

RESEARCH PAPER

Aquaporin gene expression and apoplastic water flow in bur oak (*Quercus macrocarpa*) leaves in relation to the light response of leaf hydraulic conductance

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Abstract

It has previously been shown that hydraulic conductance in bur oak leaves (*Quercus macrocarpa* Michx.), measured with the high pressure flow meter technique (HPFM), can significantly increase within 30 min following exposure to high irradiance. The present study investigated whether this increase could be explained by an increase in the cell-to-cell pathway and whether the response is linked to changes in the transcript level corresponding to aquaporin genes. Four cDNA sequences showing high similarity to members of the aquaporin gene family from other plant species were characterized from bur oak leaves and the expression levels of these cDNA sequences were examined in leaves by quantitative real-time PCR (QRT-PCR). No change was found in the relative transcript abundance corresponding to these four putative aquaporin genes in leaves with light-induced high hydraulic conductance (exposed to high irradiance) compared to leaves with low hydraulic conductance (exposed to low irradiance). However, in sun leaves that were exposed to different light levels prior to leaf collection (full sunlight, shade, and covered with aluminium foil for 16 h), the relative transcript levels of two of the putative aquaporin genes increased several-fold in shaded leaves compared to the sun-exposed or covered leaves. When the leaves were pressure-infiltrated with the apoplastic tracer dye trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (PTS₃, 0.02%), there was no change in the PTS₃ concentration of leaf exudates collected in ambient light or in high irradiance, but there was a small apoplastic acidification. There was also no change in PTS₃ concentration between the leaves infiltrated under high irradiance with 0.02% PTS₃ or with 0.1 mM HgCl₂ in 0.02% PTS₃. The results suggest that the putative aquaporin genes that were identified in the present study probably do not play a role in the light responses of hydraulic conductance at the transcript level, but they may function in regulating water homeostasis in leaves adapted to different light conditions. In addition, it is shown that high irradiance induced changes in the pH of the apoplast and that there does not appear to be a significant shift to the cell-to-cell mediated water transport in bur oak leaves exposed to high irradiance as measured by the apoplastic tracer dye.

Key words: Aquaporins, leaf hydraulic conductance, high-pressure flow meter, irradiance, transcript level, trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate.

Introduction

The ability of leaves to transport water, as measured by the hydraulic conductance of leaves, is a dynamic characteristic (Sack and Holbrook, 2006). While water flow across leaves follows the apoplastic, symplastic, and transmembrane pathways (Steudle, 2000; Maurel *et al.*, 2008), the relative

contributions of these pathways to leaf hydraulic conductance are not well characterized. Changes in hydraulic conductance can take place in the apoplastic and transmembrane pathways (Salleo *et al.*, 2001; Zwieniecki, 2001). In the transmembrane pathway, the cell membrane water permeability

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can be rapidly modulated by the abundance and activity of aquaporins (Tyerman *et al.*, 2002; Maurel *et al.*, 2008).

Aquaporins in plants constitute a large and divergent family with 35 members identified in *Arabidopsis thaliana* (Johanson *et al.*, 2001), 33 members in *Oryza sativa* (Sakurai *et al.*, 2005), and 36 members in *Zea mays* (Chaumont *et al.*, 2001). Based on sequence similarities, plant aquaporins are typically divided into four subfamilies: plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), noduline-26 like intrinsic proteins (NIP), and small basic intrinsic proteins (SIP) (Johanson *et al.*, 2001; Zardoya, 2005; Danielson and Johanson, 2008). Three new aquaporin subfamilies have also been described recently in *Physcomitrella patens*, designated GlpF-like intrinsic proteins (GIP), hybrid intrinsic proteins (HIP), and X intrinsic proteins (XIP) (Danielson and Johanson, 2008). The XIP subfamily appears to be found in a range of dicotyledonous taxa (Danielson and Johanson, 2008). The PIP subfamily can be further divided into two groups: PIP1, with low water permeability, and PIP2 with high water permeability, as determined by heterologous expression and swelling assays using *Xenopus* oocytes (Chaumont *et al.*, 2001; Katsuhara *et al.*, 2002). Heterotetramerization of PIP1 and PIP2 aquaporin isoforms may enhance the water transport activity of aquaporins (Fetter *et al.*, 2004).

The contribution of aquaporins to leaf water transport is not well understood. Aquaporin localization in leaves (Kaldenhoff *et al.*, 1995; Robinson *et al.*, 1996; Sarda *et al.*, 1997; Frangne *et al.*, 2001), the accumulation of apoplastic dyes in minor veins of transpiring leaves (Canny, 1995), temperature responses (Sack *et al.*, 2004), and the chemical inhibition of leaf hydraulic conductance (Nardini *et al.*, 2005) argue for a role of aquaporins in the bulk leaf water transport. In addition, in detached walnut (*Juglans regia*) leaves, the irradiance-dependent increase of leaf hydraulic conductance during HPFM measurements (when leaves are filled with water under constant pressure) was correlated with increased expression of two PIP2 aquaporin genes (Cochard *et al.*, 2007). However, other studies suggest that leaf aquaporins may not always play a dominant role in leaf water transport. For example, leaf hydraulic conductance was similar in wild-type *Arabidopsis* and double antisense plants with reduced expression of PIP1 and PIP2 aquaporins (Martre *et al.*, 2002). Also in *Arabidopsis*, protoplasts isolated from leaves with high rates of transpiration showed reduced osmotic water permeability while the opposite was true for the protoplasts from leaves with reduced transpiration (Morillon and Chrispeels, 2001). In tobacco, leaf hydraulic conductance was similar in wild-type and transgenic plants constitutively overexpressing PIP2;5 and PIP1;4 aquaporins under high and low irradiance conditions (Lee *et al.*, 2009). In addition, cell pressure-probe studies showed that cell hydraulic conductivity decreased in the leaves of plants in response to high irradiance, probably due to the inhibition of aquaporin-mediated water transport (Kim and Steudle, 2007, 2008; Lee *et al.*, 2008, 2009). Clearly, more studies are needed to clarify the role of aquaporins in leaf water transport.

In leaves, water flows through several order veins before leaving the xylem and entering the mesophyll. In larger veins the xylem and phloem are surrounded by parenchyma cells and by supporting cells such as collenchyma and sclerenchyma. The smallest veins are enclosed in a tightly packed layer of cells called the bundle sheath which sometimes may have lignified or suberized cell walls. Analogous to the endodermis in roots, the bundle-sheath cells may constitute a barrier that prevents water loss from the xylem (Van Fleet, 1950; Sack and Holbrook, 2006). In transpiring leaves, apoplastic dye crystals may accumulate in leaf minor veins suggesting the movement of water through the bundle-sheath cells (Canny, 1990). In a recent study (Salleo *et al.*, 2003), Phloxin B apoplastic dye was infiltrated under positive pressure through the petioles inside leaves to show that, depending on a plant species, major veins may be leaky or water is transported to increasing order veins before exiting the xylem.

It has previously been shown that the irradiance-dependent increase of leaf lamina hydraulic conductance in bur oak leaves, as measured by the HPFM method, could be modulated by various classes of chemical inhibitors, suggesting that the cell-to-cell transport may play a role in this response (Voicu *et al.*, 2008). The main objective of the present study was to examine the contribution of aquaporin gene expression at the level of transcript abundance to water transport in bur oak leaves exposed to low and high irradiance. The apoplastic fluorescent tracer dye, trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (PTS₃) was used to test the relative contributions of the apoplastic versus the cell-to-cell pathway (Voicu and Zwiazek, 2004). Consistent with the previous work (Cochard *et al.*, 2007; Voicu *et al.*, 2008) it was expected that, if high irradiance increases the expression of aquaporins in excised leaves, a similar response would be found in the sun-exposed leaves collected from trees.

