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Research paper

Light-mediated K_{leaf} induction and contribution of both the PIP1s and PIP2s aquaporins in five tree species: walnut (*Juglans regia*) case study

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Understanding the response of leaf hydraulic conductance (K_{leaf}) to light is a challenge in elucidating plant–water relationships. Recent data have shown that the effect of light on K_{leaf} is not systematically related to aquaporin regulation, leading to conflicting conclusions. Here we investigated the relationship between light, K_{leaf} and aquaporin transcript levels in five tree species (*Juglans regia* L., *Fagus sylvatica* L., *Quercus robur* L., *Salix alba* L. and *Populus tremula* L.) grown in the same environmental conditions, but differing in their K_{leaf} responses to light. Moreover, the K_{leaf} was measured by two independent methods (high-pressure flow metre (HPFM) and evaporative flux method (EFM)) in the most (*J. regia*) and least (*S. alba*) responsive species and the transcript levels of aquaporins were analyzed in perfused and unperfused leaves. Here, we found that the light-induced K_{leaf} value was closely related to stronger expression of both the *PIP1* and *PIP2* aquaporin genes in walnut (*J. regia*), but to stimulation of *PIP1* aquaporins alone in *F. sylvatica* and *Q. robur*. In walnut, all newly identified aquaporins were found to be upregulated in the light and downregulated in the dark, further supporting the relationship between the light-mediated induction of K_{leaf} and aquaporin expression in walnut. We also demonstrated that the K_{leaf} response to light was quality-dependent, K_{leaf} being 60% lower in the absence of blue light. This decrease in K_{leaf} was correlated with strong downregulation of three *PIP2* aquaporins and of all the *PIP1* aquaporins tested. These data support a relationship between light-mediated K_{leaf} regulation and the abundance of aquaporin transcripts in the walnut tree.

Keywords: aquaporin gene expression, leaf hydraulic conductance, light, trees.

Introduction

Water homeostasis is crucial to the growth and survival of terrestrial plants. The sessile nature of plants requires dynamic adjustments of hydraulic efficiency in response to changing environmental factors. Plants have evolved a series of resistances to water flow in various organs along the soil–plant–atmosphere continuum (Tyree and Zimmerman 2002). Leaves constitute

30% of this total resistance to water flow through the plant (Sack and Holbrook 2006). Early studies focused principally on the measurement (Sack and Tyree 2005, Tyree et al. 2005) and partitioning of leaf hydraulic resistance (R_{leaf} ; Sack et al. 2004). R_{leaf} is the sum of two key components: the vascular component, which includes the resistances of the petiole and major and minor veins, and the extravascular compartment, external to the

xylem (Trifilò et al. 2003, Cochard et al. 2004, Gascò et al. 2004). The respective contributions of these two components to R_{leaf} have been assessed in many species. For example, 64–80% of leaf hydraulic resistance in laurel (*Laurus nobilis* L.), sugar maple (*Acer saccharum* L.) and red oak (*Quercus rubra* L.) leaves is due to the vascular system (Zwieniecki et al. 2002, Sack et al. 2004). In this case, leaf water transport follows the apoplastic pathway. In contrast, the extravascular hydraulic resistance of leaves may account for 50–90% of whole-leaf resistance (Trifilò et al. 2003, Cochard et al. 2004), consistent with a major role of cell-to-cell water in leaves. These physiological targets indicate that water may flow across leaves by two different pathways, raising questions about the precise contribution of aquaporins to leaf hydraulic conductance.

Several studies have investigated the role of aquaporins in leaf hydraulic conductance and have yielded conflicting results. In *Arabidopsis* leaves, a negative correlation has been found between the intensity of transpiratory flux and plasma membrane intrinsic protein (PIP) abundance under conditions of strong transpiration (Morillon and Chrispeels 2001). Recently, another putative implication of aquaporins in K_{leaf} regulation was demonstrated on *Arabidopsis* bundle-sheath cells (Shatil-Cohen et al. 2011). Bundle-sheath cells are suggested to be a key checkpoint of fluxes from the xylem to stomata as proposed by Ache et al. (2010). However, *Arabidopsis* plants lacking PIP1 and PIP2 have been found to have a hydraulic conductance similar to that of wild-type plants (Martre et al. 2002). Similarly, no difference in leaf hydraulic conductance (K_{leaf}) has been seen between wild-type and transgenic tobacco plants constitutively overproducing two aquaporin isoforms (PIP2,5 and PIP1,4), under conditions of both high ($350 \mu\text{mol m}^{-2} \text{s}^{-1}$) and low ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) irradiance (Lee et al. 2009). However, NtAQP1, a tobacco PIP1 which has a notable water channel activity in protoplasts, was shown to increase water use efficiency, stomatal conductance and transpiration rate when expressed in tomato and *Arabidopsis* (Sade et al. 2010). In other species, for which there is considerable circumstantial evidence pointing to aquaporin-dependent pathways, based on the pattern of aquaporin distribution in leaf cells (Kaldenhoff et al. 1995, Robinson et al. 1996, Sarda et al. 1997, Frangne et al. 2001, Hachez et al. 2008), the dynamic nature of K_{leaf} responses to environmental factors (Sack et al. 2004, Cochard et al. 2007) and sensitivity to certain chemical components (Nardini et al. 2005, Voicu et al. 2008). A close correlation between K_{leaf} and the abundance of aquaporin transcripts has also been reported in detached walnut leaves (Cochard et al. 2007). Experiments carried out with the high-pressure flow meter (HPFM) technique have shown that K_{leaf} increases strongly and rapidly in 15 min immediately following exposure to high levels of irradiance (Sack et al. 2002, Tyree et al. 2005, Cochard et al. 2007). This light-induced increase in K_{leaf} is independent of abscisic acid (an inhibitor of stomatal

opening) and related to the upregulation of two aquaporin isoforms (*JrPIP2s*; Cochard et al. 2007). In the dark, both K_{leaf} and aquaporin abundance are low, adding support to a link between these two factors (Cochard et al. 2007). As in walnut, light increases K_{leaf} in bur oak (Voicu et al. 2008, 2009). However, no correlation has been found between light-induced K_{leaf} and the accumulation of transcripts for the four putative aquaporins isolated from leaves (Voicu et al. 2009). This suggests that light-induced K_{leaf} cannot be systemically linked to high levels of aquaporins, and additional studies are therefore required to clarify the role of aquaporins in leaf water transport.

