Planta (2005) 222: 258–268
DOI 10.1007/s00425-005-1539-z

ORIGINAL ARTICLE

Žaklina Marjanović · Norbert Uehlein · Ralf Kaldenhoff
Janusz J. Zwiazek · Michael Weiß · Rüdiger Hampp
Uwe Nehls

Aquaporins in poplar: What a difference a symbiont makes!

Received: 10 March 2005 / Accepted: 10 March 2005 / Published online: 10 May 2005

Abstract The formation of ectomycorrhizas, a tight association between fine roots of trees and certain soil fungi, improves plant nutrition in a nutrient-limited environment and may increase plant survival under water stress conditions. To investigate the impact of mycorrhiza formation on plant water uptake, seven genes coding for putative water channel proteins (aquaporins) were isolated from a poplar ectomycorrhizal cDNA library. Four out of the seven genes were preferentially expressed in roots. Mycorrhiza formation resulted in an increased transcript level for three of these genes, two of which are the most prominently expressed aquaporins in roots. When expressed in *Xenopus laevis* oocytes, the corresponding proteins of both genes were able to transport water. Together, these data indicate, that the water transport capacity of the plasma membrane of root cells is strongly increased in mycorrhized plants. Measurements of the hydraulic conductance of intact root systems revealed an increased water transport capacity of mycorrhized poplar roots. These data, however, also indicate that changes in the properties of the plasma membrane as well as those of the apoplast are responsible for the increased root hydraulic conductance in ectomycorrhizal symbiosis.

Ž. Marjanović · R. Hampp · U. Nehls (✉)
Physiologische Ökologie der Pflanzen,
Eberhard-Karls-Universität, Auf der Morgenstelle 1,
72076 Tubingen, Germany
E-mail: uwe.nehls@uni-tuebingen.de

N. Uehlein · R. Kaldenhoff
Institut für Botanik, Angewandte Pflanzenwissenschaften,
Universität Darmstadt, Schnittspahnstr. 10,
64287 Darmstadt, Germany

J. J. Zwiazek
Department of Renewable Resources, University of Alberta,
442 Earth Sciences Bldg., T6G 2E3 Edmonton, Canada

M. Weiß
Spezielle Botanik und Mykologie,
Eberhard-Karls-Universität, Auf der Morgenstelle 1,
72076 Tubingen, Germany

Keywords Ectomycorrhiza · Water transport · Aquaporin · Root hydraulic conductance · Poplar phylogeny

Abbreviations EM: Ectomycorrhiza · MIP: Membrane intrinsic protein · PIP: Plasma membrane intrinsic protein · TIP: Tonoplast intrinsic protein · NIP: Nodulin-intrinsic protein · SIP: Small intrinsic protein · P_f: Osmotic permeability coefficient · K_r: Hydraulic conductance · L_p: Hydraulic conductivity · E_a: Activation energy · PCR: Polymerase chain reaction

Introduction

Most trees of temperate and boreal forests form ectomycorrhizas, ecologically obligate symbiotic associations of plant fine roots with fungal hyphae of certain basidio- or ascomycetes (Smith and Read 1997).

Mycorrhizal plants are characterized by a higher photosynthetic rate, a larger dry mass, a higher nutrient content, and a better water-use efficiency (Parke et al. 1983; Lehto 1992; Loewe et al. 2000; Muhsin and Zwiazek 2002). In addition, several authors (Goss 1960; Parke et al. 1983; Boyle and Hellenbrand 1991; Morte et al. 2001) have shown that mycorrhiza formation often results in a better plant water supply, especially under drought conditions. One reason for this could be that mycorrhized plant roots have a larger water transport capacity, indicated by an increased root hydraulic conductivity (Muhsin and Zwiazek 2002).

Plant water balance is mostly determined by the rates of root water uptake, its transport through the plant, and water loss due to transpiration. Radial water transport in the root can be apoplastic (up to the endodermis), symplastic, or transcellular (Steudle and Peterson 1998). Due to exo- and endodermal barriers (e.g. Casparian band) in roots, water must pass through the plasma membrane before entering the xylem vessels.

Therefore, plasma membranes presumably function as check points for water uptake or loss, especially under drought conditions.

In addition to the simple diffusion of water molecules through lipid membranes, uptake and release of water by cells is mediated by membrane- intrinsic proteins called aquaporins (Kaldenhoff et al. 1998; Maurel and Chrispeels 2001; Siefritz et al. 2002). Some aquaporins are able to transport (in addition to water) also small, uncharged molecules like glycerol, urea or CO_2 (Biela et al. 1999; Weig and Jakob 2000; Gerbeau et al. 2002; Uehlein et al. 2003).

According to their protein sequence and their location within the cell, aquaporins are currently divided into four subfamilies (Johansson et al., 2001), plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-intrinsic proteins (NIPs), and small intrinsic proteins (SIPs).

Aquaporin gene expression is regulated spatially and developmentally in a cell-specific manner, via hormones, and by a wide range of environmental signals such as blue light, phytohormones, drought or salinity (Guerrero et al. 1990; Yamaguchi-Shinozaki et al. 1992; Azaizeh et al. 1992; Kaldenhoff et al. 1993; Phillips and Huttly 1994; Maurel and Chrispeels, 2001; Morillon and Lassalles 2002).

In this contribution, the impact of ectomycorrhiza formation on root hydraulic conductance of poplars, grown in a Petri dish system, was analyzed. Putative aquaporin genes, that are expressed in fully developed *Populus tremula* x *tremuloides*/*Amanita muscaria* ectomycorrhizas were isolated and investigated with respect to their transcript level in different poplar organs and the impact of mycorrhiza formation on gene expression. Furthermore, the function of selected proteins as water pores was confirmed by heterologous expression in *Xenopus laevis* oocytes.

Materials and methods

Biological material

Populus tremula x *tremuloides* (Nehls, Tübingen) cuttings were rooted in MS-medium (Sigma, St. Louis, MI, USA) under sterile conditions. Six-week-old plants were transferred into Petri dishes containing sugar-free MMN medium (Hampp et al. 1996) such that the root system was inside and the shoot outside of the Petri dish. The Petri dish cultures of plants inoculated with *Amanita muscaria* to form ectomycorrhizas and non-inoculated control plants were grown in small plastic greenhouses for additional 6 weeks at 18°C, 12 h day/night periods with 100 µmol photons m^{-2} s^{-1} illumination. Fully developed source leaves, stems, main roots, fine roots, and mycorrhizas were isolated around noontime, frozen in liquid nitrogen, and stored in RNA later (Qiagen, Hilden, Germany) at −20°C.