Materials and methods

Plant material

Oak (*Quercus macrocarpa* Michx.) leaves were collected from two mature trees growing at the University of Alberta North Campus, Edmonton, AB, Canada. After excision, the leaf petioles were quickly placed in distilled water and the leaves were brought to the laboratory. The petioles were re-cut under water, the leaves connected to the high pressure flow meter (HPFM, Dynamax Inc., Houston, TX, USA), and their hydraulic conductance measured as described below. Ten leaves per treatment were sampled from one tree (July 2007) and three leaves per treatment were sampled from the second tree (August 2007), about three weeks apart.

In July 2007, several attached, intact leaves from one of the above trees were covered at about 18.00 h with aluminium foil. The next morning at about 10.00 h, the leaves were quickly excised from the tree branch and rapidly

frozen in liquid nitrogen. At the same time, several sun leaves were also sampled as above from the same tree branch. At the time of sampling, the sun leaves were either in full sunlight (between 1300 and 1750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux, PPF), or shaded by other leaves (PPF between 50 and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The leaves were shaded due to the angle of the sun and it was not recorded for how long they were shaded. For RNA extraction, all leaf samples were stored at -80°C .

Leaf hydraulic conductance measurements

Leaf hydraulic conductance (K_{leaf}) was measured using the HPFM as previously described by Voicu *et al.* (2008). Leaves were connected to the HPFM through their petioles using compression couplings, and were perfused with water at constant pressure ranging from 350–450 kPa. A computer recorded the water flow (Q , kg s^{-1}) and the applied pressure (P , MPa) and computed K_{leaf} as Q/P every 2 s, saving mean values every 60 s. The leaves were immersed in distilled water during K_{leaf} determination to minimize temperature fluctuations at the leaf level. The flow stabilized after 30 min at ambient laboratory light (typically less than 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF). Subsequently, a subset of leaves were illuminated at about 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF supplied by white LED lamps (BL-300 series, Lamina Ceramics, Westampton, NJ, USA), while control leaves were kept at ambient laboratory light. After 30 min of differential light treatments, the leaf blade was cut and the leaf area (A , cm^2) measured with a leaf area meter (LI-3100, Li-Cor Biosciences, Lincoln, NE, USA). Leaf blades were then quickly frozen in liquid nitrogen and stored at -80°C . Following leaf blade detachment, the flow usually stabilized within 1 min and then was recorded for an additional 6 min. Petiole hydraulic conductance (K_p) was calculated as the mean of the last six readings.

Leaf lamina hydraulic conductance (K_{lam}) was determined as $K_{\text{lam}} = (1/(1/K_{\text{leaf}} - 1/K_p)) / A$ (Sack *et al.*, 2002).

RNA extraction

Frozen leaf tissue was ground to a fine powder in liquid N_2 using a prechilled mortar and pestle. The ground tissue was stored at -80°C . Total RNA was extracted using the protocol of Chang *et al.* (1993) with modifications as described in Pavy *et al.* (2008). The pellet was dried and resuspended in RNase-free water. The RNA concentration was quantified by measuring the absorbance at 260 nm. The quality of RNA preparation was checked by calculating the ratio of A_{260}/A_{280} nm. The integrity of RNA was checked on a 1% agarose gel containing ethidium bromide (0.001% v/v). Extracted RNA was stored at -80°C .

Isolation of sequences encoding putative aquaporins

Total RNA (3 μg) was reverse-transcribed using the SuperscriptII Reverse Transcriptase kit, according to the manufacturer's instructions (final reaction volume 21 μl) (Invitrogen, San Diego, CA, USA). The resulting single-

stranded cDNA was used for amplification of aquaporin cDNAs using PCR. Degenerate primers were designed against conserved regions from known plant aquaporin cDNA sequences using Vector NTI (Invitrogen). The following primers were used: for the PIP1 group 5'-CTTGKCYTTYGGTGGYATGA-3' (forward) and CT-TGAADGGRATGGCYCTGA (reverse); for the PIP2 group 5'-AAGGAYTAYSWYGAYCCWCC-3' (forward) and TBGYYGTAGATVACAGCAGC (reverse) (IDT, Coralville, IA, USA). EF1- α was cloned using the following primers: 5'-GCTGCTGAGATGAACAAGAG-3' (forward) and 5'-AACGACCCAATGGAGGATAC-3' (reverse). PCR was performed using the following conditions: 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 55°C (PIP1) or 56°C (PIP2) for 45 s, 72°C for 60 s; and the last elongation step of 72°C for 7 min. The 50 μl PCR reaction contained 1 \times ThermoPol Reaction Buffer and 2.5 U of 5 U μl^{-1} *Taq* DNA Polymerase (New England BioLabs, Beverly, MA, USA), 0.2–0.5 μM primers, 0.2 mM dNTPs (Fermentas, Burlington, ON, Canada), and 1 μl of the above cDNA preparation. PCR products were checked on a 1% agarose gel. For the PIP1 reaction, only one band was detected, which corresponded to the expected size of 580 bp. For the PIP2 reaction, the band corresponding to the expected size of 680 bp was excised from the gel. PCR products were purified using the QIAquick PCR purification or gel extraction kits (Qiagen, Chatsworth, CA, USA).

Purified PCR products were ligated into the pGEM-T plasmid according to the manufacturer's protocol (Promega, Madison, WI, USA). The vector to insert ratio was 1:1.5–3. Recombinant plasmids were used to transform *E. coli* DH5 α cells using standard protocols (Sambrook and Russell, 2001). The presence of inserts was verified by PCR using SP6 and T7 universal primers, essentially as described above. Plasmids were purified from cultures grown overnight in liquid LB using GeneJET Plasmid Miniprep Kit (Fermentas). Plasmid preparations were visualized by separating on a 1% agarose gel containing ethidium bromide. The concentration and purity of plasmid preparations were determined by reading the absorbance at 260 nm, and by calculating A_{260}/A_{280} , respectively. Plasmids were stored at -20°C .

Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing kit and the ABI 3730 DNA sequencer from Applied Biosystems (Foster City, CA, USA).

Multiple sequence alignment and phylogenetic analysis

Cloned DNA sequences were translated into deduced amino acid sequences using VectorNTI. ClustalW was used to generate multiple sequence alignments of the *Q. macrocarpa* sequences with aquaporin sequences from *Arabidopsis thaliana*, *Juglans regia*, and *Populus tremula* \times *P. tremuloides*, as follows: AtPIP1;1 (CAB71073), AtPIP1;2 (AAC28529), AtPIP1;3 (AAF81320), AtPIP1;4 (AAF02783), AtPIP1;5 (CAA20461), AtPIP2;1 (CAB67649), AtPIP2;2 (AAD18142), AtPIP2;3 (AAD18141), AtPIP2;4 (BAB09839), AtPIP2;5 (CAB41102), AtPIP2;6 (AAC79629), AtPIP2;7 (CAA17774),

AtPIP2;8 (AAC64216), JrPIP2;1 (AAO39007), JrPIP2;2 (AAO39008), PttPIP1;2 (CAH60718), PttPIP1;3 (CAH60719), PttPIP2;1 (CAH60720), PttPIP2;2 (CAH60721), PttPIP2;3 (CAH60722), PttPIP2;4 (CAH60723), PttPIP2;5 (CAH60724). Per cent similarity between different proteins was calculated as 1–distance, according to the Mega4 manual (Tamura *et al.*, 2007). The alignments were visually inspected and manually adjusted as required. Maximum parsimony phylogenetic trees were constructed in Mega4 using both the complete sequence of PIP proteins from *Arabidopsis*, *Juglans*, and *Populus* and the partial PIP sequences corresponding to the cDNAs isolated from bur oak, as well as only the region common to all sequences. As a means to test the robustness of the tree architecture obtained using maximum parsimony, these trees were compared to trees obtained by the Neighbor–Joining method, with the reliability of the resulting Neighbor–Joining trees tested using the interior branch test of phylogeny with 1000 replicates.

