is resumed for each amiRNA construct separately in Tab. 4.2. Between 18 and 23 plants per amiRNA constructs had one to two insertions of the amiRNA constructs. These plants were selected to test functionality of the construct.

Table 4.2: Summary of the number of plants at each critical step of the regeneration and selection

	Number of regenerated cali	Number of plants transferred to the greenhouse	Number of plant producing seeds	Number of plants with one or two copies of the amiRNA
AMT3;1_1	30	26	24	23
AMT3;1_2	32	29	24	18
AMT3;1_3	32	29	25	20
AMT4_1	31	25	24	19
AMT4_2	32	24	24	18
Empty vector (ROE)	32	24	23	14

Functional constructs

To test the functionality of the construct, we will set up mycorrhized and non-mycorrhized pots with the 61 AMT3;1 mutants and 37 AMT4 mutants obtained. Phenotype of colonization and gene expression will be assessed.

4.5 Acknowledgments

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