Isolation of cDNAs containing the entire reading frame of poplar aquaporins

cDNAs of all clones were obtained from an EST-project of fully developed *Populus tremula* x *tremuloides*/*Amanita muscaria* ectomycorrhizas (Nehls et al. 2001). The missing 5′-end of *PttPIP2.2* was obtained by RACE PCR, using the *SMART RACE cDNA Amplification Kit* (Clontech, Palo Alto, USA) and first strand cDNA obtained from *P. tremula* x *tremuloides*/*A. muscaria* ectomycorrhizas according to the manufacturer's instructions. The following gene specific primers were used:

PttPIP2.2: TGGCATTCTTGGTATTGC; *PttPIP2.5*: ACGGCGATGTATGTTGTGG

Semi quantitative RT-PCR

Aliquots of about 1 µg of total RNA were treated with DNAse I (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions and used for first-strand cDNA synthesis in a total volume of 20 µl, containing 50 pmol oligo-d$(T)_{18}$–primer (Amersham Biosciences) and 200 U Superscript II RNase H^- Reverse Transcriptase (Invitrogen, Groningen, Netherlands). After synthesis, 50 µl of 5 mM Tris/HCl, pH 8 were added and aliquots were stored at −80°C.

For further PCR experiments, the amount of first-strand cDNA in the different samples was calibrated such, that all samples showed identical signal intensities after amplification with specific primers for the constitutively expressed poplar gene *PttJip1* and 18S rRNA (Grunze et al. 2004) followed by agarose gel electrophoresis and ethidium bromide staining. PCR was performed using 2 U Eurogentec Taq in a total volume of 25 µl containing 5% dimethyl sulfoxide (cycling conditions: 1 min at 91°C; 30 s at 55°C; 1.5 min at 72°C). Cycle numbers were chosen such that amplification was always in the linear range.

The following primers (always the 5′primer first and both primers shown in 5′3′ orientation) were used for PCR (the 3′-primer is always originating from the non-coding 3′-end of the gene):

- *PttPIP1.1*: GTTGGACCTTTCATTGGTGC; GGACAATTTCCTTGCCAAAA
- *PttPIP1.2*: TTACATCATCATGCAGTGC; ATTCTCGAAAGGGCGAACG
- *PttPIP2.1*: CCACCTAGCCACAATCCCT; GAMAAGGAAAGGTCCRTCG
- *PttPIP2.2*: CTCTCTTGGTGGTGGTGC; CAAGGCTTTGATTGCTGC
- *PttPIP2.3*: GTTGGACCTTTCATTGGTGC; GACTAACACAGAGCACTTGC
- *PttPIP2.4*: TGGGAGCCGCTGTTATCTAC; TCCACACACCCACAAAAGAA
- *PttPIP2.5*: TGCTGAGATCATCGGTACC; TGTACATATTCGCCAGTCC

Aliquots of 6 µl of the PCR products were separated on 2% agarose gels and stained with ethidium bromide. Gels were visualized under ultraviolet light (312 nm) and images were taken using a video documentation system (Gel Doc 2000, BioRad, Munich). Signal intensities were quantified using the program package NIH Image (version 1.62; http://rsb.info.nih.gov/nih-image). All PCRs were replicated at least three times using first strand cDNA of at least two independent RNA preparations.

To quantify the differences in gene expression of a given gene in two different samples, dilution series of the first strand cDNA sample with the higher transcript level were prepared and compared to the PCR signal of the other sample after amplification using gene specific primers. The rate of dilution for obtaining identical signal intensities was then used to calculate the expression ratio.

Determination of the mRNA content of different *PIP* genes in poplar fine roots

To elucidate which of the putative PIPs is expressed most abundantly in poplar fine roots, PCR-fragments of cloned *PIP* genes were amplified using gene-specific primers. After purification, the DNA concentration was determined photometrically. Dilution series of the PCR fragments, as well as 1 µl of fine root first strand cDNA were used as a template for PCR amplification with gene-specific primers. After separation on agarose gels and ethidium bromide staining, the signal intensity of the PCR fragment obtained from root first strand cDNA was compared to that of the DNA fragments obtained from the dilution series. The sample of the dilution series that gave the same signal intensity as the root cDNA was used to calculate the number of mRNA molecules of each PIP present in the fine root RNA preparation.

Construction of *PIP* expression vectors

Open reading frames of cDNAs were amplified by PCR using the Ready To Go PCR Beads (Amersham) and gene-specific primers containing additional restriction enzyme recognition sites for *Bam*HI or *Xba*I.

Primer sequences were:

- *PttPIP1.1*: AGGATCCATGGAAGGAAAAGAA GAAG; ATCTAGACTAGGAGCGAGTTTTGA AGG
- *PttPIP2.1*: ATAGGATCCGAGAACTCCTTAGA TC; TATCTAGAGGATAGTTAAGCTCTC
- *PttPIP2.2*: ATAGGATCCACCTTGGTGATGGA GTTG; ATCTACACTTTTACTTGGCGGGGTTG
- *PttPIP2.5*: AGGATCCATGGGCAAGGACAATT GAAG; ATCTAGATAAATGTTGGAAGAGC fTCC

Amplification conditions were as follows: 34 cycles of 94°C for 30 s; 50°C for 40 s; 2 min at 72°C.

The amplified DNA fragments were cloned into the PCR 2.1-TOPO vector (Invitrogen) and used for transformation of *One-shot competent Escherichia coli*. The constructs were digested with *Bam*HI and *Xba*I and the ORFs were cloned into *Bam*HI/*Xba*I-digested pGemHe vector using T4 ligase (New England Biolabs, Beverly, MA, USA), and transformed into *One-shot* competent *E. coli*. Plasmid DNA was isolated by alkaline lysis from 20 ml overnight cultures (Sambrook et al. 1989). To ensure proper PCR amplification, the entire cDNA inserts of the clones were sequenced.

cRNA synthesis

One microgram NheI-digested plasmid DNA was used as template for cRNA synthesis using the MEGAscript in vitro cRNA synthesis kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Water uptake by oocytes

Oocytes from stage V or VI were isolated from *X. laevis* according to Zhang and Verkman (1991) and incubated in ND 96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.4) supplemented with pentamycin and streptomycin (200 U/ml each, Invitrogen) at 16°C.

