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Review

Arbuscular mycorrhizal symbiosis and plant aquaporin expression

Norbert Uehlein [a], Kerstin Fileschi [a], Martin Eckert [a], Gerd Patrick Bienert [b], Adam Bertl [a], Ralf Kaldenhoff [a,*]

[a] *Darmstadt University of Technology, Institute of Botany, Applied Plant Sciences, Schnittspahnstrasse 10, D-64287 Darmstadt, Germany*
[b] *Plant and Soil Sciences Laboratory, Department of Agricultural Sciences, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*

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Abstract

Almost all land plants have developed a symbiosis with arbuscular mycorrhizal fungi. Establishment of the association is accompanied by structural changes in the plant root. During arbuscule formation fungal hyphae penetrate the root apoplast and install highly specialized interfaces for solute transport between plant and fungus. The periarbuscular membrane which is part of the plant plasma membrane surrounding arbuscular structures was shown to harbour a high density of different transport systems. Among these also expression of aquaporins was described, which potentially can act as a low affinity transport system for ammonia or ammonium. The present study provides data for expression, localization and function of plant aquaporins in the periarbuscular membrane of mycorrhizal *Medicago truncatula* plants.

Keywords: *Medicago truncatula*; Mycorrhiza; Aquaporin; Ammonia; Ammonium; Nodulin; Periarbuscular membrane

Contents

1. Introduction

1.1. Mycorrhiza

Some 80% of land plant species can form a symbiosis with arbuscular mycorrhizal (AM) fungi (Newman and Reddell, 1987). Establishment of arbuscular mycorrhiza

* Corresponding author. Tel.: +49 6151 163805; fax: +49 6151 163836.
E-mail address: kaldenhoff@bio.tu-darmstadt.de (R. Kaldenhoff).

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induces complex changes in both morphology and nutritional status of the host plant. During the formation of the symbiotic association fungal hyphae penetrate roots and form arbuscule-like structures in host cortical cells. Although these arbuscules invade a host cell, they are surrounded by a periarbuscular plant plasma membrane, assuring the apoplastic location of fungal structures. The periarbuscular membranes in the host cells are the sites were bidirectional exchange of nutrients between the symbiotic partners takes place (Sanders et al., 1977; Smith et al., 2001). The AM fungus supports the plant with macronutrients like phosphorus while it receives photosynthetically fixed carbon from the plant. Phosphorus and carbohydrates are the most important nutrients that are exchanged between the symbiotic partners (Smith and Read, 1997), but there is also a significant nitrogen uptake by the fungus and transfer to the plant (He et al., 2003). Furthermore, plants show a higher drought stress tolerance and a higher stomatal conductance (Auge, 2001) when associated with mycorrhizal fungi. As the mechanisms of the nutrient exchange between the symbionts are not well understood, the study of membrane proteins located in the periarbuscular membrane can give insights to the preferred way of nutrient exchange during the AM symbiosis.

1.2. Aquaporins

Aquaporins represent a class of the major intrinsic protein superfamily (MIP), which mediate the passive water flux across biomembranes down an osmotic gradient. The proteinacious transport of water increases the water permeability of a lipid bilayer to a considerable extend. The growing number of reports dealing with plant aquaporins indicates for the progress in research in this field. In addition to the extracellular water movement in the apoplast of the plants, the aquaporin-mediated intracellular and cell-to-cell movement of water plays an important role in plant water relations. High levels of aquaporin expression were shown in tissues with high water fluxes across membranes, e.g. in fast growing regions, in shoots and leaves, but also in roots where water uptake occurs (Kaldenhoff et al., 1995; Otto and Kaldenhoff, 2000). In addition to this tissue or organ specificity, expression of aquaporins is also linked to physiological stages, such as seed germination or flower initiation. Abiotic factors like drought and salt stress have been shown to influence aquaporin expression, most probably via phytohormones like abscisic acid or gibberelic acid (Mariaux et al., 1998; Siefritz et al., 2001).