Since the identification of the first aquaporins (*AtTIP*) in *Arabidopsis*, many studies have investigated their role in many fundamental plant processes (Maurel et al. 2008, Heinen et al. 2009). More than 30 major intrinsic proteins (MIPs) have been isolated from *Arabidopsis* (Johanson et al. 2001), maize (Chaumont et al. 2001) and rice (Sakurai et al. 2005). Plant aquaporins are classified into five main subfamilies on the basis of their location within the cell and sequence similarities: tonoplast intrinsic proteins (TIPs), PIPs, nodulin 26-like intrinsic membrane proteins (NIPs), small basic intrinsic proteins (SIPs) and X-intrinsic proteins (XIPs) (Danielson and Johanson 2008, Lopez et al. 2012). The PIP family has two main subgroups: PIP1s and PIP2s. The PIP1s differ from the PIP2s in having a longer N-terminal extension and a shorter C-terminal end. The PIP2s have a stronger effect on water conductance than PIP1s in *Xenopus laevis* oocytes (Chaumont et al. 2001, Katsuhara et al. 2002), whereas some PIP1s could be involved in CO_2 diffusion (Maurel 2007, Maurel et al. 2008). Aquaporins play a key role in plant water status. Their activity is therefore finely regulated at the post-translational level, by phosphorylation, intracellular pH and cations (Chaumont et al. 2005, Maurel 2007). Aquaporins are also amenable to transcriptional regulation, particularly in response to environmental factors, such as water deficit (Quist et al. 2004, Alexandersson et al. 2005, Liu et al. 2006, Porcel et al. 2006), freeze–thaw events (Sakr et al. 2003) and light (Cochard et al. 2007).

Light is one of the most important environmental factors governing many aspects of plant growth and development (Kendrick and Kronenberg 1994) and the K_{leaf} of many plants (Sack et al. 2003, 2005, Lo Gullo et al. 2005, Nardini et al. 2005, Tyree et al. 2005, Sack and Holbrook 2006, Cochard et al. 2007, Sellin et al. 2008, Scoffoni et al. 2008, Voicu et al. 2008, 2009, Lee et al. 2009, Savvides et al. 2012). The aim of this study was to investigate the contribution of aquaporins to light-induced K_{leaf} by analyzing the accumulation of aquaporin transcripts. We explored the effects of light on K_{leaf} (HPFM approach) and aquaporin expression in the same experimental condition, for five species (*Fagus sylvatica*, *Juglans regia*, *Quercus robur*, *Salix alba* and *Populus tremula*) from the same location (Clermont-Ferrand, France), some of which had been studied before (Cochard et al. 2007, Voicu et al. 2008, 2009). With the

exception of *S. alba*, the species studied displayed increases of various magnitudes in hydraulic conductance in light-exposed leaves. A significant correlation between light-induced K_{leaf} and the levels of transcripts for both *PIP1* and *PIP2* aquaporins was found only in walnut. We further investigated the role of walnut aquaporins in light-induced K_{leaf} identifying eight new plasma membrane aquaporin isoforms (4PIP2 and 4PIP1) and studying their transcript expression in conditions of high irradiance and darkness. Generally, *JrPIP2s* seemed to contribute more than *JrPIP1s* to light-induced K_{leaf} and changes in light quality decreased K_{leaf} by decreasing aquaporin expression. Lastly, conflicting results were recently obtained on *Q. rubra* K_{leaf} suggesting that HPFM experiments could lead to misinterpretation of the phenomenon and the contribution of each leaf compartment to bulk conductance (Rockwell et al. 2011). Here we also used evaporative flux method (EFM) technology to evaluate leaf conductance, which endorsed our HPFM dataset. In the same line, aquaporin gene expression primarily obtained on HPFM-perfused leaves was compared with unperfused leaves in the same irradiance conditions to eliminate uncertainty about any possible artifactual HPFM-induced aquaporin expression modulation, giving analogous results on five tree species. All these findings indicate that (i) HPFM is a robust technology to assess K_{leaf} on tree and (ii) aquaporins are a major target in the upregulation of K_{leaf} in response to light in walnut, and that this tree may be an appropriate model for dissection of the regulatory gene network involved in this process.

Material and methods

Plant material

The experiments were performed during the summers of 2008 and 2009, on leafy branches sampled from 15-year-old *Juglans regia* (L.) cv. Franquette (walnut), *Salix alba* (L.) (white willow), *Populus tremula* (L.) (aspen), *Fagus sylvatica* (L.) (beech) and *Quercus robur* (L.) (oak) trees growing in the INRA (Institut National de la Recherche Agronomique) arboretum near Clermont-Ferrand (France). Leafy branches were sampled at random from the part of the tree exposed to sunlight, and immediately re-cut under water. They were then enclosed in black plastic bags and kept in total darkness, at a high relative humidity, for 24 h before use. Only the mature, developed leaves from the branches were used for experiments.

Leaf hydraulic conductance measurements

Leaf hydraulic conductance was measured by the HPFM method, as previously described by Cochard et al. (2007). Briefly, degassed pressurized water was forced into the petiole of an excised leaf under positive pressure (P , MPa), and the flow of water into the petiole was measured. Light was provided by two 400 W high-pressure sodium lamps (SON-T pia, Philips

France, Suresne) delivering $\sim 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf level. Water flow values (F , mmol s^{-1}) were recorded at room temperature (25°C), every 30 s, with a computer connected to an HPFM, and leaf hydraulic conductance (K_{leaf} , $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$) was calculated as $K_{\text{leaf}} = F/(P \times \text{LA})$, where LA is the total leaf area (m^2). Leaf hydraulic conductance was measured on leaves exposed to light for 120 min, and left in the dark for 120 min.

To ensure our HPFM results, another technique was used to determine K_{leaf} experimentally under the same light conditions using the EFM (Sack et al. 2002, Cochard et al. 2007). Unlike HPFM, this method allows free leaf transpiration under high irradiance. Walnut and willow shoots were harvested before dawn and enclosed in moist plastic bags to ensure high humidity around leaves. The bags were sealed at shoot base and kept dipping in distilled water until experimentation. To ensure the overnight rehydration, leaves were measured for initial water potential ($>0.2 \text{ MPa}$, $n = 5$) using a pressure chamber (Model 600, Plant Moisture Stress). For the measurements, leaves were sampled from stems kept in the plastic bags. Petioles were rapidly re-cut with razor blades under water in order to prevent air bubbles in the xylem and triggering of embolism. They were maintained in water until they were connected to plastic tubing using compression fittings. The hydraulic circuit was filled with ultrapure degassed water. Instead of using a scale, the flow rate (F) was recorded using liquid mass flow meters ($5\text{--}20 \text{ g h}^{-1}$ LIQUI-FLOW, Bronkhorst, The Netherlands). After being connected with the hydraulic circuit and under low irradiance ($<20 \mu\text{mol m}^{-2} \text{s}^{-1}$), flow stabilization was reached after 10–15 min. A batch of five leaves was retrieved at this moment to obtain night conductance after water potential and area measurement. For another batch of five leaves, conductance after 120 min under high irradiance was measured. The light source, placed above the samples was then turned on, supplying $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf level. A fan was also used in order to limit heating and also to favor a high transpiration rate. The water flow was measured for 120 min and final leaf water potential was measured at the end of the experiment to evaluate the driving force (Ψ_{leaf}) and calculate K_{leaf} from $K_{\text{leaf}} = F/(\Psi_{\text{leaf}} \times \text{LA})$.