Transmembrane helices were predicted with the on-line TopPred program (<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred>), which predicts transmembrane regions using hydrophobicity analysis according to Claros and von Heijne (1994).

Quantitative real-time PCR (QRT-PCR)

To test the hypotheses that aquaporin expression will increase in the leaves which show a high irradiance-induced increase in hydraulic conductance and in the leaves exposed to full sunlight while on a tree crown, QRT-PCR experiments were performed. Plant material was collected and total RNA was extracted as described above. For each sample, 6 µg of DNaseI-treated total RNA was reverse transcribed using M-MuLV reverse transcriptase, and the cDNA was treated with RNaseH to remove the RNA using the manufacturer's protocol (New England Biolabs), with a final reaction volume of 20 µl. The cDNA was diluted 1:10, and 2.5 µl of the dilution was used for each QRT-PCR reaction. QRT-PCR analysis of the putative aquaporin genes was carried out in 96-well plates using the Applied Biosystems 7500 Fast Real-Time PCR System. The primers used for QRT-PCR were designed using Primer Express 3 (Applied Biosystems). The following gene-specific primers (5'–3') were used: for *QmPIP1;1* gene AAA AAG GTG GAG CCA ACT TCG (forward) and ACA TGG GAG TCC CTA GCG C (reverse); *QmPIP2;2* gene GTG GTG GCG CCA ACT CTC (forward) and AGG AAC GTG TGA ATC TCT AGC TTT C (reverse); for *QmPIP2;3* gene TAA TGG TGG TGG TGC TAA CCT TG (forward) and CGT GCG CTT CTC TTT GGG (reverse); for *QmPIP2;1* gene GGT GGA GCC AAC GGG C (forward) and GAA CAT GGG AAT CCC TTG CA (reverse); for *EF1-α* gene CTC ACG GGT CTG TCC ATC CT (forward) and TGT GCC GTC CTC ATT ATT GAC T (reverse). Specificity of the amplification was confirmed by melting curve analysis for the PCR products at the end of each QRT-PCR run. For the quantitative PCR reaction, 2.5 µl of the cDNA dilution was used in a total volume of 10 µl containing 0.4

µM of each forward and reverse gene-specific primer, 0.2 mM dNTPs, 0.25× SYBR Green, 0.1× ROX, and 0.3 U Platinum *Taq* (Invitrogen). The cycling conditions were: Step 1, 95 °C for 2 min; Step 2, 40 cycles of 95 °C for 15 s and 60 °C for 60 s; Step 3, 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s, and 60 °C for 15 s. Data collection was carried out at the end of each round in Step 2. Quantification of transcript abundance was performed using standard curves generated with a dilution series of amplicons produced from each of the aquaporin cDNAs, using plasmid inserts as template and SP6 and T7 universal primers for amplification in PCR reactions as described in previous sections. The five different amplicons were pooled prior to making a serial dilution series spanning from 4×10^7 to 4×10^1 copies for each amplicon. *EF1-α* was used as the reference gene. The amplifications were done on three to four independent samples for each treatment (biological replicates), and triplicate reactions were carried out for each sample (technical replicates). All samples that were to be compared statistically were assayed on the same 96-well plate. Therefore, each 96-well plate was divided into two standard curves (for the PIP and *EF1-α* genes) occupying 20 wells, two wells as blanks, and six wells for each sample (three for the PIP gene and three for the *EF1-α* gene). By assaying all biological replicates of an experiment for PIP expression and *EF1-α* expression on the same 96-well plate, the effect of plate-to-plate variation, that would have been encountered if multiple 96-well plates were used to accommodate additional biological replicates, was minimized. The amount of each amplicon (expressed as copy number) was determined using its corresponding standard curve. The relative abundance of each investigated gene was calculated by dividing the copy number of the transcript of interest with the copy number of *EF1-α* transcript. The mean values of the technical replicates were used for statistical analysis.

Relative apoplastic water flow and HgCl₂ effects, pH changes in bur oak leaves

Changes in apoplastic water flux through leaves were investigated using a fluorescent tracer dye, trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (PTS₃) (Hanson *et al.*, 1985; Voicu and Zwiazek, 2004). The dye is membrane impermeable and does not bind to cell walls (Hanson *et al.*, 1985; Moon *et al.*, 1986). In leaves, water moving out of the xylem can flow either around the cells or enter the symplast at the bundle-sheath level. Since PTS₃ is confined to the apoplast, it may be used to assess the relative contribution of apoplastic versus cell-to-cell pathways for water movement in leaves. Therefore, if irradiance increases the aquaporin activity in leaves and the contribution of the cell-to-cell pathway increases, a decline in the dye concentration from ambient light to high irradiance would be expected. Using pressure chambers (PMS Instruments, Corvallis, OR, USA), leaves were infused with a solution of 0.02% (w/v) PTS₃ under 0.4 MPa pressure. The first droplets coming out of the stomata were wiped with paper towels, then leaves were enclosed in plastic bags, placed on

the bench under ambient light ($PPF < 15 \mu\text{mol m}^{-2} \text{s}^{-1}$), and the solution was collected in the bags. After about 0.5 ml had been collected (about 1.5 h), the leaves were wiped dry with paper towels, enclosed in new plastic bags, and placed under high irradiance (PPF of about $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$) provided by LED lamps as described above and a new fraction collected for about 30 min. Then the tertiary veins, secondary veins, and leaf blade were successively severed and the corresponding exudates collected in order to investigate if accumulation of PTS_3 had occurred in the leaf veins and if there are apoplastic pH changes as water moves through the leaves. A small amount of the infusion solution was also collected. The pH of the different fractions collected was then measured with a micro pH probe (Jenco Electronics, Taipei, Taiwan). The concentration of PTS_3 was determined using a Sequoia-Turner 450 fluorometer (Apple Scientific, Chesterland, OH, USA) using an excitation wavelength of 405 nm and an emission wavelength of 515 nm. Concentrations of PTS_3 in different fractions were calculated from a standard curve prepared from known PTS_3 concentrations.

To measure changes in apoplastic flow induced by mercury treatment, two solutions were forced inside different leaves: control solution consisting only of 0.02% PTS_3 , and 0.1 mM HgCl_2 in 0.02% PTS_3 . The leaves were enclosed in plastic bags and placed under the high irradiance source as described above. The leaf exudates were collected every hour for 3 h. Before a new exudate was collected, leaves were wiped dry with paper towels and enclosed in new plastic bags. It was expected that if water moves mostly through the cell-to-cell pathway under high irradiance, mercury treatment would block the aquaporins and cause an increase in the dye concentration of the exudate, since more water would now move mostly through the apoplast.

Statistical analysis

Analysis of variance was performed using the GLM or Mixed procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC, USA). Data were checked to meet the normality and homogeneity of variances assumptions.