Although, many aquaporins are highly selective for water, uptake experiments with *Xenopus laevis* oocytes, expressing injected aquaporin-cRNA, clearly showed certain aquaporins to be permeable to small solutes, such as glycerol, urea, amino acids or even small peptides and ions (Biela et al., 1999; Eckert et al., 1999; Ikeda et al., 2002; Yasui et al., 1999). Recently, the human aquaporin AQP1, the tobacco aquaporin NtAQP1 and aquaporins from *Triticum* and *Arabidopsis* have been demonstrated to transport CO_2 and/or NH_3/NH_4^+ (Nakhoul et al., 1998; Uehlein et al., 2003; Jahn et al., 2004; Loque et al., 2005). In line with the different cellular compartments in plants, aquaporins seem to be mainly localized either in the plasma membrane or in the tonoplast. Accordingly, on the sequence level, several subfamilies are distinguishable, which seems to reflect different localization or functional properties. Sequence analysis of the *Arabidopsis* genome revealed 35 aquaporin full length genes (Johanson et al., 2001) divided into the subfamilies of the tonoplast intrinsic proteins (TIPs), the plasma membrane intrinsic proteins (PIPs), the Nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs) (Chaumont et al., 2001).

Based on structural information from protein crystal analysis the general biochemical transport- and selectivity-mechanisms were analysed (Murata et al., 2000; Walz et al., 1997). Aquaporins exhibit a characteristic topology with six transmembrane helices and both N- and C-terminal domains protruding into the cytoplasm. A highly conserved aminoacid-motif, asparagine–proline–alanine (NPA), appears twice in the polypeptide. In the functionally folded molecule both are in close proximity and directly involved in the mechanism of water permeation. For the human aquaporin 1 (HsAQP1) water has been shown to permeate in single-file mode. A fine-tuned rotation of the water dipole during passage of each molecule is essential for water selectivity (de Groot and Grubmuller, 2001; Murata et al., 2000). The proteins act as two-stage filters. One stage is build up by the conserved fingerprint

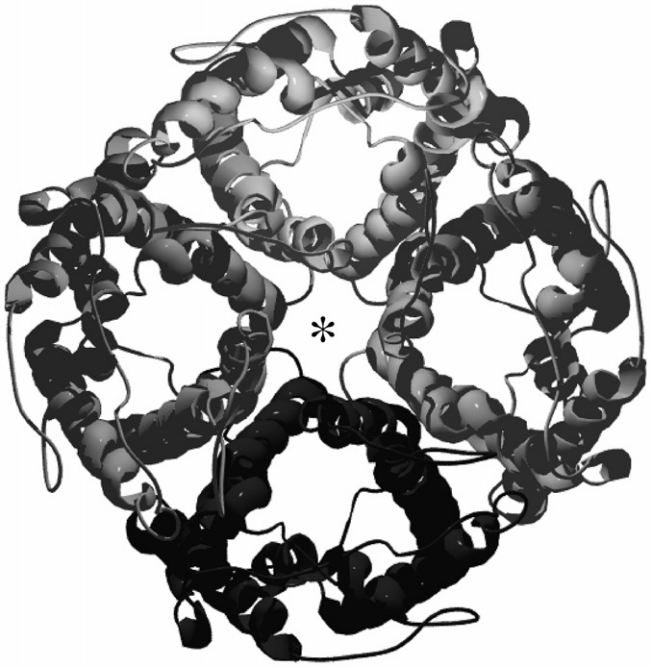

Fig. 1. Top view on an aquaporin tetramer possessing four individual pores and a putative central pore (*). The aquaporin model was produced using Modeller 7v7, Swiss-PdbViewer v3.7 and POV-Ray™ v3.5 for Windows.

motifs (NPA) forming the region for the selective passage of the substrate (water) and another by an aromatic/arginine-region (ar/R). The ar/R region forms the narrowest part of the pore and prevents permeation of protons. Hydrophobic regions near the NPA motifs are rate-limiting water barriers and reduce interactions between water molecules, thus disrupting the single-file mode and enabling the water dipole rotation (de Groot and Grubmuller, 2001).

Functional aquaporins reside in the respective membranes in a tetrameric arrangement (Fig. 1) consisting of four individually pore forming monomers, which line a putative fifth pore in the center of the tetramer.