Forty nl of water containing 30-40 ng cRNA (or no cRNA) were injected into oocytes. After incubation in ND 96 solution (220 mOsm) for 2-3 days at 16°C, oocytes were transferred to hypotonic ND 96 (70 mOsm). Changes in oocyte diameter were monitored at room temperature for a period of 3 min using a video microscope (one frame every 5 s). The volume increase of the oocytes was calculated from changes in the surface area of each oocyte using ScionImage (Scion Corporation, Frederick, Maryland, USA). The osmotic permeability coefficient (P_f) was calculated according to Zhang and Verkman (1991). P_f ($[1/(A\ \Delta\pi)]$ where A is the area and $\Delta\pi$ is the osmolality gradient at zero time) is the slope of the linear fit in the plot of oocyte surface (taken by a video camera) versus time in hyposmotic solution.

To determine mercury sensitivity, oocytes were incubated prior to water uptake measurements for 10 min at room temperature in ND 96 solution supplemented with 0.5 mM $HgCl_2$.

Construction of the phylogenetic tree

The protein alignment was constructed using CLUSTALW (Thompson et al. 1997). Ambiguous alignment positions were excluded from the phylogenetic analysis.

To estimate phylogenetic relationships, the alignment was analyzed using a Bayesian approach based on Markov chain Monte Carlo as implemented in the computer program MrBayes 3.0b4 (Huelsenbeck et al. 2002). This approach allows estimation of the a posteriori probability for the monophyly of sequence groups, i.e. the probability that a group is monophyletic given the sequence alignment.

We ran four incrementally heated simultaneous Monte Carlo Markov chains over 2 million generations using random starting trees and assuming a percentage of invariable alignment sites with gamma-distributed substitution rates of the remaining sites. Rather than specifying an amino acid substitution model, we allowed the process to sample randomly from the substitution models implemented in MrBayes. Trees were sampled every 100 generations resulting in an overall sampling of 20,000 trees, from which the first 8,000 trees were discarded. The remaining 12,000 trees were used to compute a majority rule consensus tree (including also compatible groups of lower frequencies) to get estimates for the posterior probabilities. Branch lengths were averaged over the sampled trees. Stationarity of the process was controlled using the Tracer software, version 1.0 (http://evolve.zoo.ox.ac.uk/beast).

The Bayesian Markov chain Monte Carlo phylogenetic analysis was repeated, always using random starting trees, to test the independency of the results from topological priors (Huelsenbeck et al. 2002).

Root hydraulic properties

Root hydraulic conductance (K_r) was measured with a high-pressure flow meter (Dynamax Inc., Houston, TX, USA) (Tyree et al. 1997) in the excised undisturbed root systems in the solid agar medium that was used to grow plants. Root flow rates were measured at steadily increasing pressures between 0 MPa and 0.4 MPa (Muhsin and Zwiazek 2002). Root hydraulic conductance was calculated as the slope of the linear portion of the graph produced by the high-pressure flow meter software, and was expressed against root surface area to calculate root hydraulic conductivity, L_p (kg m^{-2} s^{-1} MPa^{-1}).

The activation energy (E_a) for root water transport was calculated from the slope of Arrhenius plots obtained by plotting the natural logarithm of mean K_r values against the inverse of absolute temperatures. For the Arrhenius plot determinations, K_r measurements were obtained as above after exposing the roots to temperatures increasing from 5°C to 25°C in 5°C steps (Muhsin and Zwiazek 2002). Roots were exposed to 5°C for 15 min before the first L_p measurement was obtained. Root systems of 18 mycorrhizal and 18 non-mycorrhizal plants were taken for the K_r and L_p measurements, and six plants per treatment for the E_a determinations.

Root surface areas were calculated according to Wan and Zwiazek (1999) by using Sigma Scan 3.0 (Jandel Scientific, San Rafael, CA, USA) after scanning the root systems with a ScanMaker III scanner (Microtek Laboratory Inc., Redondo Beach, CA, USA).

Miscellaneous

PCR fragments were separated on agarose gels, stained with ethidium bromide, and excised with a scalpel. DNA was isolated from the gel pieces using the *NucleoSpin* gel extraction kit (Macherey & Nagel, Düren, Germany).

Overlapping sequencing was performed using M13 universal and reverse primers (Stratagene, Heidelberg, Germany) as well as internal primers (Invitrogen) and the *ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit* (Applied Biosystems, Forster City, CA, USA) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

For analysis of DNA and protein sequences, the program package GeneJockey II (Biosoft, Cambridge, UK) was used. The sequence data was compared to gene libraries using Blastx (Altschul et al. 1997) and further analyzed using Clustal X (Thompson et al. 1997).

Results

Impact of ectomycorrhiza formation on the root hydraulic properties of poplars

To get an integrative view about the impact of mycorrhiza formation on the water transport capacity of poplar roots, hydraulic conductance was measured according to Tyree et al. (1997) using excised undisturbed root systems of 12-week-old mycorrhized and non-mycorrhized plants grown in a Petri dish system according to Hampp et al. (1996).

Mycorrhizal plants had about 40% higher root hydraulic conductance values (K_r) compared to non-mycorrhized plants.

Since the root system of mycorrhized plants had a 17% smaller surface area (895.3 mm^2 κ 49.4 mm^2) compared to that of non-mycorrhized plants (1081.8 mm^2 κ 44.6 mm^2), the difference in the root hydraulic conductivity (L_p; Fig. 1) was, compared to K_r, even higher, indicating a 57% larger water transport capacity of mycorrhized roots.

Arrhenius plots revealed that mycorrhizal plants maintained higher L_p values throughout the range of examined temperatures from 5°C to 25°C. The E_a values calculated from the slopes of Arrhenius plots were 7.0 kcal mol^{-1} and 4.0 kcal mol^{-1} for mycorrhizal and non-mycorrhizal root systems, respectively.