Light treatments

The effect of light quality on K_{leaf} was investigated in leaves exposed to light from which the blue light component had been removed with a blue light filter (Geste Scénique®, La Creche, France). We tested the effect of blue light on K_{leaf} by illuminating leaves with blue-free light immediately after the dark phase. The leaves were then illuminated with white light, after removal of the blue light filter. White light was provided by two 400 W high-pressure sodium lamps (Philips SON-T pia) delivering $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf level, and the leaves were placed at a distance of 28 cm from the lamp in the absence of the blue light filter, and 24 cm from the lamp in the presence of the blue light filter (distances determined with a neutral filter).

Comparison of PIP1 and PIP2 aquaporin abundance in the five species studied

We investigated the relationship between the effects of light on K_{leaf} and aquaporin abundance in these five species, by first identifying the PIP aquaporins in the light-exposed leaves of the various species, most likely to be involved in this physiological process. For this purpose, we explored the PIP subfamilies in each species using a panel of PIP1- and PIP2-related sequences as queries against the non-redundant and the expressed sequence tag (EST) databases available in the Molecular GenBank databases at the National Center for Biological Information, NCBI (<http://www.ncbi.nlm.nih.gov/>). As genomic databases were limited for the target species, very few PIP sequences had been obtained. To enrich this information, we set out to design degenerate oligonucleotide primers for PIP1s (PIP1DF and PIP1DR) and PIP2s (PIP2DF/PIP2DR) (Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). Amplicons were cloned, and several clones were sequenced. We then designed generic oligonucleotide primer sets for PIP1s (PIP1GF/PIP1GR) and PIP2s (PIP2GF/PIP2GR), each binding to the most conserved coding regions of the aquaporin isoforms previously isolated from the five species (Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) amplification was performed in a 30- μ l reaction mixture containing 0.5 U of platinum *Taq* polymerase (Clontech, Saint-Germain-en-Laye, France), 1 \times reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 0.2 μ M of each primer, 0.3 μ l of SYBR green I (1/10,000, Sigma, Saint Quentin Fallavier, France) and 3 μ l of RT product diluted 1:20. The PCR conditions were: 94 °C for 3 min, then 40 cycles of 94 °C for 20 s, 52–60 °C for 20 s (depending on the optimal annealing temperature of the primer used) and 72 °C for 20 s. The *J. regia* 18S ribosomal RNA gene (accession No. AF399876) was used as internal standard. The relative changes in aquaporin transcript expression (Q_r) were calculated by comparison with the expression of the 18S rRNA gene using the delta–delta method mathematical model (Livak and Schmittgen 2001). The biological dark control corresponded to HPFM-perfused leaves sampled just before illumination. Values are shown as $\log_2 Q_r$. As sampled leaves were under flooded physiological conditions, a simultaneous molecular analysis was carried out on unperfused leaves harvested from leafy branches in similar light- and time-course conditions.

Isolation of PIP1 and PIP2 sequences from walnut tree

JrPIP1- and *JrPIP2*-related ESTs from walnut were identified in the Molecular GenBank databases at NCBI, with *Arabidopsis thaliana* (L.) *AtPIP1* and *AtPIP2* protein sequences and *J. regia* *JrPIP2*;1 (AY189973) and *JrPIP2*;2 (AY189974) used as direct queries with the tBLASTn algorithm (Altschul et al. 1997).

A multiple alignment of amino acid sequences was generated with ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). If a set of ESTs could be identified, manual contigs were generated in conserved regions for further analysis using complete sequences. Redundant entries, including sequences with random point mutations or polymorphisms (similarities >98%), and single incomplete sequences with hypothetical lengths of less than 75% of their complete homologs were excluded from the analysis. For each putative isoform retrieved, full-length *J. regia* aquaporin clones were first generated with primer sets binding to the 5'/3' untranslated regions. The amplicons were sequenced and specific new primer sets were designed for each isoform and used for RT-qPCR analyses.

The cDNAs generated by the reverse transcription of mRNA were amplified in an iCycler iQ (Bio-Rad Laboratories, Hercules, CA, USA), in 50 μ l of reaction mixture containing 2 μ l of a 1:40 dilution of cDNA, 0.5 U of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 10 μ M primers (Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). The PCR cycling program consisted of heating at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52–60 °C (Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online) for 30 s, 72 °C for 90 s and a final elongation step at 72 °C for 15 min. The PCR products were checked by electrophoresis in a 1.5% agarose gel. Bands of the expected size were excised from the gel and purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR products were ligated into the pGEM[®]-T Easy plasmid (Promega, Madison, USA), and the resulting plasmid was introduced into *Escherichia coli* (thermo-competent JM109 cells), according to the standard protocols supplied by the manufacturer. The presence of inserts was checked by PCR with the SP6-T7 universal primers, essentially as described earlier. For each insert, we carried out restriction analysis on 10 μ l of the resulting recombinant plasmids, selected at random and the DNA inserts from clones with different restriction patterns were sequenced on both strands (MWG Biotech, Courtaboeuf, France). Sequence data were analyzed with the NCBI BLAST server.

Bioinformatic analysis

The open reading frames (ORFs) of each cloned DNA sequence were translated into deduced amino acid sequences with the ExPASy proteomics server (<http://www.expasy.org/tools/dna.html>). These sequences were aligned with each other and with the complete sequences of the PIP1 and PIP2 aquaporin proteins from *A. thaliana*, *Populus trichocarpa* (Torr and Gray ex Hook) (PIP1 and PIP2 nomenclature according to Almeida-Rodriguez et al. 2010), *P. tremula*, *Q. robur*, *S. alba* and *Vitis vinifera* (L.). Partial sequences from *Quercus macrocarpa* Michx. were also included in this analysis, as they had been studied in different light conditions (Voicu et al. 2009). All multiple

alignments of the amino acid sequences were generated with ClustalW (Thompson et al. 1994, <http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The unrooted phylogenetic trees were constructed using the maximum likelihood (ML) method implemented in the PhyML program v3.0 (Guindon and Gascuel 2003). Specifically, PhyML analyses were conducted with the Jones–Taylor–Thornton (JTT) substitution matrix and the stability for degree of support for each internal branch in the resulting trees was confirmed by 500 bootstrap trials. The Tree View program (Page 1996) was used to display the phylogenetic tree and bootstrap values >50% were reported. Percentages of amino acid similarity and identity were calculated using the NCBI *bl2seq* algorithm.