2. AQPs and mycorrhiza

First hints on the involvement of aquaporins in the altered water uptake and transport capacities of mycorrhized plants were reported by Roussel et al. (1997) and Krajinski et al. (2000), who found mycorrhiza-induced expression of TIP aquaporins in parsley and *Medicago truncatula*. Krajinski et al. (2000) analysed the transport properties of the tonoplast intrinsic aquaporin MtAQP1 (see Fig. 2) and showed its function as a water conducting pore. They suggested that the increased expression of MtAQP1 is important for optimizing water transport after the changes that occur in the plant cell due to the formation of the symbiosis.

Recently, also expression of aquaporins of the PIP subfamily has been reported to increase during formation of the ectomycorrhizal symbiosis in poplar (Marjanovic et al., 2005). Since mycorrhizal plants show an increased drought stress tolerance Ruiz-Lozano (2003) suggested that the investigation of aquaporins in mycorrhizal plants under drought stress conditions might be of a special interest. In tobacco it was shown that the expression of NtAQP1 is affected under drought stress conditions in non mycorrhized plants (Siefritz et al., 2002). The effect of reduced

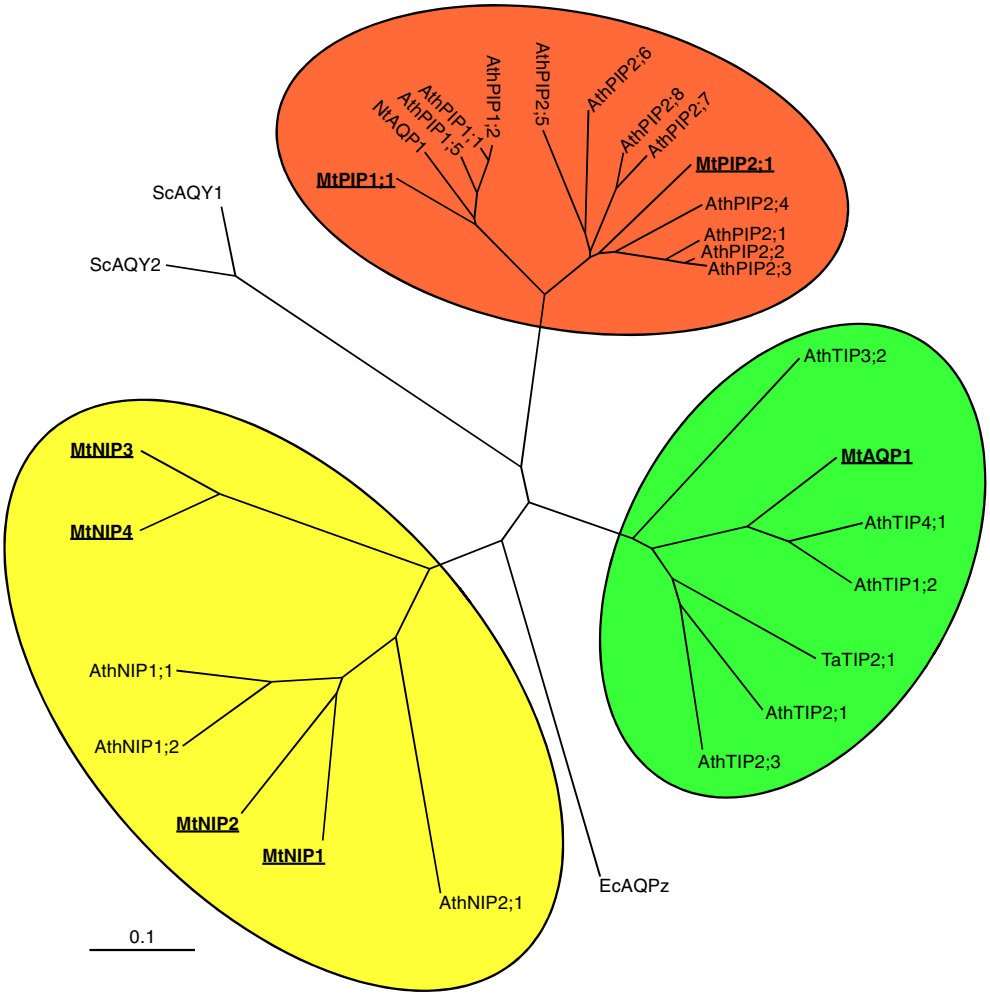

Fig. 2. Phylogenetic tree showing the *Medicago truncatula* (Mt) aquaporins identified within the Focus Programm "Mycorrhiza" (unpublished) in relation to selected aquaporins from the three model organisms *Arabidopsis thaliana* (Ath), *Saccharomyces cerevisiae* (Sc) and *Escherichia coli* (Ec). Also included are NtAPQ1 (*Nicotiana tabacum*) and TaTP2;1 (*Triticum aestivum*) which served as controls in the yeast complementation assay described in Fig. 8. The tree shows a clear grouping of the *Medicago* aquaporins into the subfamilies PIP1, PIP2, NIP and TIP. The tonoplast intrinsic aquaporin MtAQP1 was described by Krajinski et al. (2000).

expression of NtAQP1 was also investigated in mycorrhized NtAQP1 antisense plants under both, drought stress and well-watered conditions (Porcel et al., 2005). Reduction of NtAQP1 expression had no effect on the colonization of the plant root by mycorrhizal fungi. However, under drought stress conditions the shoot dry weight and the root fresh weight of the wild type tobacco plants was found to be higher than that in the NtAQP1-antisense plants. So the water transport mediated by NtAQP1 seems to be important for the efficiency of the symbiosis under drought.

In contrast to the report of Krajinski et al. (2000), who worked under well-watered circumstances, Porcel et al. (2006) provided evidence for a decrease in the expression in non-mycorrhizal and more severe in mycorrhizal soybean and lettuce plants under drought stress conditions (Porcel et al., 2006). Data from Ouziad et al. (2005) indicated for a decrease in the expression of PIP and TIP aquaporins induced by mycorrhizal colonization and salt stress in tomato. Porcel et al. (2006) proposed that the reason for the reduction in the PIP expression might be to minimize the water loss of the cells.