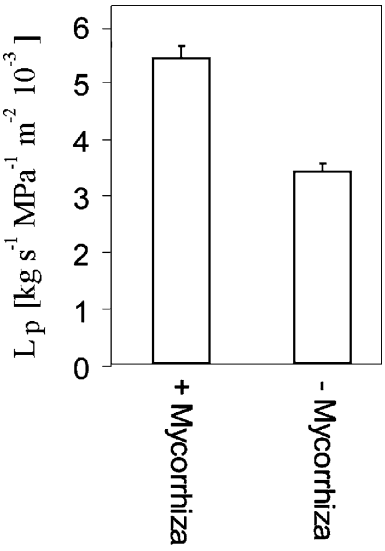

Fig. 1 Measurements of the root hydraulic conductivity of mycorrhized and non-mycorrhized poplar root systems. Root hydraulic conductance was measured with a high-pressure flow meter in the excised undisturbed root systems of mycorrhized and non-mycorrhized poplar plants and used (together with the size of the root systems) to calculate the root hydraulic conductivity (L_p)

Isolation of putative poplar aquaporin genes and phylogenetic analysis of the deduced proteins

Since water transport in roots is, in addition to cell wall properties, dependent on the plasma membrane transport capacity, putative aquaporin genes were isolated from fully developed *P. tremula* x *tremuloides*/*A. muscaria* ectomycorrhizas obtained from plants grown in a Petri dish system (Hampp et al. 1996). A total of seven cDNAs encoding putative poplar aquaporins were obtained as a result of a PCR approach using degenerated primers and an EST project of a mycorrhizal cDNA library (data not shown). All cDNAs encode members of the PIP subfamily (*PttPIP1.1*: Accession number AJ849323; *PttPIP1.2*: Accession number AJ849322; *PttPIP2.1*: Accession number AJ849324; *PttPIP2.2*: Accession number AJ849325; *PttPIP2.3*: Accession number AJ849326; *PttPIP2.4*: Accession number AJ849327; *PttPIP2.5*: Accession number AJ849328).

A detailed phylogenetic analysis of the relationship of the newly identified poplar aquaporins with all known PIP proteins from *Arabidopsis thaliana*, *Zea mays*, and tree species as well as other selected plants was performed (Fig. 2). The results are well compatible with predictions directly inferred from the protein alignment (Fig. 3).

Consistent with the literature, the deduced poplar PIP proteins were divided into the two groups, PIP1 and PIP2. However, PIP2 proteins were further divided into two subgroups (Fig. 2), which correlates with the length of their loopA region (located between the first and the second transmembrane domain; Fig. 3). Subgroup I (containing PttPIP2.1 and PttPIP2.2, conifer PIP2 proteins, AtPIP2.7 and AtPIP2.8, BoPIP3, VbrPIP2.2 and SoPm28) has a loopA region that is even one amino acid shorter than that of PIP1 proteins. Subgroup II (containing PttPIP2.3, PttPIP2.5, AtPIP2.1 to AtPIP2.6) has a loopA region that is seven (five in the case of PttPIP2.4) amino acids longer than that of proteins of the first PIP2 subgroup.

Expression analysis of poplar PIPs in different poplar organs

The expression of the identified poplar PIP genes was determined by semi-quantitative RT-PCR using primers from the non-coding 3′-ends of the cDNAs. Since some of the cDNAs were quite similar, the obtained PCR fragments were subjected to direct sequencing or gene-specific restriction analysis. No cross amplification of a similar PIP gene was ever observed. Furthermore, no PCR signal was ever observed with fungal cDNA, indicating (together with PCR fragment sequencing and restriction analysis) that no cross hybridization with fungal cDNA occurred in mycorrhizal samples.

The expression profiles of PIP genes were investigated in different organs of non-mycorrhized (source leaves, stems, main and fine roots) and mycorrhized (main roots and fine roots) poplar plants obtained from a Petri dish system (Fig. 4, Table 1).

PttPIP1.1 was expressed in all poplar organs revealing the highest transcript level in fine roots (ten-fold higher transcript level when compared to leaves). During mycorrhiza formation, the transcript level increased in fine roots but decreased in main roots by a factor of three.

With the exception of leaves, *PttPIP1.2* was constitutively expressed in all poplar organs. Mycorrhiza formation increased gene expression by a factor of 1.5 only in main roots.

PttPIP2.1 was also constitutively expressed in all organs. Gene expression increased in main roots and decreased in fine roots by mycorrhiza formation.

PttPIP2.2 was mainly expressed in fine roots (sixfold higher transcript level compared to leaves). Mycorrhiza formation resulted in a two-fold increase of the transcript level in main roots and a two-fold reduction in mycorrhized fine roots.

PttPIP2.3 was preferentially expressed in main roots (fourfold higher transcript level compared to leaves). Mycorrhiza formation led to a two-fold increase in gene expression in main and a three-fold increase in fine roots.

PttPIP2.4 was expressed in all organs but revealed its highest transcript level in fine roots. Mycorrhiza formation had no impact on gene expression in main roots and decreased expression by a factor of two in fine roots.

The transcript level of *PttPIP2.5* was twofold higher in fine roots compared to leaves and only barely