Lamina hydraulic conductance from different trees, PTS_3 concentration, HgCl_2 effects, and pH changes in bur oak leaves apoplast were analysed using a repeated measures analysis of variance. The Variance-Covariance Matrix chosen for each model was an iterative process and based on the Schwarz's Bayesian Criterion (SAS 2000). Petiole hydraulic conductivity was analysed with a t test. Transcript levels of different genes in leaves with K_{lam} determined were analysed using the sampled tree as a random factor. For the *QmPIP2;2* gene, the data were log transformed to meet the ANOVA assumptions. Data for *QmPIP2;1* gene were analysed using ANOVA with unequal variance followed by mean separation with Tukey adjustment. For all different statistical models, Least Square Means (lsmeans) and standard errors were calculated for treatments and the lsmeans separated using a pdiff option. All differences were

tested at the $P \leq 0.05$ level. For the log transformed data mean values of the raw data were used for plotting.

Results

Cloning of new PIP genes expressed in *Q. macrocarpa* leaves

The above approach produced four unique sequences that showed high similarity to PIP genes after a BLAST analysis (Altschul *et al.*, 1997; <http://www.ncbi.nlm.nih.gov/BLAST>). These partial cDNA clones have lengths between 578 bp and 677 bp, corresponding to 192–225 amino acids, respectively. The deduced amino acid sequence of these partial cDNA clones corresponded to approximately two-thirds to four-fifths of the full coding region of the *Arabidopsis* PIPs. A multiple alignment of the deduced amino acid sequences of *Q. macrocarpa* and aquaporins identified from other plant species indicated that one of the four sequences exhibits greater sequence similarity to the PIP1 class of aquaporins, while the other three sequences exhibit greater sequence similarity to the PIP2 class. Accordingly, these cDNAs were named *QmPIP1;1*, *QmPIP2;1*, *QmPIP2;2*, and *QmPIP2;3*, with accession numbers FJ495147, FJ495155, FJ495161, and FJ495163, respectively.

Structure and phylogenetic analysis of PIP genes from *Q. macrocarpa*

The deduced amino acid sequences of the identified cDNAs from *Q. macrocarpa* included features typical of MIP proteins. The partial sequences obtained in this study contained residues conserved in other PIP proteins, as well as the repeated NPA motif, a typical MIP family signature (Fig. 1). Hydrophobicity analysis of the putative partial polypeptides as determined by TopPred, indicated five membrane spanning putative α -helices (Fig. 1). All of the identified putative aquaporin amino acid sequences contain the four amino acid residues of the ar/R (aromatic/Arg) filter, thought to govern the water transport specificity of aquaporin-type MIP (Forrest and Bhave, 2008; Wallace and Roberts, 2004). They also contain histidine residues shown to be involved in pH sensing in other aquaporins (Luu and Maurel, 2005).

At the amino acid level, the *Q. macrocarpa* sequences share a high percentage similarity with different aquaporin proteins. The predicted amino acid sequence of *QmPIP2;2* gene has the highest sequence similarity with PttPIP2;4 (87.9%), AtPIP2;5 (85.3%), JrPIP2;1 (85.3%), and JrPIP2;2 (85.3%). For the *QmPIP2;1* sequence the highest similarity is shared with JrPIP2;1 (90.2%), JrPIP2;2 (90.2%), PttPIP2;5 (88.8%), PttPIP2;4 (86%), PttPIP2;3 (84.4%), AtPIP2;1 (84.4%), and AtPIP2;2 (84.4%). The *QmPIP2;3* sequence has the highest amino acid similarity with AtPIP2;8 (88.9%), AtPIP2;7 (88.5%), PttPIP2;1 (87.6%), and PttPIP2;2 (87.6%). The *QmPIP1;1* sequence has the highest sequence similarity with AtPIP1;3 (90.6%),

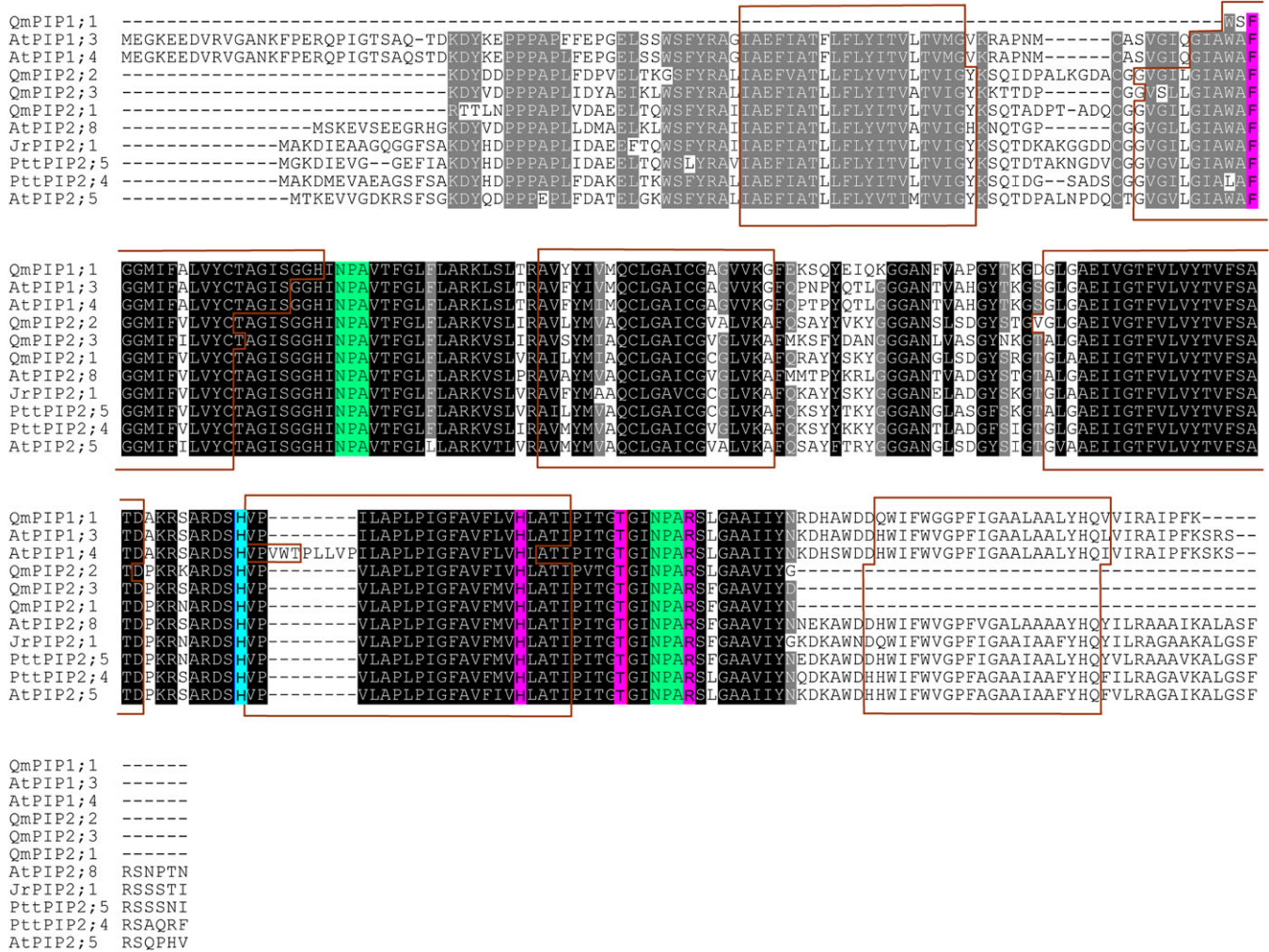


Fig. 1. Multiple sequence alignment of PIP sequences from *Q. macrocarpa*, *A. thaliana*, and *P. tremula* × *P. tremuloides*. Predicted amino acid sequences were aligned using ClustaW. Identical residues in all sequences are shaded in black, while similar residues are shaded in grey. The conserved NPA motives are shaded in green. The ar/R selectivity filter residues are shaded in magenta. Histidine residues involved in pH sensing are shaded in blue. Predicted transmembrane helices are shown in brown boxes.