In contrast to the tonoplast, the periarbuscular membrane which is part of the plasma membrane controls the direct fluxes of substances between plant and fungus. The role of aquaporins with regard to transport processes across this membrane has to be elucidated. The focus of the presented work is on the identification and functional characterization of plasma membrane intrinsic aquaporins as putative candidates for important substrates in symbiotic water and nutrient exchange between arbuscular mycorrhizal partners.

As already mentioned before also significant nitrogen transfer from the fungus to the plant occurs during mycorrhizal symbiosis (He et al., 2003). Inorganic nitrogen is taken up by the fungal extraradical mycelia as nitrate from the soil and assimilated via nitrate reductase and the GS-GOGAT cycle, which in the end leads to formation of arginine. Arginine is transported from the extraradical to the intraradical mycelia where it is catabolized again producing amongst other substances ammonia, which equlibrates with ammonium according to the pH. These processes are consistent with increased expression of enzymes involved in primary nitrogen fixation in the extraradical mycelia, whereas enzymes involved in arginine catabolism are upregulated in the intraradical mycelia (Govindarajulu et al., 2005). Only little is known about the transfer of nitrogen from the fungus to the plant, but Govindarajulu et al. (2005) proposed an involvement of ammonium transporters (AMT). Two systems, displaying different apparent affinities for transport of ammonia have been described in plants: a high affinity (HATS) and a low affinity transport system (LATS; Wang et al., 1993). The high affinity transport, which is mediated by the AMT/MEP/Rh family of membrane proteins (Ludewig et al., 2002) follows a Michaelis–Menten kinetic and is saturated at ammonium concentrations below 1 mmol (Wang et al., 1993), whereas the low affinity ammonium transport is not saturated even at ammonium concentrations as high as 50 mmol (Wang et al., 1993) and this transport was shown to follow a linear kinetic. Such linearity characterizes facilitated diffusion through a pore system as it is for example provided by aquaporins. Aquaporin mediated membrane transport of ammonia is already well analysed. Nakhoul et al. (2001) showed that expression of the human aquaporin 1 in *Xenopus* oocytes enhances the ammonia permeability of the oocyte plasma membrane. Jahn et al. (2004) analysed the ammonia permeability of three tonoplast intrinsic aquaporins from *Triticum aestivum* and of the mammalian AQP8. Recently, the ammonia permeability of two tonoplast intrinsic aquaporins from *Arabidopsis thaliana* was reported (Loque et al., 2005). The mammalian aquaporins 3, 8, and 9 were shown to support significant membrane fluxes of NH_3/NH_4^+ besides being water conducting channels. These aquaporins could have physiological importance for liver and kidney function (Holm et al., 2005). Taken together, aquaporins could be at least a component of the low affinity ammonia transport system.