Transcript accumulation

The patterns of expression of the genes encoding the PIP1 and PIP2 aquaporins were analyzed in leaves subjected to various periods of white light (dark, 15 min, 1 h, 2 h and then 2 h after return to darkness) or after 1 h of exposure to blue-free light (corresponding to the maximum value of K_{leaf}). Samples were disconnected from the HPFM, immediately immersed in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from 200 mg of leaves in cetyl trimethylammonium bromide extraction buffer, as described by Chang et al. (1993). First-strand cDNA was synthesized from 1 μg of t_xRNA with SuperScript III (Invitrogen), according to the manufacturer's instructions. Quantitative PCR amplification was then carried out in an iCycler iQ (Bio-Rad) machine, in 30 μl of reaction mixture containing 3 μl of cDNA (1:20 dilution), 0.5 U of platinum *Taq* DNA polymerase (Invitrogen), 10 μM specific primers and a 1:1000 dilution of SYBR green I (Sigma). The PCR conditions were as follows: initial denaturing by heating at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 20 s, annealing at $54/58^{\circ}\text{C}$ for 20 s and polymerization at 72°C for 20 s. The relative quantity (Q_r) of aquaporin (AQP) transcripts using the *18S ribosomal* RNA gene as internal standard was calculated with the delta–delta method mathematical model (Livak and Schmittgen 2001); the biological dark controls were HPFM-perfused leaves sampled just before illumination. Values are shown as $\log_2 Q_r$. For each of the genes studied, we analyzed three independent biological replicates, and every run was carried out in triplicate. The values shown are means \pm standard deviations. Primers were designed with the Primer3plus program (<http://www.bioinformatics.nl/primer3plus>; Rozen and Skaletsky 2000). The amplification efficiencies of all the primer sets were routinely checked (data not shown).

Statistical methods

The effect of the various treatments on leaf hydraulic conductance was assessed by one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD)

post hoc test. For qPCR, only statistically different results with $P < 0.05$ (Student's *t*-test) were considered.

Results

Light response of K_{leaf} and aquaporin transcript levels in *J. regia*, *F. sylvatica*, *Q. robur*, *S. alba* and *P. tremula*

We investigated the link between light-increased K_{leaf} values and the accumulation of aquaporin transcripts, by analyzing these parameters together, in the same experimental conditions, in five species (*J. regia*, *F. sylvatica*, *Q. robur*, *S. alba* and *P. tremula*) growing at the same site. For all the species studied, K_{leaf} response was measured initially in the dark and then after 2 h of illumination using HPFM (Figure 1a).

In the dark, the K_{leaf} values of *J. regia*, *F. sylvatica* and *Q. robur* were very low (6, 3 and 2 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$, respectively), well below those obtained after 2 h of illumination (K_{leaf} values of 26, 7 and 14 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$, respectively; Figure 1a). Light-stimulated K_{leaf} increased only slightly for *P. tremula*, and no significant difference between light and dark conditions was found for *S. alba* using HPFM. To confirm the reliability of the HPFM approach, we used an independent method, EFM, to

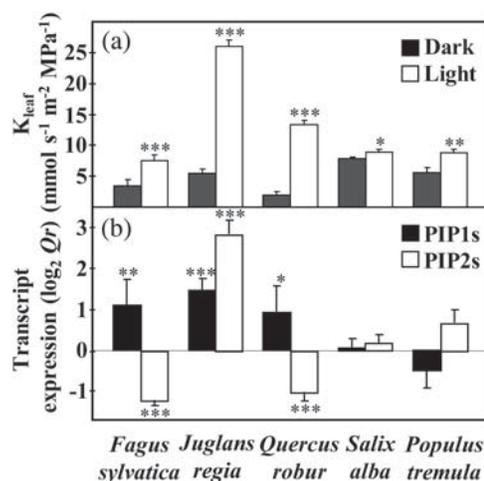


Figure 1. Leaf hydraulic conductivity (K_{leaf} , $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$), (a) and relative transcript expression of *PIP1s* and *PIP2s* aquaporin groups ($\log_2 Q_r$) (b) for five tree species in dark condition and after 2 h white light exposure. Leaf hydraulic conductance data were recorded using an HPFM method. Changes in transcript levels of the *PIP1s* (black) and *PIP2s* (clear) were monitored in leaves of the five tree species after 2 h exposure to white light. Dark leaves were used as controls for relative expression calculations. The *18S rRNA* gene expression was used as endogenous control. Expression levels (\log_2) are presented as expression ratio (treated to dark control). Relative transcript expressions were determined by real-time quantitative RT-PCR using generic *PIP1* or *PIP2* primer sets designed within conserved regions between selected species. Data correspond to means of three technical repeats from three independent biological experiments, and bars represent standard error ($n=9$). Asterisks represent significant differences (Student's *t*) (* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$).

evaluate K_{leaf} . Using concurrently HPFM and EFM, we measured *J. regia* and *S. alba* leaf conductance since these two species showed, respectively, the highest and lowest response under high irradiance in our initial HPFM assay. Flow measurements were recorded over the same duration. *Juglans regia* K_{leaf} increased upon illumination from 6.4 ± 0.5 to 26.7 ± 0.2 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ after 120 min measured with EFM, which corresponds to a 313% increase and is the same range as found with HPFM (333%). Both measurements gave a fourfold increase for this species (Figure 2). For the same treatment, *S. alba* K_{leaf} measured using EFM increased from 6.4 ± 0.3 to 8.9 ± 0.3 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ ($n = 5$), which is equivalent to a 39% increase, that is slightly greater than 11% obtained with HPFM.

This diversity of the K_{leaf} response to light provided a physiological framework for further investigations of the relationship between K_{leaf} profile and the relative abundance of aquaporin transcripts. To this end, we monitored the patterns of *PIP1* and *PIP2* transcripts simultaneously in the leaves of these five species, with generic oligonucleotide primers specific for *PIP1s* (*PIP1GF/PIP1GR*) and *PIP2s* (*PIP2GF/PIP2GR*).

In the dark, all these species had low K_{leaf} values associated with a low abundance of both *PIP1* and *PIP2* transcripts (data not shown). In the light, they displayed distinct profiles of aquaporin gene expression. In *J. regia*, light-induced K_{leaf} was associated with an increase in the abundance of *JrPIP1* and *JrPIP2* transcripts (three- and sevenfold) (Figure 1b). In *F. sylvatica* and *Q. robur*, light-stimulated K_{leaf} was associated with the accumulation of *PIP1* transcripts only (doubling), the expression of *PIP2* genes being repressed. No significant difference was found between light and dark conditions for *SaPIP1*, *SaPIP2* and *P. tremula PIP2* transcripts, and only a slight difference was found for *PoptrePIP1* transcripts (Figure 1b).