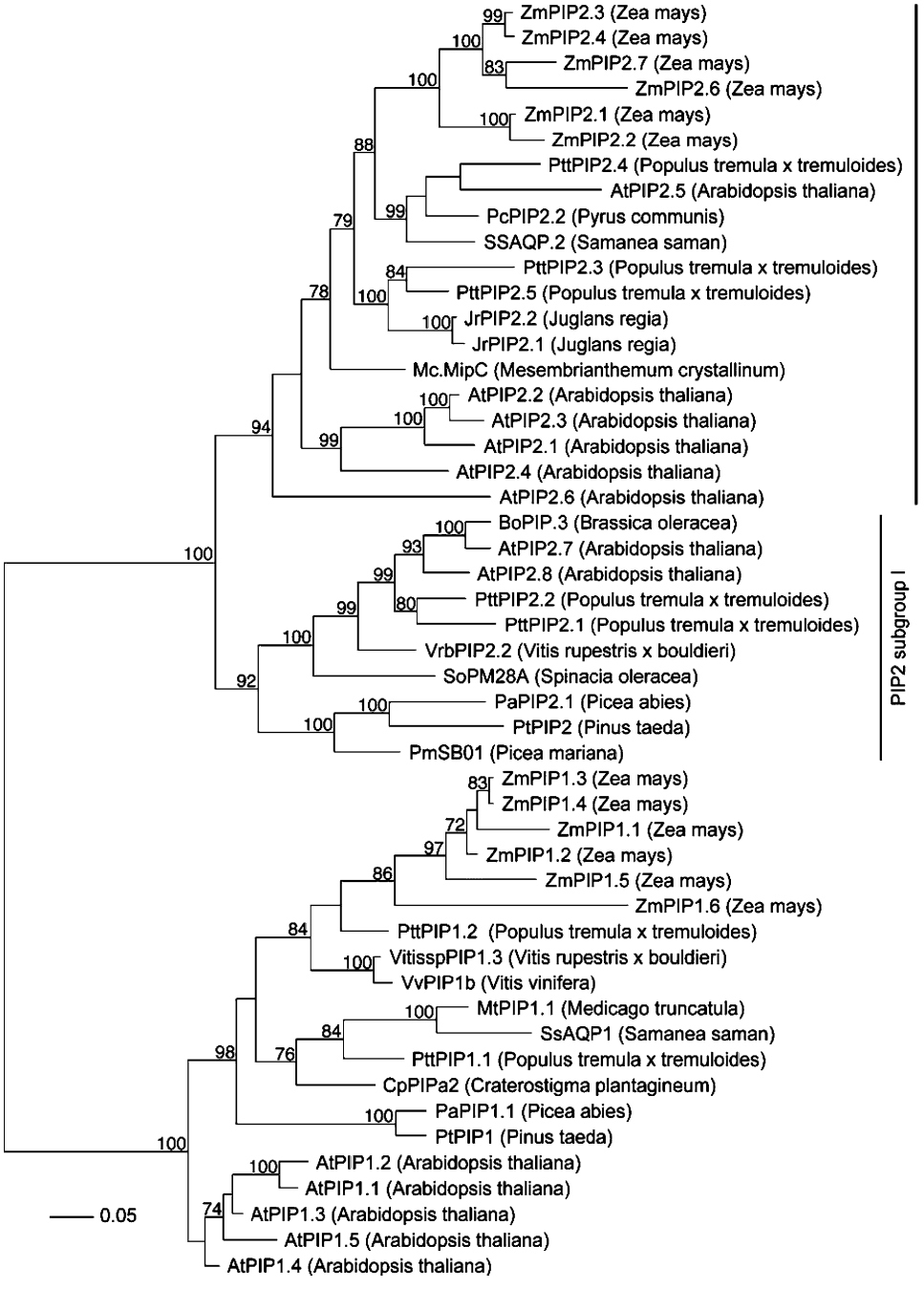

Fig. 2 Phylogenetic relationships between PttPIP proteins and those of *A. thaliana*, *Z. mays*, and other selected plants. Bayesian Markov chain Monte Carlo analysis was performed using four incrementally heated chains, random starting trees, and assuming a percentage of invariable alignment sites with gamma-distributed substitution rates of the remaining sites. The process ran over two million generations; trees were stored every 100th generation. Majority rule consensus from 12,000 trees that were sampled after the Markov chains had reached stationary. Branch lengths were averaged over the sampled trees and scaled in terms of expected numbers of amino acid substitutions per site. Numbers at branches are estimates for a posteriori probabilities that the respective groups of proteins are monophyletic given the sequence alignment (values below 70% not shown). (Accession number of genes used for tree formation: AtPIP1.4, Q39196; AtPIP1.2, Q06611; AtPIP2.6, Q9ZV07; AtPIP1.3, Q08733; AtPIP2.8, Q9ZVX8; AtPIP1.5, Q8LAA6; AtPIP2.4, Q9FF53; AtPIP2.1, P43286; AtPIP1.1, P43285; AtPIP2.5, Q9SV31; AtPIP2.2, P43287; AtPIP2.3, P43287; AtPIP2.7, P93004; SoPM28B, CAB56217; McMipC, AAA93521; VvbPIP2.2, AAF71820; VitisspPIP1.3, AAF71818; PaPIP2.1, T14889; PaPIP1-1, T14863; PmSB01, AAC32107)

detectable in the stem. Mycorrhiza formation resulted in a fivefold increase in transcript level in fine roots and a reduction in main roots by a factor of two.

To get an impression on the impact of single poplar PIPs on the water transport capacity of fine roots, the relative abundance of transcripts was determined for each gene. The following order, listed from strongly expressed to weakly expressed genes, was obtained: *PttPIP2.5* > *PttPIP2.2* > *PttPIP2.3* > *PttPIP1.2* > *PttPIP1.1* > *PttPIP2.4* > *PttPIP2*. The transcript levels of *PttPIP2.5* and *PttPIP2.2* were at least ten-times higher than those of all other investigated *PIP* genes.

Functional analysis of PttPIPs in *Xenopus laevis* oocytes

Two couples of proteins, PttPIP2.1/PttPIP2.2 and PttPIP2.3/PttPIP2.5, revealed a very similar protein sequence but differing expression profiles in poplar organs and with respect to mycorrhiza formation. Thus, the ability of these proteins to perform water transport in *X. laevis* oocytes was investigated.

Expression of *PttPIP2.1* and *PttPIP2.2* enabled only very low water fluxes, not significantly different from that of a negative control (Fig. 5). While expression of *PttPIP2.3* revealed a low, but significantly higher water

Fig. 3 Alignment of the loopA region of plant PIPs. Protein sequences of the loopA region (located between the first and the second transmembrane domain) of *A. thaliana*, *Z. mays*, and selected other plants PIPs were aligned using Clustal X. Colored frames separate PIP1 and PIP2 proteins as well as the two suggested PIP2 subgroups. The end of transmembrane domain 1 and the begin of domain 2 (calculated according to Kyte and Doolittle 1982) are underlined

transport rate than the control, expression of *PttPIP2.5* resulted in the highest osmotic permeability coefficient (P_f value 250) measured so far for plant aquaporins, indicating a very high water transport capacity of the protein. Since these results were confirmed using different cRNA and oocyte batches, technical problems to explain the different rates of water uptake of PIP proteins can be ruled out.

The water transport activity of none of the investigated PIP proteins was inhibited by mercury (data not shown).

PIP1 proteins were not investigated since they usually reveal much smaller water uptake rates than PIP2 proteins when expressed in oocytes.