Hints on the involvement of aquaporins in membrane ammonia permeability came from another type of symbiosis: the interaction between N_2-fixing rhizobia and *Glycine max*. The ammonia permeability of the peribacteroid membrane, which is a membrane type with a function similar to the periarbuscular membrane, was analyed (Niemietz and Tyerman, 2000). Infection of soybean roots by nitrogen-fixing rhizobia leads to expression of plant nodule-specific genes. Nodulin 26, a member of the aquaporin water channel family was shown to be a major protein component of the symbiosome membrane. In functional tests in the heterologous *Xenopus* oocyte expression system Nodulin 26 was shown to be permeable to water and glycerol (Dean et al., 1999; Rivers et al., 1997). Both ammonia and water transport across the membrane were reversibly inhibited by treatment with the general protein inhibitor mercury chloride, indicating that ammonia might potentially permeate through Nodulin 26 (Niemietz and Tyerman, 2000).

3. Aquaporins in *Medicago*

3.1. Expression and localization

Six aquaporins were identified from *M. truncatula*. Based on the amino acid sequence, they were classified as members of the aquaporin subfamilies PIP1, PIP2 and NIP (Fig. 2 and Table 1) and named MtPIP1;1, MtPIP2;1, MtNIP1, MtNIP2, MtNIP3 and MtNIP4 (GenBank accession nos.: AF386739; AY059380; AY059381; AY539750; AY539749; AY605123), according to the proposed nomenclature for major intrinsic proteins in plants. Expression of the identified aquaporins in *Medicago* plants was studied under well watered conditions. When *Medicago* plants were associated with the mycorrhizal fungus *Glomus mossae*, two of the six aquaporins identified from *Medicago* clearly

Table 1
Medicago aquaporins identified during the Focus Programm "Mycorrhiza"

GenBank accession no.	Synonym	Reference
AJ251652	MtAQP1 (TIP)	Krajinski et al. (2000)
AF386739	MtPIP1;1	This study
AY059380	MtPIP2;1	This study
AY059381	MtNIP1	This study
AY539750	MtNIP2	This study
AY539749	MtNIP3	This study
AY605123	MtNIP4	This study

showed increased expression, as revealed by virtual northern analysis (Fig. 3). The effect turned out to be specific to roots, as exemplified for MtNIP1 in Fig. 4. A similar increase was also observed for MtPIP2;1, whereas transcript abundance of the other candidate genes was not affected by mycorrhization (data not shown). To confirm a causal relationship between mycorrhiza-induced expression of the corresponding mRNA and localization of aquaporins in the tissue during mycorrhization, immunolocalization experiments were performed using mycorrhized and non-mycorrhized *M. truncatula* root fragments. The level of arbuscule colonization of root fragments was approximately 26% related to root length. In preliminary experiments a specific antibody against the *Arabidopsis* aquaporin PIP1;2 (Biela et al., 1999; Otto and Kaldenhoff, 2000) was tested for aquaporin recognition on a root protein extract. As shown in Fig. 5a this antibody was found to hybridize with *Medicago* proteins giving a clear signal at about 30 kDa. Immunolocalization showed the accumulation of aquaporin protein in mycorrhized root fragments in the vascular part and especially in the root cortex (Fig. 5b). In this root area formation of arbuscules is expected. Immunolocalization on non-mycorrhized *Medicago* roots showed the typical aquaporin expression only in the area of the vascular bundles, as described previously (Otto and Kaldenhoff, 2000).