To ensure that the aquaporin expression trends were related to illumination but not to any flooding physiological state of the HPFM water-perfused leaves, a molecular analysis was subsequently carried out on unperfused leaves sampled from branches exposed to similar light conditions. Differences in

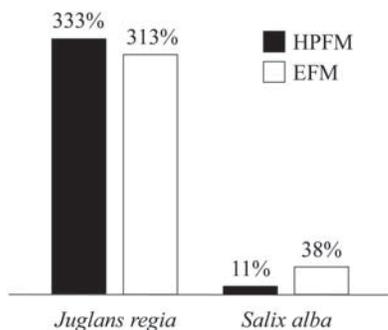


Figure 2. Methodological comparison of HPFM and EFM for measuring K_{leaf} after 2 h under high irradiance (%) relative to dark control in *J. regia* and *S. alba* ($n = 5$).

relative changes emerged in terms of absolute values, but these differences were not statistically significant, and these data confirmed for each tree species the light-dependent transcript expression trends of *PIP1* and *PIP2* aquaporins (Figure 3).

Identification and analysis of the sequence of *J. regia* *PIP* proteins

We investigated the expression of aquaporin genes in the light and its effects on K_{leaf} in greater detail by identifying eight new full-length aquaporins from the NCBI database, using the *JrPIP2;1* (AY189973) and *JrPIP2;2* (AY189974) and aquaporin protein sequences of *A. thaliana* as queries (see the Materials and methods section). The deduced amino acid sequences of these newly identified aquaporins displayed the characteristic features of MIPs. Phylogenetic analysis of the deduced protein sequences clearly demonstrated that four of these sequences were more closely related to the *PIP1* group of aquaporins, while the other four were more closely related to the *PIP2* group (Figure 4). The four *JrPIP1* sequences contained 287, 290, 285 and 292 amino acids and had, on average, 87% amino acid similarity (Supplementary Figure S1 available as Supplementary Data at *Tree Physiology* Online). Regarding the four new *JrPIP2* sequences, they contained 284, 280, 281 and 239 amino acids, and displayed a mean 80% similarity to the amino acid sequences of *JrPIP2;1* and *JrPIP2;2* (Sakr et al. 2003). The level of amino acid sequence similarity between the *JrPIP1* and *JrPIP2* groups was less than 70%, consistent

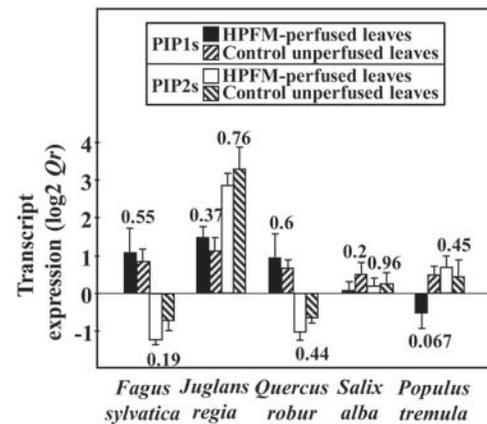


Figure 3. Comparison of transcript expression changes of *PIP1* and *PIP2* aquaporin groups for five tree species in dark condition and after 2 h white light exposure in HPFM water-perfused and control-unperfused leaves. Dark leaves were used as controls for relative expression calculations. The *18S rRNA* gene expression was used as endogenous control. Expression levels (\log_2) are presented as expression ratio (treated to dark control). Relative transcript expressions were determined by real-time quantitative RT-PCR using generic *PIP1* or *PIP2* primer sets designed within conserved regions between selected species. Numbers represent Student's *t*-test *P* values for average HPFM-perfused expression versus control-unperfused levels (no difference in expression if $P < 0.05$).

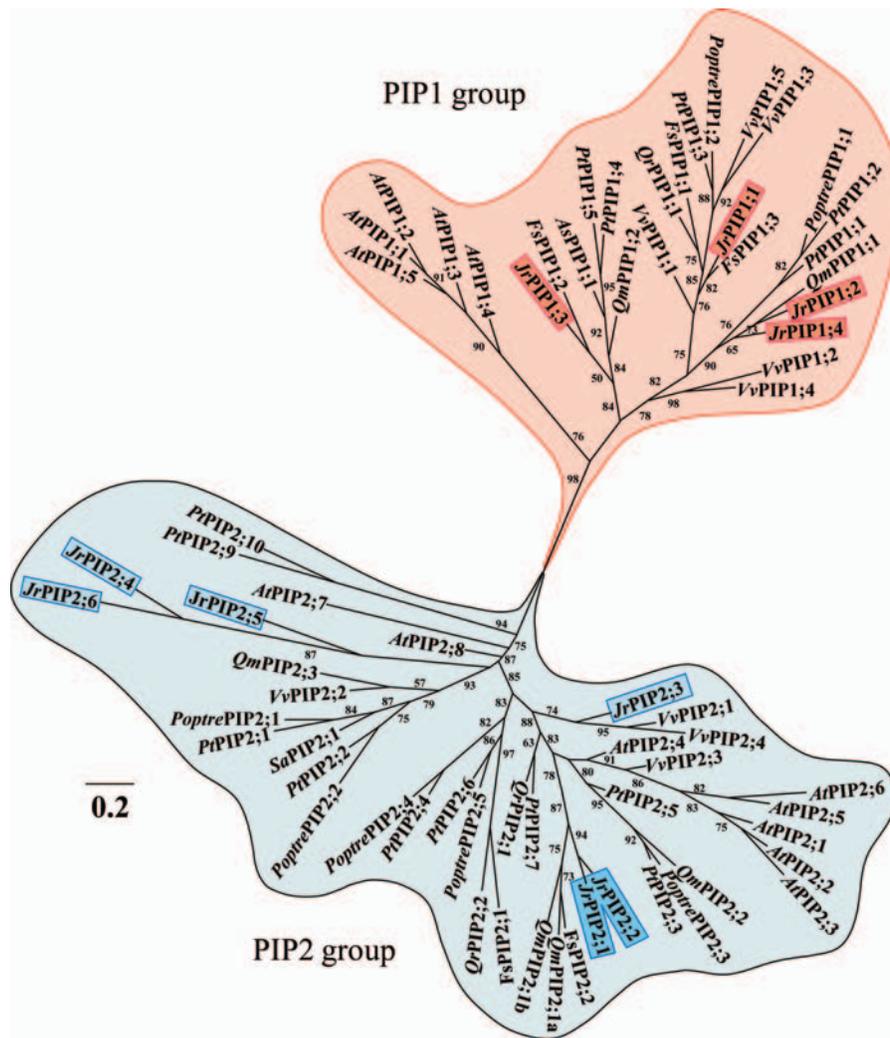


Figure 4. Phylogenetic tree analysis of *J. regia* (*Jr*) PIP1 and PIP2 aquaporin proteins with *A. thaliana* (*At*), *P. tremula* (*Poptre*), *P. trichocarpa* (*Pt*), *Q. macrocarpa* (*Qm*), *Q. robur* (*Qr*), *S. alba* (*Sa*) and *V. vinifera* (*Vv*), and PIP1 and PIP2 aquaporin proteins. The unrooted phylogeny of the PIP protein sequences was inferred using maximum likelihood. The tree was produced using PhyML with a genetic distance calculated by the JTT model of amino acid change. The numbers at the nodes represent percent of bootstrap values (above 50%) based on 500 replicates. The bar length is proportional to the number of amino acid substitutions per site. Species and accession numbers of each sequence are listed in Supplementary Table S2 available as Supplementary Data at *Tree Physiology* Online.