Discussion

Unlike animals, sessile organisms like plants are forced to endure an unstable environment and must adopt mechanisms to withstand drastic environmental changes (e.g. drought) or a limitation of resources. A common

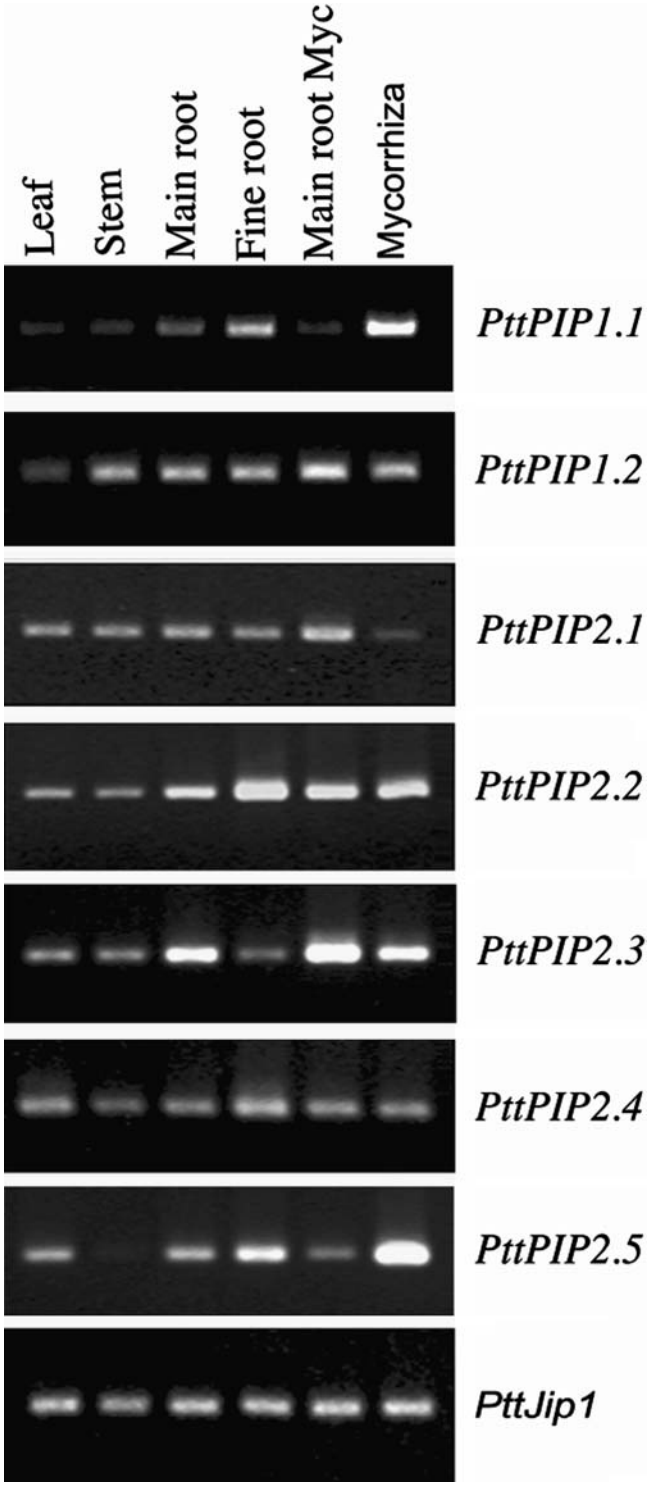

Fig. 4 Expression profile of *PttPIPs* in poplar organs and ectomycorrhizas. Total RNA was isolated from leaves, stems and roots (separated into fine and main roots) of non-mycorrhized poplar plants and mycorrhizas (mycorrhized fine roots). Aliquots of about 1 µg were DNAse I treated and used for first-strand cDNA synthesis. The RNA concentration in the different samples was equalized according to the banding intensities obtained with 18S rRNA primers (data not shown) and the constitutively expressed poplar gene *PttJip1* (Grunze et al. 2004). PCRs were performed with gene specific primers from the non-coding 3′-end of the genes

way to reduce the impact of these factors is the association of plant roots with microorganisms, especially certain soil fungi, resulting in the formation of mycorrhizas (Smith and Read 1997).

Since long distance nutrient transport in soil towards the roots is driven by plant transpiration, water is of special importance not only for the maintenance of cell turgor but also for plant nutrition. Mycorrhized plants do not only reveal an increased nutrient content but also an increased CO_2 fixation rate (Loewe et al. 2000; Wright et al. 2000), indicating a lower stomatal resistance in symbiosis. Due to the fact that plant roots have apoplastic barriers, water has to pass the plasma membrane either at the cortex or the endodermis in order to reach the vascular system. Thus, water pores (aquaporins) within the plasma membrane could control the water flux into (and out of) the plant root.

In this study, seven genes encoding plasma membrane intrinsic proteins (PIP) were isolated from an ectomycorrhizal cDNA library obtained from fully developed *P. tremula* x *tremuloides*/*A. muscaria* ectomycorrhizas (Nehls et al. 2001). All PIP proteins contained six hydrophobic membrane-spanning domains, two conserved NPA motifs as well as accompanying amino acids supporting the selectivity for water transport. Furthermore, a characteristic putative phosphorylation site was present in all PttPIP2 members.

Like PIP proteins of other plants (Johansson et al. 2001), poplar PIP proteins can be divided into two main branches (PIP1 and PIP2). Chaumont et al. (2001) concluded that both PIP1 and PIP2 groups could additionally be divided into species-specific subgroups and that intensive plant PIP differentiation probably happened some time after monocot–dicot separation. However, in contrast to PIP1 proteins of *Arabidopsis* and *Z. mays* which cluster together, the two poplar PIP1 proteins are located in different subbranches. This result indicates that the subdivision within the PIP1 branch is not exclusively dependent on the phylogenetic relationship of plants.

According to the phylogenetic tree presented in this contribution, PIP2 proteins can be divided into two subgroups, subgroup I containing PttPIP2.1 and PttPIP2.2, and subgroup II containing PttPIP2.3, PttPIP2.4, and PttPIP2.5. This grouping correlates well with the length of loopA (located between the first and the second transmembrane domain). As the loopA region of subgroup I resembles that of PIP1 proteins and as this subgroup contains all known conifer PIP2 members (accepted as evolutionarily older than dicots), it could be supposed that proteins of subgroupI are evolutionarily older and could originate from a PIP1 ancestor. Subgroup II has a loopA region that is seven (five in the case of PttPIP2.4) amino acids longer than that of subgroupI. Since subgroupII contains all *Z. mays* members and monocots are accepted as evolutionarily younger, the separation of subgroupI and II might have occurred during dicot formation and could indicate the specialization of certain organs occurring during the

Table 1 Relative expression rates of *PttPIP* genes in poplar organs of non-mycorrhized (leaf, stem, main and fine root) and mycorrhized (main and fine root = mycorrhiza) plants obtained by semi-quantitative RT-PCR (Fig. 4)

Non-mycorrhized plant				Mycorrhized plant		Gene
Leaf	Stem	Main root	Fine root	Main root	Fine root (mycorrhiza)	
1.0	1.4	3.3	10.2	1.1	30.7	*PttPIP1.1*
1.0	3.5	3.5	3.5	4.9	3.6	*PttPIP1.2*
2.7	2.6	3.3	2.7	5	1.0	*PttPIP2.1*
1.1	1.0	2.0	6.0	3.7	3.4	*PttPIP2.2*
1.2	1.1	4.0	1.0	8.1	3.1	*PttPIP2.3*
2.3	1.0	1.4	3.7	2.1	1.9	*PttPIP2.4*
1.6	-	1.8	4.1	1.0	19.5	*PttPIP2.5*

development of dicotelydons (e.g. pulvini). A further diversification of subgroupII probably happened after the monocot–dicot separation, since *Z. mays* PIP2 proteins (that reveal a loopA region with additional four amino acids) still cluster in one branch separated from the other PIP2 proteins of this subgroup.