For plant–bacteria symbioses, it has been shown that Nodulin 26-like aquaporins are expressed at sites of solute transfer between the plant and the symbiotically associated bacteria. The data presented here indicate that this might be the case for the interaction between plant and fungus as well.

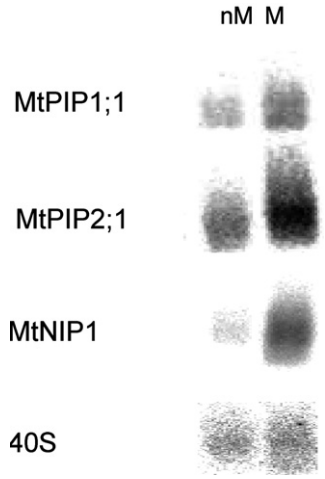

Fig. 3. Virtual Northern blot showing expression pattern of different *Medicago truncatula* aquaporin genes indicating higher expression of aquaporins in mycorrhized plant (unpublished data). Full length probes against MtPIP1;1, MtPIP2;1 and MtNIP1 were used. The expression signal was compared to the expression of a ribosomal 40S protein S8-like fragment as standard. nM, non-mycorrhized; M, mycorrhized.

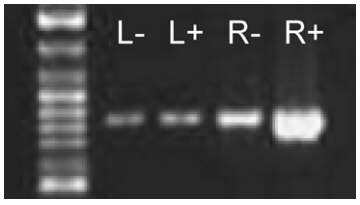

Fig. 4. Quantitative RT-PCR: expression of MtNIP1 in leaves (L) and roots (R) of mycorrhized (+) and non-mycorrhized (−) *Medicago* plants showing clear increase in transcript abundance in mycorrhized roots (unpublished data). No mycorrhiza induced effect is evident in leaves.

3.2. Function

Function of the identified aquaporin gene products was addressed using two different approaches. Studies addressing water and glycerol permeability were performed in the *Xenopus* oocyte expression system and aquaporin facilitated cellular ammonia uptake was analysed via functional complementation of a yeast mutant defective in ammonia uptake. After injection of in vitro-transcribed cRNA and a three-day expression period, oocytes were transferred into hypotonic medium and swelling of the cells was recorded. The permeability coefficients (P_f), calculated from the swelling rates of oocytes expressing the respective aquaporin indicated a clear increase in water permeability of MtPIP2;1 and a significant but not substantial increase

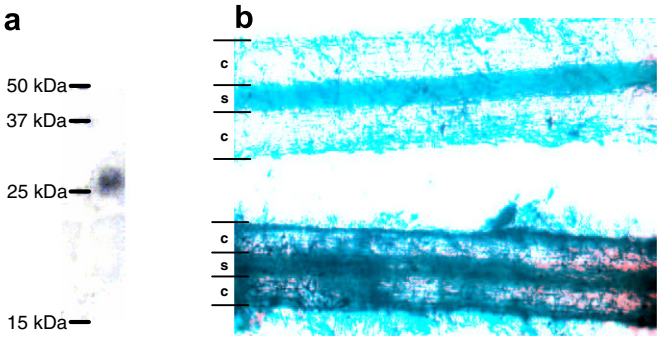

Fig. 5. (a) Western blot using protein extract from mycorrhized *Medicago* roots with an *Arabidopsis* PIP1-aquaporin-specific antibody identifies a protein of about 27 kDa. (b) Immunolocalization of non-mycorrhized (upper) and mycorrhized (lower) *Medicago* root fragments with *Arabidopsis* PIP1-antibody used in a, indicating strong expression of aquaporins in cortical (c) and stelar (s) regions of the mycorrhized root fragment (unpublished data).