AtPIP1;5 (90.6%), AtPIP1;1 (89.6%), and Ptt1;2 (89.6%). Among them, the deduced amino acid sequences of these partial cDNAs share between 77.5% and 88.5% sequence similarity. At the nucleotide level, the percentage similarity varies from 71.3% to 80.4%. Bur oak QmPIP2;1 and QmPIP2;2 also show a longer A loop region.

Phylogenetic analysis of the *Q. macrocarpa* PIP partial sequences was performed together with selected characterized aquaporin sequences from *A. thaliana*, *J. regia*, and *P. tremula* × *P. tremuloides* hybrid (Fig. 2). Figure 2 depicts a consensus maximum parsimony tree (of 212 trees) obtained using the portion of sequence common to all sequences. The maximum parsimony consensus tree (of eight trees) obtained using full-length sequences for *Arabidopsis*, *Juglans*, and *Populus* together with the partial *Q. macrocarpa* sequences showed identical topology and similar branch support to the tree obtained using only the common sequence region (data not shown). Neighbor-Joining trees obtained using either full-length sequences for *Arabidopsis*, *Juglans*, and *Populus* or the common sequence

region also showed very similar topology (data not shown). The maximum parsimony tree showed clustering of the encoded proteins with different aquaporin subgroups, verifying that QmPIP1;1 belongs to the PIP1 class, while QmPIP2;1, QmPIP2;2, and QmPIP2;3 belong to the PIP2 class. According to the phylogenetic analysis, QmPIP1;1 is a potential orthologue of one *Arabidopsis* (AtPIP1;5) and two hybrid poplar (PttPIP1;1 and PttPIP1;2) aquaporins; QmPIP2;1 is a potential orthologue of AtPIP2;4, PttPIP2;3, PttPIP2;5, JrPIP2;1, and JrPIP2;2; QmPIP2;2 is a potential orthologue of AtPIP2;5; and QmPIP2;3 is a putative orthologue of AtPIP2;7, PttPIP2;1, and PttPIP2;2.

K_{lam} light response and transcript level of putative leaf aquaporins

Lamina hydraulic conductance increased significantly in leaves from both trees upon exposure to high irradiance (Fig. 3A, B). For the same leaves, there were no significant differences in the transcript level of putative aquaporin

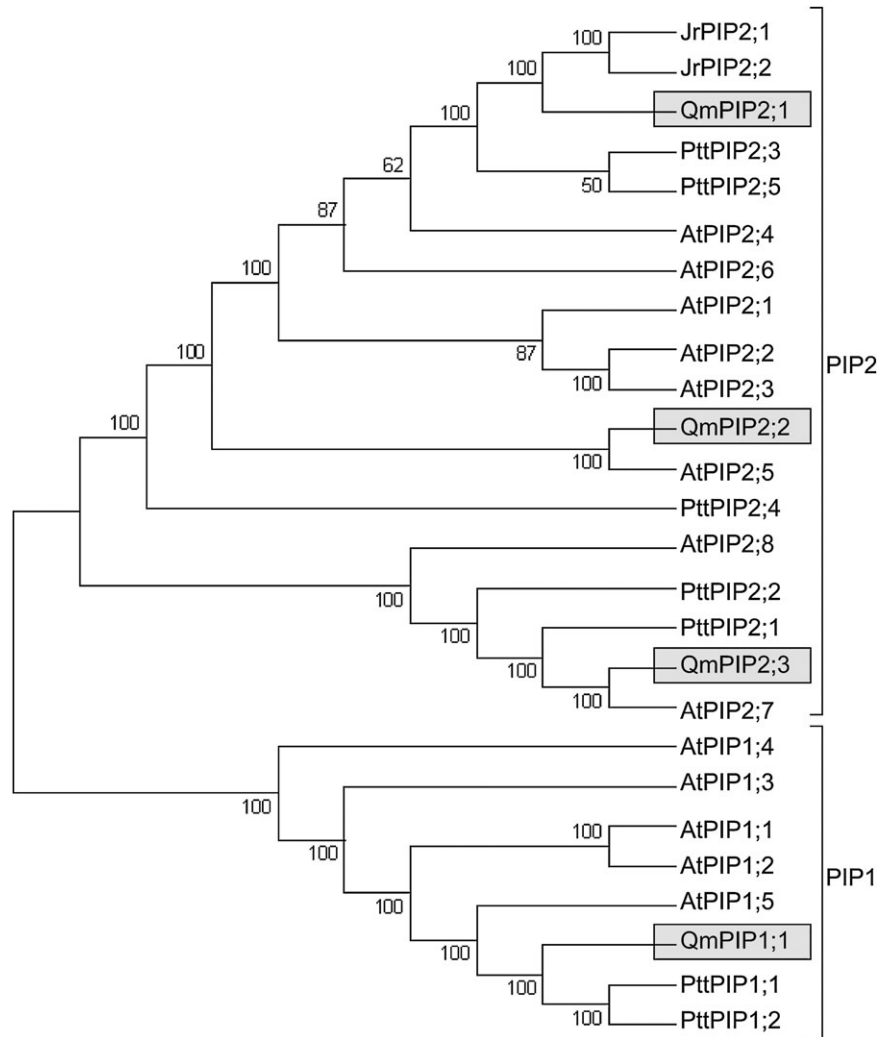


Fig. 2. Phylogenetic relationship between *QmPIP* deduced amino acid sequences and sequences of *A. thaliana*, *P. tremula* × *P. tremuloides*, and *J. regia*. Sequences were aligned using ClustalW, and the region common to all sequences was selected for analysis by maximum parsimony. The maximum parsimony tree was obtained using Mega4. The percentage of parsimonious trees in which the associated taxa clustered together are shown next to the branches.

genes between the treatments (Fig. 4). However, there were some significant differences ($P=0.021$) between trees in terms of the expression level of putative aquaporin genes (Fig. 4). As required before assessing gene expression, the suitability of EF1- α as a reference gene was assessed by performing ANOVA with the EF1- α C_T values of each sample. There was no significant difference in the mean C_T value of EF1- α between light treated (22.38 ± 0.41) or control leaves (22.44 ± 0.41) ($P=0.894$).

Aquaporin expression and the light responses within a tree crown

Different putative aquaporin genes showed different expression profiles with the light regime. The EF1- α was also used as a reference gene for this experiment since there were no significant difference in the mean C_T value of EF1- α among shade-exposed (23.74 ± 0.32), light-exposed (23.89 ± 0.32), and dark-treated leaves (23.85 ± 0.32) ($P=0.947$).

There was no significant difference in transcript abundance for *QmPIP1;1* and *QmPIP2;3* as a function of the different treatments (Fig. 5A, C). For *QmPIP2;2*, although the differences were not significant at the 0.05 level, there was a tendency for an increasing level of expression in the shade-exposed leaves (overall ANOVA $P=0.073$; dark-shade $P=0.078$; light-shade $P=0.159$; and light-dark $P=0.888$) (Fig. 5C). Different light regimes significantly influenced the expression of *QmPIP2;1*. The highest level of expression was found in shade-exposed leaves, followed by light exposed leaves, and dark-treated leaves (Fig. 5D).