The increased length of the loopA region of PIP2s of subgroupII might result in a larger flexibility of the proteins, leading perhaps to increased water transport capacities. Interestingly, two PIP2 proteins, causing rather large rates of water transport when expressed in the *X. laevis* oocyte system (PttPIP2.5, this contribution, and SSAQP2, Moshelion et al. 2002), belong to subgroupII.

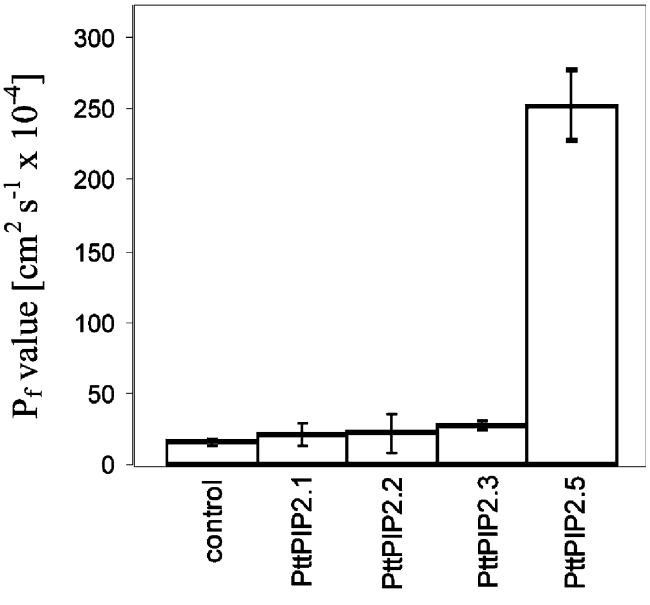

Fig. 5 P_f-values of selected PIP2 proteins heterologously expressed in *X. laevis* oocytes. The increase rate of oocytes expressing *PttPIP2.1*, *PttPIP2.2*, *PttPIP2.3*, or *PttPIP2.5 or* containing no cRNA (control) was calculated from the changes in the surface area of single oocytes using NIH Image. The osmotic permeability coefficient (P_f) was calculated according to Zhang and Verkman (1991). The average of ten experiments and the standard deviations are shown

PIP genes, investigated in this study, were detectable in almost all poplar organs. This result is supported by "electronic Northern blot" analysis of EST library data. ESTs for all investigated PIP genes could be found by a BlastN search (http://poppel.fysbot.umu.se) in public cDNA libraries of leaves, stems and roots. However, even when expressed in all organs, *PttPIP1.1, PttPIP2.2, PttPIP2.3*, and *PttPIP2.5* are preferentially expressed in roots. With the exception of *PttPIP2.2*, the expression levels of all these *PIP* genes increased during ectomycorrhiza development, indicating an increased water transport capacity of the plasma membrane of mycorrhized plants.

While PttPIP2.1 and PttPIP2.2 (both belonging to the presumably older PIP2 subgroupI) revealed no significant water transport when expressed in *X. laevis* oocytes, two transporters of subgroupII, PttPIP2.3 and PttPIP2.5, showed water transport in the heterologous system. While PttPIP2.3 induced a low (P_f value of 22) but significant water uptake, PttPIP2.5 caused a very high water transport activity (P_f value of 252).

It has to be taken into account, that the swelling behavior of oocytes does not necessarily reflect the situation in planta since the transport properties of the foreign protein as measured in oocytes depends on the translation efficiency of the cRNA, the efficiency of protein targeting to the plasma membrane, and the phosphorylation state of the protein. However, aquaporins, that proved to be highly efficient in the oocyte system, mainly originate from tissues with a requirement for high water flux (Kaldenhoff and Eckert 1999; Moshelion et al. 2002). The extremely high water transport capacity of PttPIP2.5 could thus indicate the importance of this protein for water transport in poplar roots.

For white spruce (*Picea glauca)* and *Ulmus*, Muhsin and Zwiazek (2002) observed a larger $HgCl_2$ sensitivity of non-mycorrhized compared to mycorrhized roots, indicating a modulated composition of aquaporins in the plasma membrane. Since the expression of *PttPIP2.5* in poplar was strongly increased upon mycorrhization, the corresponding protein revealed a large water transport capacity, and was insensitive to mercury, it could be supposed, that a homology of this protein might be responsible for the changes observed in white spruce and *Ulmus*.

The increased expression rate of most of the preferentially root-expressed PIP genes in ectomycorrhizas, together with the high rate of water transport of PttPIP2.5 (the most abundantly expressed PIP gene), indicates a strongly enhanced water transport capacity of the plasma membrane of poplar roots in ectomycorrhizal symbiosis. Similarly to previous reports with white spruce and *Ulmus* (Muhsin and Zwiazek 2002; Landhäusser et al. 2002), an increased root hydraulic conductance of mycorrhized poplar plants, reflecting an increased water transport capacity of the root system in symbiosis, was observed in this contribution. However, as in white spruce and *Ulmus*, the larger activation energy for root water transport (E_a) of mycorrhized

compared to non-mycorrhized poplar fine roots also indicates that not only the transport capacity of the plasma membrane but also that of the apoplast is enhanced in ectomycorrhizal symbiosis.

The impact of root anatomy on hydraulic conductance has been shown for a number of different plants (Rieger and Litvin 1999). Changes in root anatomy, which are well documented for mycorrhized fine roots (fungal hyphae are present in the root apoplast and cell wall properties are modulated; for review see Smith and Read 1997), could thus explain, together with an increased plasma membrane transport capacity, the increased root hydraulic conductance that is observed for mycorrhized roots. The reduced size of the root system of mycorrhized poplar plants might be a further consequence of the increased root hydraulic conductance.

In summary, mycorrhized plants exhibit a higher capacity for water transport from (and to) the soil. This might be one reason for the increased nutrient uptake and a lower stomatal resistance of mycorrhized plants, allowing increased rates of CO_2 fixation and thus, a more efficient photosynthesis.