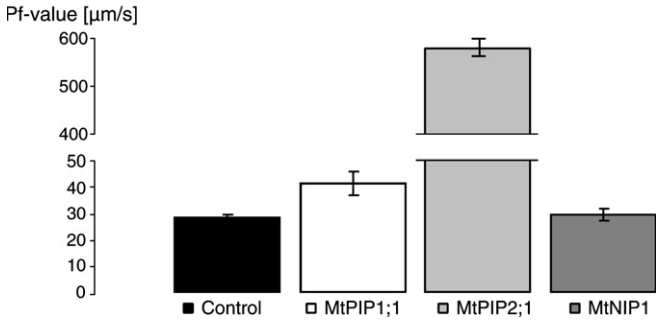

Fig. 6. Water permeability coefficient obtained by *Xenopus* oocyte swelling assay (unpublished data). Oocytes expressing the indicated *Medicago* aquaporins were transferred into hypoosmotic medium and swelling of the cells was recorded. MtPIP2;1 and MtPIP1;1 induce increased water permeability of the oocyte membrane, whereas no change in water permeability could be detected in MtNIP1 expressing oocytes. Bars, standard error; Control, water injected oocytes.

for MtPIP1;1 compared to water-injected oocytes (controls). Interestingly, no facilitated water uptake could be realized in cells expressing the NIP genes, as exemplified by MtNIP1 expressing cells (Fig. 6).

Additional information about aquaporin selectivity was obtained from uptake experiments in *Xenopus* oocytes using ^{3}H-labelled glycerol. Here, only MtPIP2;1 expressing oocytes showed a significantly increased uptake of glycerol compared to the control (Fig. 7).

Nodulin 26-like aquaporins have been described as good candidates for cellular ammonia transport (Niemietz and Tyerman, 2000; Tyerman et al., 2002) and could as such establish the low affinity ammonia transport system (Wang et al., 1993). Ammonia permeability of the NIP aquaporins was studied by means of the yeast complementation assay. For that, a triple deletion yeast strain (31019b; Δmep1Δmep2Δmep3; Marini et al., 1997) was transformed with plasmids carrying the genes coding for the NIP aquaporins or a tonoplast intrinsic protein (TaTIP2;1), which has been shown to be permeable to ammonia by Jahn et al. (2004). Growth of the transformed yeast strains was compared to growth of the triple deletion strains harbouring the

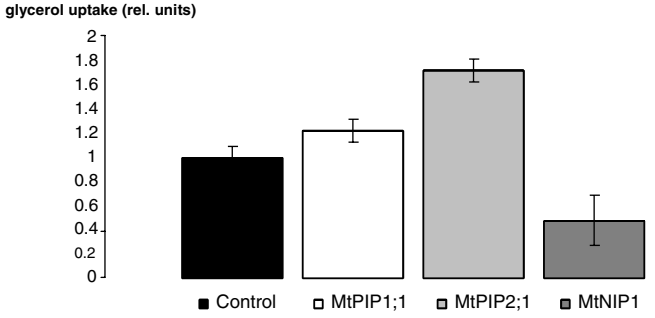

Fig. 7. Glycerol uptake of aquaporin expressing oocytes (unpublished data). Single oocytes were incubated in [^{3}H] glycerol containing medium (final activity 3.7 MBq/ml) for 10 min. Absorbed radioactivity was recorded by scintillation counting. Glycerol uptake is significantly increased only in MtPIP2;1 expressing oocytes. Bars indicate standard errors. Control, water injected oocytes.