Changes in pH, PTS₃ concentration and HgCl₂ effects in leaf exudates

When leaves were exposed to high irradiance, the pH of the leaf exudate significantly decreased ($P=0.04$) (Table 1). The pH increased in the exudates collected after tertiary veins and secondary veins were severed. When the leaf blade was

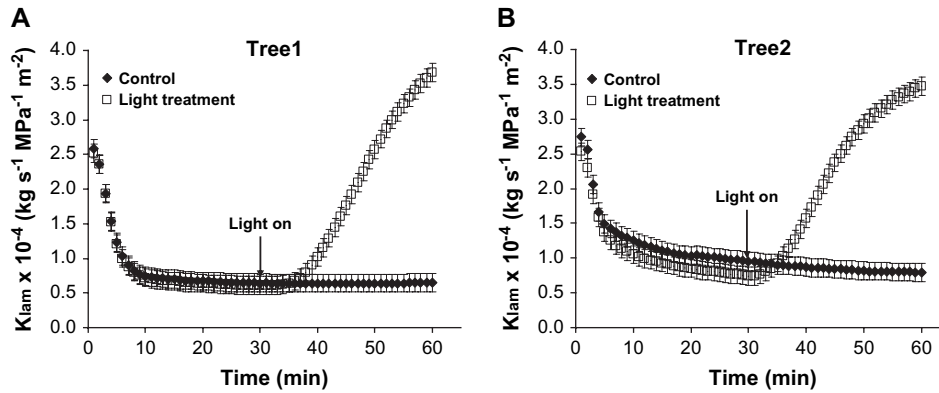


Fig. 3. Time-course of lamina hydraulic conductance (K_{lam}) of leaves from two bur oak trees: (A) tree 1 ($n=10$), and (B) tree 2 ($n=3$). Control leaves were exposed only to ambient laboratory lights. Light treated leaves were exposed for 30 min to high irradiance, following low irradiance exposure. Values are least square means. Mean standard error bars are also shown. At the end of K_{lam} determination, leaves were quickly frozen and used for gene expression analysis.

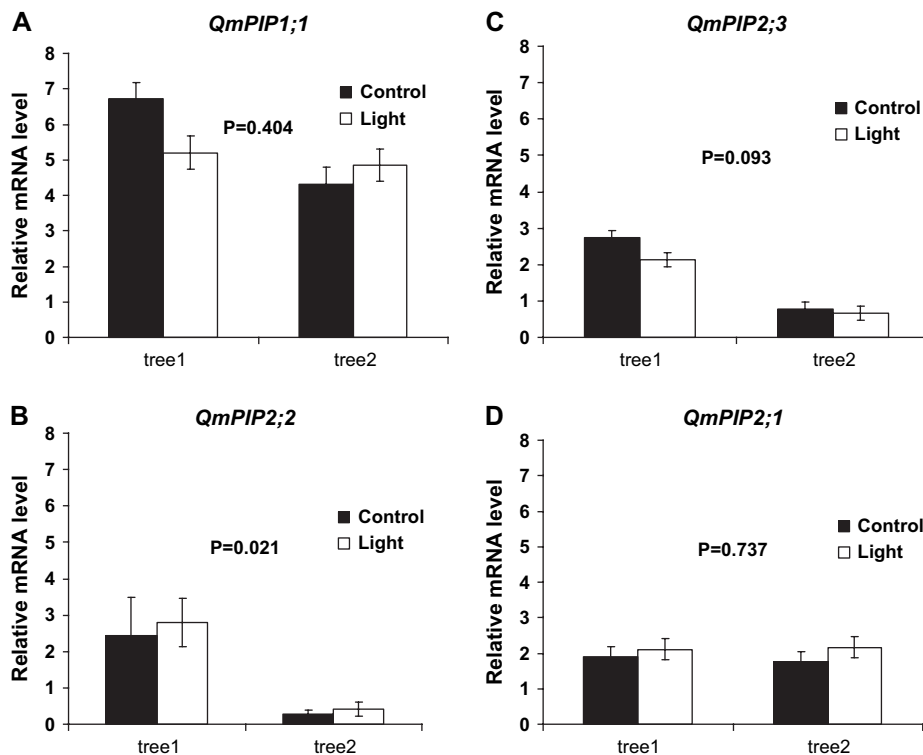


Fig. 4. Relative expression level of putative aquaporin genes *QmPIP1;1* (A), *QmPIP2;2* (B), *QmPIP2;3* (C), and *QmPIP2;1* (D), between the leaves with light-induced high hydraulic conductance (light treatment) and leaves with low hydraulic conductance (control), shown separately for each tree. Mean values \pm standard errors are shown ($n=3$). The P values assess the statistical difference ($P \leq 0.05$) between trees.

removed, the pH of the petiole exudates was similar to the pH of the infusion solution (Table 1).

The concentration of the apoplastic dye PTS_3 significantly decreased ($P=0.002$) by 20% in leaf exudates collected in ambient light and did not change in the exudates collected in high irradiance (Table 1). When the leaves were subjected to high irradiance, the PTS_3 concentration remained significantly lower than the concentration of the infusion solution. The concentration of PTS_3 in-

creased slightly in the exudates collected after vein severance (Table 1). However, the concentration of PTS_3 in ambient light or high irradiance was not significantly different than that of vein exudates (Table 1).

When leaves were pressure-infiltrated with a solution of PTS_3 in combination with 0.1 mM $HgCl_2$, the concentration of PTS_3 in leaf exudates after 1 h significantly decreased by about 10% in both PTS_3 ($P=0.048$) and PTS_3+HgCl_2 ($P=0.03$) treatments (Fig. 6). However, there were no

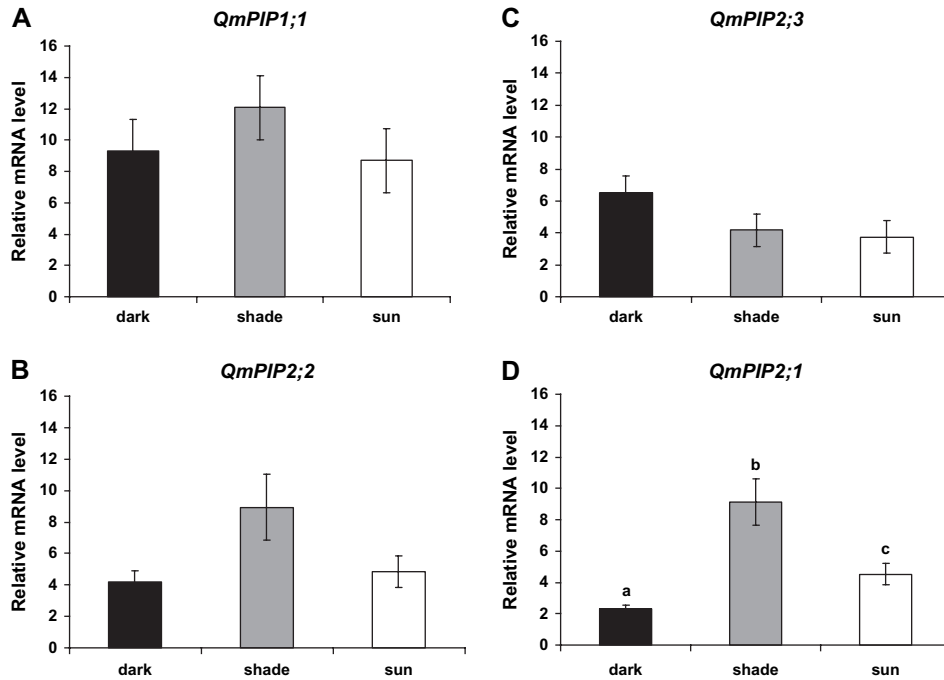


Fig. 5. Relative expression level of putative aquaporin genes *QmPIP1;1* (A), *QmPIP2;2* (B), *QmPIP2;3* (C), and *QmPIP2;1* (D), in leaves exposed to different light levels *in situ* (full sunlight, sun-adapted; shade, shade-adapted; and covered with aluminium foil, dark-adapted leaves). Mean values \pm standard errors are shown ($n=4$). Bars with different letters represent significant ($P \leq 0.05$) differences.