Acknowledgements We are indebted to Margret Ecke, Tawfik Muhsin and Andrea Bock for excellent technical assistance and to Dr. Nina Grunze for critical reading of the manuscript. This work was financed by the Deutsche Forschungsgemeinschaft (Ha 970/10-1 and Ne 332/7-1) as part of the focus program SSP1084 "Molekulare Grundlagen der Mykorrhiza Symbiose".

References

Altschul S, Thomas L, Alejandro A, Jinghui Z, Zheng Z, Webb M, David J (1997) Gapped BLAST and PSI-Blast: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Azaizeh H, Gunse B, Steudle E (1992) Effects of NaCl and $CaCl_2$ on water transport across root cells of maize (*Zea mays* L.) seedlings. Plant Physiol 99:886–894

Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R (1999) The *Nicotiana tabacum* plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. Plant J 18:565–570

Boyle CD, Hellenbrand KE (1991) Assessment of the effect of mycorrhizal fungi on drought tolerance of conifer seedlings. Can J Bot 69:1764–1771

Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R (2001) Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 125:1206–1215

Gerbeau P, Amodeo G, Henzler T, Santoni V, Ripoche P, Maurel C (2002) The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. Plant J 30:71–81

Goss RW (1960) Mycorrhizae of ponderosa pine in Nebraska grassland soils. Res Bull 192:35–47

Grunze N, Willmann M, Nehls U (2004) Impact of ectomycorrhiza formation on monosaccharide transporter gene expression in poplar roots. New Phytol 164:147–156

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsible gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15:11–26

Hampp R, Ecke M, Schaeffer C, Wallenda T, Wingler A, Kottke I, Sundberg B (1996) Axenic mycorrhization of wild type and transgenic hybrid aspen expressing T-DNA indolacetic acid-biosynthesis genes. Trees 11:59–64

Huelsenbeck JP, Larget B, Miller RE, Ronquist F (2002) Potential applications and pitfalls of Bayesian inference of phylogeny. Syst Biol 51:673–688

Johansson U, Karlsson M, Johansson I, Gustavsson S, Sjövall S, Fraysee L, Weig A, Kjellbom P (2001) The complete set of genes encoding major intrisic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinstic proteins in plants. Plant Physiol 126:1358–1369

Kaldenhoff R, Eckert M (1999) Features and function of plant aquaporins. J Photochem Photobiol 52:1–6

Kaldenhoff R, Kolling A, Richter G (1993) A novel blue light- and abscisic acid-inducible gene of *Arabidopsis thaliana* encoding an intrinsic membrane protein. Plant Mol Biol 23:1187–1198

Kaldenhoff R, Grote K, Zhu JJ, Zimmermann U (1998) Significance of plasmalemma aquaporins for watertransport in *Arabidopsis thaliana*. Plant J 14:121–128

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Landhäusser SM, Muhsin TM, Zwiazek J (2002) The effect of ectomycorrhizae on water relations in aspen (*Populus tremuloides*) and white spruce (*Picea glauca*) at low soil temperatures. Can J Bot 80:684–689

Lehto T (1992) Mycorrhizas and drought resistance of *Picea sitchensis* (Bong) Carr I. In condition of nutrient deficiency. New Phytol 122:661–668

Loewe A, Einig W, Shi L, Dizengremel P, Hampp R (2000) Mycorrhiza formation and elevated CO_2 both increase the capacity for sucrose synthesis in source leaves of spruce and aspen. New Phytol 145:565–574

Maurel C, Chrispeels MJ (2001) Aquaporins. A molecular entry into plant water relations. Plant Physiol 125:135–138

Morillon R, Lassalles JP (2002) Water deficit during root development: effects on the growth of roots and osmotic water permeability of isolated root protoplasts. Planta 214:392–399

Morte A, Diaz GR, P. Alarcon JJ, Sanchez-Blanco MJ (2001) Growth and water relations in mycorrhizal and nonmycorrhizal *Pinus halepensis* plants in response to drought. Physiol Plant 44:263–267

Moshelion M, Becker D, Biela A, Uehlein N, Hedrich R, Otto B, Levi H, Moran N, Kaldenhoff R (2002) Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. Plant Cell 14:727–739

Muhsin TM, Zwiazek JJ (2002) Ectomycorrhizas increase apoplastic water transport and root hydraulic conductivity in *Ulmus americana* seedlings. New Phytol 153:153–158

Nehls U, Bock A, Ecke M, Hampp R (2001) Differential expression of hexose-regulated fungal genes within *Amanita muscaria Populus tremula* x *tremuloides* ectomycorrhizas. New Phytol 150:583–589

Parke JL, Linderman RG, Black CH (1983) The role of ectomycorrhizas in drought tolerance of Douglas-fir seedlings. New Phytol 95:83–95

Phillips AL, Huttly AK (1994) Cloning of two giberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: Expression of the tonoplast water channel, TIP, is increased by GA3. Plant Mol Biol 24:603–615

Ramahaleo T, Morillon R, Alexandre J, Lassalles JP (1999) Osmotic water permeability of isolated protoplasts. Modifications during development. Plant Physiol 25:885–896

Rieger M, Litvin P (1999) Root system hydraulic conductivity in species with contrasting root anatomy. J Exp Bot 50:201–209

Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York

Siefritz F, Tyree MT, Lovisolo C, Schubert A, Kaldenhoff R (2002) PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. Plant Cell 14:869–876

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis, vol 2. Academic, London

Steudle E, Peterson CA (1998) How does water get through roots. J Exp Bot 49:775–788

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nuc Acids Res 24:4876–4882

Tyree MT (1997) The cohesion-tension theory of sap ascent: current controversies. J Exp Bot 48:1753–1765

Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R (2003) The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 425:734–737

Wan X, Zwiazek JJ (1999) Mercuric chloride effects on root water transport in aspen (*Populus tremuloides*) seedlings. Plant Physiol 121:939–946

Weig AR, Jakob C (2000) Functional identification of the glycerol permease activity of *Arabidopsis thaliana* NLM1 and NLM2 proteins by heterologous expression in *Saccharomyces cerevisiae*. FEBS Let 481:293–298

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. Plant Cell Env 23:39–49

Yamaguchi J, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsible to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 33:217–224

Zhang R, Verkman AS (1991) Water and urea permeability properties of *Xenopus oocytes*: expression of mRNA from toad urinary bladder. Am J Physiol 260:C26–C34