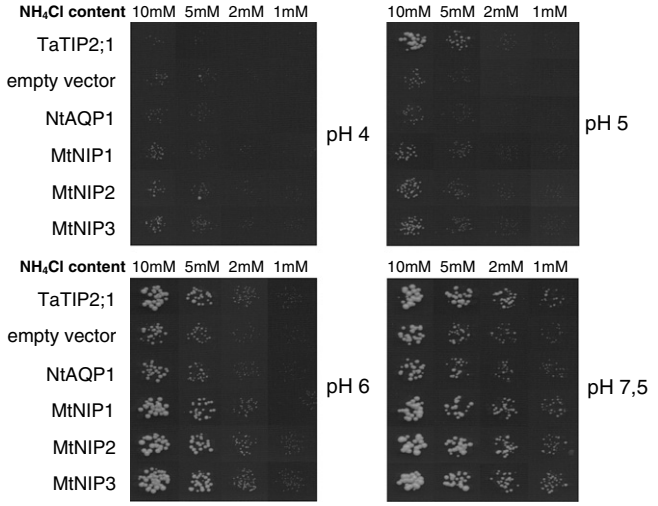

Fig. 8. Yeast complementation assay (unpublished data). 8 µl of yeast cells (Saccharomyces cerevisiae strain 31019b; OD_{600} 1.4 ± 0.1, dilution 1:10,000) expressing *Nicotiana tabacum* aquaporin 1 (NtAQP1), *Medicago truncatula* NIPs (MtNIP1, MtNIP2, MtNIP3) and controls (TaTIP2;1 or empty vector) were dropped on solid medium and the cells grown in the presence of different concentrations of NH_4Cl as single nitrogen source and at different pH for 6 days.

empty vector on solid media containing NH_4Cl as the sole nitrogen source. Expression of MtNIP1 to 3 was sufficient to complement the incapability in ammonia uptake compared to the respective controls (Fig. 8). Ammonia permeability is not necessarily a common property of plant aquaporins, as is shown in Fig. 7 for NtAQP1, a plasma membrane intrinsic aquaporin from *Nicotiana tabacum*, which did not restore the ability of the mutant yeast cells to take up ammonia. By contrast, NtAQP1 was described before to be permeable to water and glycerol (Biela et al., 1999) and, which is of major importance for plant physiology, to facilitate the cellular uptake of CO_2 and promote plant growth (Uehlein et al., 2003).

4. Outlook

In this report newly identified aquaporins of *M. truncatula* are described with regard to expression, localization and function. The involvement of aquaporins in plant water relations was demonstrated by us and others (Kaldenhoff et al., 1998; Marjanovic et al., 2005; Martre et al., 2002; Siefritz et al., 2002) and the contribution of aquaporins to symbiotic interactions in the plant kingdom is of special interest. The periarbuscular membrane surrounds the arbuscle and keeps it entirely apoplastic. Therefore, in order to maintain the symbiosis, it must be a site of highly efficient transmembrane water and solute transport from the fungus into the root cell and vice versa. The strong induction of MtPIP2;1 and MtNIP1 expression during mycorrhization indicate physiological changes, i.e. adaptation of water and solute transport systems in the root cells, which are indeed the sites where plant and

fungus interact. However, the functional features of these two aquaporins are quite different. MtPIP2;1 is highly permeable to water and, interestingly, has an additional permeability to glycerol in the heterologous oocyte system – a combination of different functions which has never been described for a plant PIP2 aquaporin before. Usually plant aquaporins, which promote glycerol uptake, only exhibit a low or no water permeability in *Xenopus* oocytes, whereas aquaporins facilitating high water fluxes are typically highly selective for water. MtNIP1 – as well as the non-mycorrhiza induced aquaporins MtNIP2 and MtNIP3 – possibly facilitate the cellular uptake of ammonia, as it was indicated by the yeast complementation test. The putative plasma membrane localization (Bienert and Kaldenhoff, unpublished observations) suggests an involvement of both aquaporins in symbiotic exchange processes located at the periarbuscular membrane. Although the function of this NOD26-like aquaporin is not entirely resolved, the strong transcriptional activity points to a common regulative pathway shared by nodulation and mycorrhization specific processes. Experiments with the increasing number of *M. truncatula* mutants impaired or blocked in different stages of symbiotic colonization would allow further investigation of such regulative pathways.

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Ralf Kaldenhoff studied biology and obtained his doctoral degree at Hanover University, Institute of Botany, where he worked on blue light- and abscisic acid-inducible genes of *Arabidopsis thaliana*. Subsequently, he worked as a postdoctoral fellow at the MPI for Molecular Genetics, Berlin. In 1991, Ralf Kaldenhoff became an assistant professor at the University Hanover, Institute of Botany. He moved to Würzburg University, Institute of Biophysics and Molecular Plant Physiology, where he was appointed a full professor and started extensive work on expression and function of plant aquaporins. Since 2003 Ralf Kaldenhoff is professor at Darmstadt University of Technology, Institute of Botany. He is director of the department of Applied Plant Science and Dean of Biology Study.