Table 1. pH and PTS_3 concentration changes in 0.02% PTS_3 forced through bur oak leaves

The leaves were exposed to ambient light ($\text{PPF} < 15 \mu\text{mol m}^{-2} \text{s}^{-1}$) followed by high irradiance ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF), then tertiary veins and secondary veins were successively severed. At the termination of the experiment, the leaf blade was detached and the petiole exudates collected. The pH and % PTS_3 of the infusion solution are presented. ANOVA P values for differences among different treatments are also given. The values represent least square means \pm SD ($n=5-6$). The numbers followed by different letters represent significant differences ($P \leq 0.05$) after Tukey adjustment.

Treatment	pH	% $\text{PTS}_3 \times 100$
Infusion solution	5.07 ± 0.13 a	2.06 ± 0.04 a
Ambient light leaf exudates	5.09 ± 0.19 a	1.66 ± 0.24 b
High irradiance leaf exudates	4.72 ± 0.17 b	1.63 ± 0.30 b
Tertiary veins exudates	4.74 ± 0.53 ab	1.76 ± 0.41 ab
Secondary veins exudates	5.04 ± 0.41 ab	1.68 ± 0.45 ab
Petiole	5.24 ± 0.20 a	1.73 ± 0.35 ab
ANOVA P value	0.014	0.003

differences between treatments over time, while the PTS_3 concentrations in both treatments increased after 2–3 h from the beginning of the treatment (Fig. 6).

Discussion

The present study identified four putative aquaporin genes from bur oak leaves, and characterized the response of these genes to light at the level of transcript abundance. Contrary to our hypothesis, the transcript abundance corresponding to these four genes was not correlated with the irradiance-

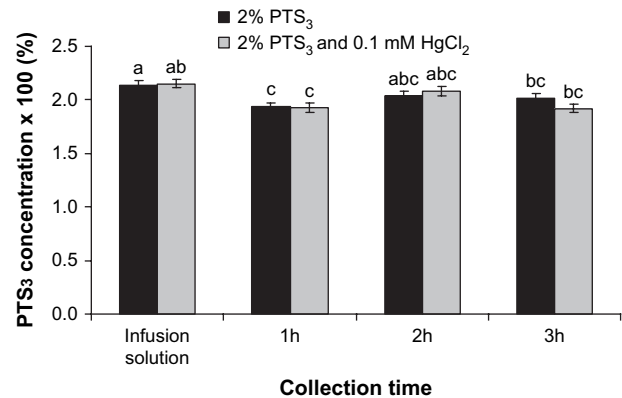


Fig. 6. Changes in the concentration of a solution of 0.02% PTS_3 pressure-infiltrated into oak leaves, alone or in combination with 0.1 mM HgCl_2 . Least square means \pm SE are presented ($n=6$). Bars with different letters represent significant ($P \leq 0.05$) differences after Tukey adjustment.

dependent increase in the lamina hydraulic conductance during the HPFM measurements. The experiment using leaves exposed to full sunlight and shade, showed that at least one aquaporin, *QmPIP2;1*, was differentially expressed when the leaves were exposed *in situ* to different light regimes. The study also showed that light induced changes in the pH of the apoplast and that there does not appear to be a significant shift to the cell-to-cell mediated water transport in bur oak leaves exposed to high irradiance.

The cDNA sequences identified from the bur oak leaves belong to the PIP subfamily of aquaporins, three to the

PIP2 group and two to the PIP1 group. The presence of the highly conserved NPA motif and the ar/R selectivity filter residues (Fig. 1) suggest that the identified PIPs are likely to be functional aquaporins (Wallace and Roberts, 2004; Forrest and Bhavé, 2008). The ar/R filter is thought to govern the water transport specificity of aquaporin-type MIP (Wallace and Roberts, 2004; Forrest and Bhavé, 2008). In addition, the amino acid sequences contain the histidine residue involved in pH sensing (Luu and Maurel, 2005). Most of the putative orthologues of QmPIP2;1 and QmPIP2;2 suggested by phylogenetic analysis, with the exception of PttPIP2;3 and AtPIP2;5 have been shown to be highly conductive to water when expressed in oocytes (Sakr *et al.*, 2003; Marjanovic *et al.*, 2005). In addition, QmPIP2;1 and QmPIP2;2 also show a longer loop A region, which was suggested to increase water transport capacities (Marjanovic *et al.*, 2005). All plant PIP2 proteins tested so far exhibit high water-channel activity, while PIP1 proteins have either low or no water-channel activity (Chaumont *et al.*, 2005). However, PIP1 and PIP2 may form heterotetramers which may further enhance their water transport activities (Maurel *et al.*, 2008).

The contribution of aquaporins to water transport in leaves is not very well understood. The rapid irradiance-dependant increase of leaf hydraulic conductance is thought to involve increased aquaporin activity (Tyree *et al.*, 2005; Voicu *et al.*, 2008). However, in the present study, there was no change in the relative expression level of four putative aquaporin genes between leaves with light-induced high hydraulic conductance (high irradiance) and those with low hydraulic conductance (low irradiance) (Figs 3, 4). By contrast, Cochard *et al.* (2007) showed a large increase in the JrPIP2;1 and JrPIP2;2 transcript levels in walnut leaves with light-induced high hydraulic conductance measured by the HPFM technique. There are several possible explanations for this apparent discrepancy. For example, although in our study the putative aquaporin genes were isolated from leaves of bur oak, it is possible that there are other aquaporins that are also expressed in leaves. Further, in *Arabidopsis* leaves, all of the PIP genes are expressed but at different levels (Alexandersson *et al.*, 2005). Consequently, it is possible that one or more other leaf-expressed aquaporins contributes to leaf hydraulic conductance. It is also possible that the expression of aquaporins involved in regulating leaf hydraulic conductance is controlled at the post-translational rather than transcriptional level, since aquaporin activity can be regulated by post-translational modifications such as phosphorylation, protonation, and heterotetramer formation (Maurel *et al.*, 2008). Finally, there may be species-specific differences in aquaporin expression and activity. In the present study, the difference in the aquaporin expression levels between the two different trees may be related to the different microenvironmental conditions of the sampled trees or to the time difference between sampling of trees (about 3 weeks).