with the current PIP nomenclature (Johanson et al. 2001). Finally, as observed in classical plant PIP families, all *JrPIP1* sequences had a longer N-terminal sequence and a shorter C-terminal sequence than *JrPIP2* protein sequences. These sequences were thus named *JrPIP1*;1 (FJ971053), *JrPIP1*;2 (FJ971054), *JrPIP1*;3 (FJ971055), *JrPIP1*;4 (FJ970489) and *JrPIP2*;3 (FJ971056), *JrPIP2*;4 (FJ971057), *JrPIP2*;5 (FJ971058), *JrPIP2*;6 (FJ971059).

Expression patterns of the various *JrPIP1* and *JrPIP2* genes during irradiance

The expression patterns of the 10 *JrPIP* genes were monitored at five time points (0 dark; 15 min light; 1 h light; 2 h light and 2 h after return to the dark). When leaves were exposed to light, their K_{leaf} immediately began to increase, reaching a

maximum after 2 h of irradiance (Figure 5a). In addition, for all the *JrPIP1* and *JrPIP2* genes investigated, transcript abundance was transiently upregulated by light and peaked after 1 h (*JrPIP1*;1, *JrPIP1*;2, *JrPIP1*;3, *JrPIP2*;1 and *JrPIP2*;4) or 2 h (*JrPIP1*;4, *JrPIP2*;2 and *JrPIP2*;6) of irradiance (Figure 5b and c). Only *JrPIP2*;3 peaked earlier, with transcript levels peaking within the first 15 min of irradiance. Finally, K_{leaf} gradually declined toward the initial value when leaves were returned to the dark, as did the abundance of most of the *JrPIP1* and *JrPIP2* transcripts. The strongest upregulation (eightfold) was observed for *JrPIP2*;1, after 1 h of illumination.

Effect of blue light on *J. regia* K_{leaf} and *JrPIP* transcripts

Light may exert its effects through its intensity or its quality. We therefore investigated whether the K_{leaf} response to light

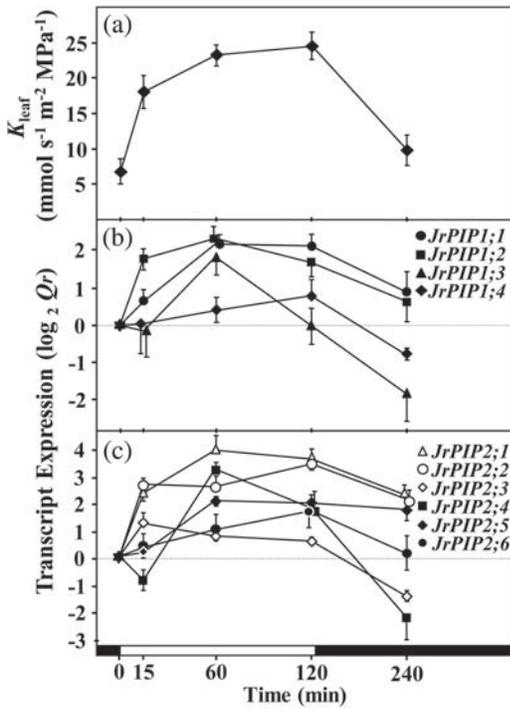


Figure 5. Time courses of leaf hydraulic conductance response (K_{leaf}) (a) and kinetic changes in transcript expression ($\log_2 Q_r$) of *JrPIP1*s (b) and *JrPIP2*s (c) in leaves of *J. regia* under conditions of light (white bar) and darkness (black bar). Leaf hydraulic conductance data were recorded using an HPFM method. Relative transcript expressions were determined by real-time quantitative RT-PCR using *PIP1* or *PIP2* specific primer sets for each isoform. Dark leaves were used as controls for relative expression calculations, and *18S rRNA* gene expression was used as endogenous control. Expression levels (\log_2) are presented as expression ratio (treated to dark control). Black and white bars on the x-axis correspond to the dark and light periods, respectively. Data correspond to means of three technical repeats from three independent biological experiments, and bars represent standard error ($n = 9$).

was dependent on light quality. The use of a blue light filter (excluding blue light) resulted in a K_{leaf} value 65% lower than that obtained in control conditions (8 and 23 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ for minus blue light and white light, respectively; Figure 6a). K_{leaf} increased reversibly when the same leaf was returned to white light after removal of the blue light filter, with the maximum value reached at 160 min. A summary of the light quality-dependent K_{leaf} statistical values is shown in Figure 6b. The *JrPIP* transcript levels were modified by blue light deprivation (Figure 7). Transcript levels were much lower for *JrPIP2;3*, *JrPIP2;4* and *JrPIP2;5* and slightly but significantly lower for *JrPIP2;2* in the absence of blue light. All the *JrPIP1* aquaporins tested displayed a downregulation of transcript abundance in the absence of blue light, suggesting that *JrPIP1*s are more sensitive to a lack of blue light than *JrPIP2*s.

Discussion

The K_{leaf} stimulation by light has been reported to differ between species (Sack and Tyree 2005, Sack and Holbrook 2006).