During the HPFM measurements of K_{lam} water is pushed under constant pressure inside detached leaves and quickly floods leaf air spaces. An infiltrated leaf apoplast resulted in

cytoplasmic acidification and apoplastic alkalization (Felle, 2006) and may decrease the rate of water transport through aquaporins by cytosolic proton gating (Tournaire-Roux *et al.*, 2003). In the present study, a small acidification has been detected in response to high irradiance in leaf exudates and that possibly started in tertiary veins (Table 1). Light-induced acidification of the leaf apoplast has previously been reported (Mühling *et al.*, 1995; Shabala and Newman, 1999; Stahlberg and Van Volkenburgh, 1999) and shown to be associated with leaf veins (Shabala *et al.*, 2002). In addition, changes in apoplastic pH may accompany changes in leaf hydraulic conductance (Aasamaa and Söber, 2001). Therefore, while leaf infiltration may cause cytosolic acidification and, subsequently, aquaporin blockage by cytosolic protons, high irradiance may promote the pumping of protons out of the cytosol and activation of the aquaporin activity. This may be reflected in an increased K_{lam} under high irradiance. At the same time, pH decreases in leaves may also increase the rate of water flow out of leaf veins through pit membranes (Zwieniecki, 2001). While only a change in the pH of leaf exudates was detected, the influence of apoplastic pH on leaf hydraulic conductance should be investigated further.

The contribution of gene expression of putative aquaporins to water transport in bur oak in sun leaves that were exposed to different light levels *in situ* (full sunlight, shaded, and covered leaves) was investigated further. To our knowledge, this study is the first to report expression of aquaporin genes in leaves growing and harvested under natural field conditions. The relative transcript level of the *QmPIP2;1* gene increased significantly in shaded leaves as opposed to the leaves kept in darkness or in full sunlight (Fig. 5D), contrary to our original hypothesis that aquaporin expression will increase in full sunlight. Transcript levels corresponding to *QmPIP2;2* also showed a similar trend (Fig. 5B). Our results are in agreement with those from *Arabidopsis* showing that protoplasts isolated from leaves with high levels of transpiration had reduced osmotic water permeability, while the opposite was true for the protoplasts isolated from the leaves with reduced transpiration (Morillon and Chrispeels, 2001). In addition, cell-pressure-probe measurements of leaf midrib cells showed that, in intact plants, there is a decrease of leaf cell hydraulic conductivity under high transpirational demand induced by high irradiance (Kim and Steudle, 2007, 2008; Lee *et al.*, 2008, 2009). Our previous study showed that, in bur oak, the light-induced enhancement of K_{lam} saturates at around $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF which corresponds to shade-like conditions (Voicu *et al.*, 2008). Therefore, at higher irradiances, as experienced in the field and beyond those that are typically achievable in growth chambers, aquaporin transcripts and activity are likely to be down-regulated. When stomata partially closed in shaded leaves, there was a moderate enhancement in leaf water potential experienced by *Q. macrocarpa* leaves within the tree crown, (Knapp, 1992; Hamerlyncx and Knapp, 1994). This may trigger aquaporin up-regulation which may allow for

a faster leaf rehydration and restoration of the water homeostasis.

The relative expression levels of the PIP genes appear to be different in the leaves that were measured with the HPFM (Fig. 4) as compared with the leaves for which no HPFM measurements were performed (Fig. 5). Since the leaves for both experiments were sampled only two days apart, the mRNA content may have decreased after leaf excision and during HPFM measurements as a result of hypoxia being imposed on the leaf.

In the present study, when the concentration of the fluorescent apoplastic tracer PTS₃ was assessed, it showed no difference among different experimental treatments that were expected either to enhance (high irradiance versus ambient light – decline in PTS₃ concentration; Table 1) or reduce (HgCl₂ versus distilled water – increase in PTS₃ concentration; Fig. 6) the rate of water flow through the cell-to-cell pathway. This is not consistent with an increase in the cell-to-cell pathway as suggested for the K_{lam} light response (Cochard *et al.*, 2007; Voicu *et al.*, 2008). Mercury reduced the bur oak K_{lam} irradiance response in our previous study (Voicu *et al.*, 2008), but in the present study, the PTS₃ concentration was not affected by mercury (Fig. 6). The use of PTS₃ as a quantitative apoplastic tracer has been criticized because, in roots, PTS₃ is mainly retained by the endodermis and around 0.3% of the initial concentration is recovered in the xylem when the roots were pressure-infiltrated with PTS₃ (Zimmermann and Steudle, 1998; Zimmermann *et al.*, 2000; Voicu and Zwiazek, 2004). However, it has previously been found that, in detached aspen roots, an increase in the relative PTS₃ concentration was correlated with an overall reduced water flow and a concomitant decline in the abundance of aquaporin proteins caused by a treatment with cycloheximide (Voicu and Zwiazek, 2004). Therefore, in the present study, PTS₃ was used to assess the relative contributions of the apoplastic and cell-to-cell pathways. However, it was noticed that there was very little difference in the PTS₃ concentration of the infusion solution and that of leaf exudates. When tertiary or secondary veins were cut open or when the lamina was detached, the increase in PTS₃ concentration was small, indicating that there was probably no high accumulation of the dye in the apoplast of leaf xylem. Since some non-specific binding of PTS₃ in the leaf may have occurred, the leaf treatment applied in the present study did not result in any difference in the PTS₃ concentration. Under the conditions of the present study the dye moved relatively fast past the veins xylem. The possibility that PTS₃ has accumulated in leaf minor veins (Canny, 1995) and moved by diffusion outward cannot be discounted. By the time the leaf exudates were collected in ambient light (which took about 1.5 h), the diffusion may have equalized the PTS₃ concentration in veins with that of leaf exudates. However, the diffusion of PTS₃ out of veins through the cell walls is probably a very slow process in ambient light; when light intensity of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF was used, it took about 8 h for the dye to reach the abaxial epidermis (Fitzgerald and Allaway, 1991). In one instance when leaves

were viewed under an epifluorescent microscope, PTS₃ moved very fast even across the suberized bundle-sheath tissues (Eastman *et al.*, 1988). In the present study, leaves experienced similar conditions during PTS₃ applications as during the HPFM measurements of K_{lam} . Therefore, it is possible that, during the HPFM measurements of K_{lam} , the cell-to-cell pathway of water movement is largely bypassed (Sack *et al.*, 2002), or that the main pathway for water movement in leaves exposed to high irradiance is through the apoplast (Kim and Steudle, 2007; Lee *et al.*, 2008, 2009). In the latter case, the rapid change of K_{lam} under high irradiance may be accomplished by the increase in water permeability of the pit membranes at the bundle-sheath–xylem interface or of cell-walls as determined by changes in the ionic composition of the xylem sap (Zwieniecki, 2001). Light may induce large ionic fluxes in the vascular bundles of the leaf (Shabala *et al.*, 2002) and xylem sap ionic composition and pH may increase or decrease the xylem hydraulic conductance through the swelling and shrinking of the pectin hydrogel of the pit membranes (Zwieniecki, 2001). In twigs, the effect appears to be mediated by phloem (Zwieniecki *et al.*, 2004). However, the involvement of phloem or bundle-sheath cells in regulating the water permeability of the surrounding apoplast should be further tested.

In conclusion, the putative aquaporin genes that were identified in the present study probably do not play a role in the light responses of hydraulic conductance at the transcript level as measured by the HPFM technique and at irradiance levels greater than 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF. However, at least one of these aquaporins could be important in regulating water homeostasis in leaves under varying light conditions. These results and those of the apoplastic fluorescent tracer experiments demonstrate that the pathways for water movement in leaves are far from being understood and require more follow-up studies. Future experiments should investigate the interplay among aquaporin transcripts and protein levels in leaves from different species exposed to a combination of different water potentials, air relative humidity and light intensities.

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