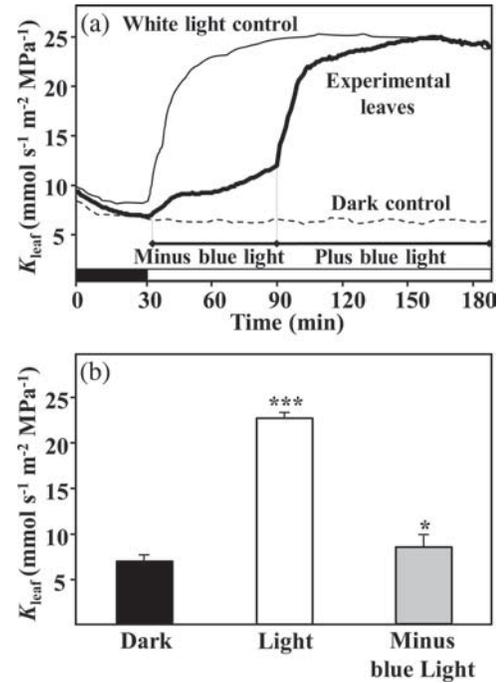


Figure 6. (a) Typical time courses of leaf hydraulic conductance (K_{leaf}) response of *J. regia* under white light and dark conditions (control mean), and under filtering for blue light wavelengths (minus blue light) followed by a return to ambient full spectrum white light condition (plus blue light) (experimental leaves). Leaf hydraulic conductance data were recorded using an HPFM method. Black bar on the x-axis corresponds to the dark periods. (b) Mean K_{leaf} values (\pm SE) in control dark, control light, and minus blue light leaves. Asterisks represent significant differences (Student's *t*) (* $0.01 < P < 0.05$; *** $P < 0.001$).

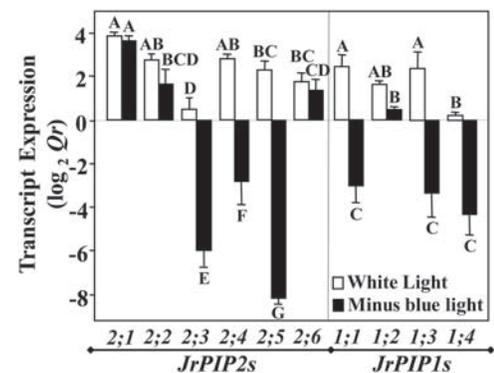


Figure 7. Changes in transcript levels of the *JrPIP1*s and *JrPIP2*s in leaves of *J. regia* after 2 h exposure to white light or with blue light wavelengths filtered out (minus blue light). Dark leaves were used as controls for relative expression calculations. The *18S rRNA* gene expression was used as endogenous control. Expression levels (\log_2) are presented as expression ratio (treated to dark control). Relative transcript expression of indicated genes was determined by real-time quantitative RT-PCR using specific primer sets for each isoform. Data correspond to means of three technical repeats from three independent biological experiments, and bars represent standard error ($n = 9$). Levels not connected by same letters are significantly different (Tukey HSD).

It may be weak (<1.5-fold increase) or stronger (two- to seven-fold), depending on the species studied (Sack et al. 2003, Tyree et al. 2005, Cochard et al. 2007, Voicu et al. 2008). Using HPFM, we found that light strongly increased K_{leaf} in *Q. robur*, *F. sylvatica* and *J. regia* (seven-, three- and fourfold), with a smaller increase observed in *P. tremula* (about 1.5-fold) (Figure 1). Although significant, a smaller difference was observed in *S. alba* K_{leaf} with HPFM (11%) than with EFM (38%). The maximum values attained by K_{leaf} differed between these species, ranging from 7 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ in *F. sylvatica* to 26 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ in *J. regia*. Previous data for 107 species showed that the maximal value of light-induced K_{leaf} ranged between 0.76 and 49 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$ (Sack and Tyree 2005). The reasons for these differences remain unclear. Some studies have suggested that K_{leaf} intensity depends on the distribution of the xylem and extra-xylem pathways within the leaf (Sack and Holbrook 2006). The resistance of the leaf xylem (R_{xylem}) is a major component of R_{leaf} in *Q. rubra* and *A. saccharum* leaves (Zwieniecki et al. 2002, Sack et al. 2004), and differences in vein architecture between species may be reflected in differences in R_{leaf} . However, if R_{xylem} is negligible with respect to extra-xylem resistance ($R_{\text{outside xylem}}$), the difference in R_{xylem} between species will have little impact on overall R_{leaf} (Sack and Holbrook 2006). Other studies have reported that the hydraulic resistance of the leaf xylem is of about the same magnitude as that in the extra-xylem pathways (Cochard et al. 2004, Gascò et al. 2004, Sack et al. 2004, 2005, Nardini et al. 2005), and that species tend to vary in terms of partitioning. Water conductance may be higher in leaves with a higher minor vein density, due to the existence of a greater surface area for the exchange of xylem water with the surrounding mesophyll (Cochard et al. 2004) and the transport of water over smaller distances outside the xylem (Roth-Nebelsick et al. 2001, Sack and Frole. 2006).

There is also evidence to suggest that aquaporins contribute to leaf water transport in detached *J. regia* leaves (Cochard et al. 2007). In this species, the irradiance-dependent increase in leaf hydraulic conductance during HPFM measurements has been shown to be correlated with higher levels of *JrPIP2;1* and *JrPIP2;2* aquaporin gene expression (Cochard et al. 2007). These findings are entirely consistent with the data shown in Figure 5, clearly indicating that the light-induced K_{leaf} may be related to increases in the expression of many newly identified *J. regia* aquaporin genes from both the PIP1 (*JrPIP1;1*, *JrPIP1;2* and *JrPIP1;3*) and PIP2 (*JrPIP2;1*, *JrPIP2;2*, *JrPIP2;3*, *JrPIP2;4*, *JrPIP2;5* and *JrPIP2;6*) subfamilies. The accumulation of transcripts for these genes was observed between 1 and 2 h after exposure to light, except for *JrPIP2;3*. Thus, the effect of light on *J. regia* K_{leaf} involves both PIP2 and PIP1 aquaporins. However, based on the patterns of transcript accumulation observed, light had a much stronger effect on PIP2 than on PIP1 isoforms (Figure 5). The PIP1 aquaporins therefore

appear to make less contribution to light-induced K_{leaf} than PIP2 aquaporins. Similar observations have been reported for other processes. Both PIP1 and PIP2 subfamilies are presented together for all species and organisms, e.g., in leaves of *A. thaliana* (Jang et al. 2004, Postaire et al. 2010), rice (Sakurai et al. 2005, 2008), maize (Hachez et al. 2008), *Q. macrocarpa* (Voicu et al. 2009) and *P. trichocarpa* (Secchi et al. 2009). These aquaporins may have different complementary functions within the leaf. For example, *NtAQP1* is involved not only in glycerol transport (Biela et al. 1999) and CO_2 diffusion (Uehlein et al. 2003) but also WUE and potentially water permeation (Sade et al. 2010). As for PIP2s, they are reportedly shown to play a key role in the flux of water across the plasma membrane. Lastly, aquaporins may increase water transport through the heterotetramerization of PIP1 and PIP2 aquaporin isoforms in the leaf (Fetter et al. 2004, Zelazny et al. 2007).

The relationship between light-induced K_{leaf} and aquaporin transcript levels does not extend to all herbaceous and woody species tested to date. In *A. thaliana*, leaf hydraulic conductance was similar in wild-type and double-antisense plants with only low levels of PIP1 and PIP2 aquaporins (Martre et al. 2002). In the same species, protoplasts isolated from leaves with high rates of transpiration had low osmotic water permeability whereas protoplasts from leaves with low levels of transpiration had high osmotic water permeability (Morillon and Chrispeels 2001). In tobacco plants, leaf hydraulic conductance is similar in wild-type and transgenic plants constitutively over-expressing the *PIP2;5* and *PIP1;4* aquaporin genes in conditions of both high- and low irradiance (Lee et al. 2009). In addition, cell pressure-probe studies have shown that cell hydraulic conductivity in leaves decreases in response to high levels of irradiance, probably due to the inhibition of aquaporin-mediated water transport (Kim and Steudle 2007, 2009, Lee et al. 2008, 2009). A recent study in *A. thaliana* leaves showed that darkness increased the transcript abundance of several *PIP* genes, including *AtPIP1;2* [initially referred to as *AthH2* or *PIP1b* (Kaldenhoff et al. 1995)], which accounts for a significant proportion of the aquaporin-mediated leaf water transport in plants grown in the dark for extended periods (Postaire et al. 2010). In woody species, aquaporins do not seem to contribute to leaf hydraulic conductance in some of the plants tested. Light increased K_{leaf} in *Q. robur*, *F. sylvatica*, *P. tremula* and *J. regia*, consistent with previous findings (Cochard et al. 2007, Voicu et al. 2008, Voicu and Zwiazek 2010). The strongest effects were observed for *Q. robur* and *J. regia*, at 14 and 24 $\text{mmol s}^{-1} \text{m}^{-2} \text{MPa}^{-1}$, respectively. Under the same experimental conditions, no correlation was found between K_{leaf} magnitude and PIP gene expression in any of the species other than *J. regia* (Figure 1a and b). In *Q. robur*, light strongly stimulated K_{leaf} , but *PIP2* transcript levels were downregulated and those of *PIP1*s remained similar to those in the control (T_0) (Figure 1b). Similar patterns of *PIP* gene expression were observed for *F.*

sylvatica. The absence of a link between the response of K_{leaf} to light and *PIP* expression was recently reported for *Q. macrocarpa* (Voicu et al. 2009). The authors suggested that aquaporins might function by regulating water homeostasis in leaves adapted to different light conditions. However, to date, in contrast to in vitro assays or root systems (Martinez-Ballesta et al. 2003, Coskun et al. 2012), knowledge about modulation of the cell homeostasis in relationship with aquaporins in leaves related with irradiance is deficient (Luu and Maurel 2005, Sack and Holbrook 2006). Similarly, the moderate light-induced increase in K_{leaf} in *P. tremula* is independent of *PIP* gene expression (Figure 1a and b), and seems instead to be linked to leaf metabolism (Voicu and Zwiazek 2010).

Lastly, it could be reasonably suspected from our molecular assays that the aquaporin transcript levels would be connected with the flooding physiological state of the HPFM water-perfused leaves (revealing any flooding tolerance phenotype of these tree species), and not to light exposure as such. To this end, for each tree species we also monitored the transcript expression patterns of *PIP1* and *PIP2* in unperfused leaves, harvested in leafy branches before dawn and then placed in similar light conditions. When relative changes are viewed strictly in value terms, minor differences in *PIP1* and *PIP2* transcript changes were apparent between analyses, but the expression trends of these analyses all significantly pointed in the same direction (Figure 3). These findings confirm that changes in aquaporin expression were rather due to light exposure than to flooding physiological state of the HPFM experimentation. For leaf hydraulic conductance, HPFM measurements were supported by an independent method (EFM), which is not reported to induce flooding (Sack and Tyree 2005). Both gave similar values on *J. regia* and *S. alba*, respectively, the most and least light-responsive tested species according to HPFM. As reported, these methods were shown to give similar results on several woody species (Sack et al. 2002, Scoffoni et al. 2008) and on crop plants (Tsuda and Tyree 1999). Under high irradiance, increase in conductance was in the same range for *J. regia* (+333% for HPFM versus +313% for EFM), but HPFM might underestimate change in conductance for *S. alba* (+11%) relative to the 38.6% measured by EFM (Figure 2). In our experimental conditions, we assume HPFM to be a suitable assay method for accurately obtaining K_{leaf} .

Light is known to affect gene expression through changes in its intensity or quality (Girault et al. 2008, Voicu et al. 2008). When *J. regia* leaves were exposed to light rendered blue free light by a blue light filter, K_{leaf} was about 65% lower than that in the presence of white light (8 mmol s⁻¹ m⁻² MPa⁻¹ versus 23 mmol s⁻¹ m⁻² MPa⁻¹) (Figure 6b). The only other comprehensive study investigating the effect of light quality on K_{leaf} reported similar results for *Q. macrocarpa* (Voicu et al. 2008). In this earlier study, the increase in leaf hydraulic conductance was greater in response to blue and green light than to visible light of longer

wavelengths. Interestingly, the response of K_{leaf} to white light was also greater than to light of any single wavelength, suggesting that each wavelength acts individually (Voicu et al. 2008).

Under white light, all *J. regia* aquaporin transcripts were upregulated within 1 h of irradiance (except for *JrPIP1;4*). By contrast, in the absence of blue light, only *JrPIP2;1* and *JrPIP2;6* transcript levels were similar to those obtained under white light (Figure 7). The expression of these aquaporin genes is blue light independent, potentially accounting for the 35% higher K_{leaf} values obtained in these conditions than in the dark. Further studies with filters blocking other wavelengths of light (e.g., red) would be needed to investigate the contribution of different wavelengths to light-induced K_{leaf} and aquaporin levels. In contrast, aquaporin transcript accumulation was moderately lower for *JrPIP2;2* and much lower for the other aquaporins (*JrPIP2;3*, *JrPIP2;4*, *JrPIP2;5*, *JrPIP1;1*, *JrPIP1;3* and *JrPIP1;4*) in the absence of blue light. These findings suggest that blue light makes a major contribution to light-induced K_{leaf} , possibly related to the downregulation of *PIP1s* and *PIP2s*. *A. thaliana* AthH2, a blue light-responsive aquaporin, is upregulated in expanding and differentiated cells when plants are exposed to blue light (Kaldenhoff et al. 1995). In tobacco plants, Lorenz et al. (2003) have identified abundant flavin-binding sites in NtAQP1, an aquaporin of the *PIP1* subfamily. The binding of flavin to the protein can be induced photochemically by blue light, providing evidence for a possible role of the riboflavin-binding protein *PIP1* as a photoreceptor.

In conclusion, our data provide a cornerstone for the role of aquaporins (*PIP1s* and *PIP2s*) in the light-modulated K_{leaf} in the walnut tree. This species could be a useful model for gaining a better understanding of the molecular basis of such regulation.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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