Putative Role of Aquaporins in Variable Hydraulic Conductance of Leaves in Response to Light[1]

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Molecular and physiological studies in walnut (*Juglans regia*) are combined to establish the putative role of leaf plasma membrane aquaporins in the response of leaf hydraulic conductance (K_{leaf}) to irradiance. The effects of light and temperature on K_{leaf} are described. Under dark conditions, K_{leaf} was low, but increased by 400% upon exposure to light. In contrast to dark conditions, K_{leaf} values of light-exposed leaves responded to temperature and 0.1 mM cycloheximide treatments. Furthermore, K_{leaf} was not related to stomatal aperture. Data of real-time reverse transcription-polymerase chain reaction showed that K_{leaf} dynamics were tightly correlated with the transcript abundance of two walnut aquaporins (*JrPIP2,1* and *JrPIP2,2*). Low K_{leaf} in the dark was associated with down-regulation, whereas high K_{leaf} in the light was associated with up-regulation of *JrPIP2*. Light responses of K_{leaf} and aquaporin transcripts were reversible and inhibited by cycloheximide, indicating the importance of de novo protein biosynthesis in this process. Our results indicate that walnut leaves can rapidly change their hydraulic conductance and suggest that these changes can be explained by regulation of plasma membrane aquaporins. Model simulation suggests that variable leaf hydraulic conductance in walnut might enhance leaf gas exchanges while buffering leaf water status in response to ambient light fluctuations.

Terrestrial plants face the contradictory demand of maximizing carbon dioxide uptake for photosynthesis while minimizing water loss to the atmosphere. Plants dynamically and finely adjust their hydraulic efficiency by offering a series of resistances in different organs along the soil-plant-atmosphere continuum (Tyree and Zimmermann, 2002). For instance, the leaf seems to contribute 50% of the hydraulic resistance of the aerial part of plants, which represents about 30% of the whole plant resistance (Nardini and Tyree, 1999; Tsuda and Tyree, 2000; Sack et al., 2003), but this share would, of course, vary if leaf resistance is itself variable (Sack and Holbrook, 2006). Evidence linking hydraulic conductance to plasma membrane aquaporins (plasma intrinsic proteins [PIPs]), which facilitate water transport through cell membranes, has been well documented for roots (Javot and Maurel, 2002; Tyerman et al., 2002). Conversely, the full significance of PIPs in leaf conductance to water is widely lacking.

Water movement through a leaf can follow an apoplastic (through the cell walls) or a cell-to-cell route. The latter includes symplastic (through plasmodesmata) and transcellular (across cell membrane) routes (Canny 1988, 1995). Even though increasing attention has been paid to characterize leaf hydraulic conductance, the importance of PIPs in this process remains unclear. Under high transpiration conditions, a negative correlation between the transpiration rate and PIP abundance has been reported in Arabidopsis (*Arabidopsis thaliana*) leaves (Morillon and Chrispeels, 2001). Similarly, antisense inhibition of PIP1 and PIP2 expression did not affect leaf hydraulic conductance in Arabidopsis (Martre et al., 2002). These results may suggest that high water flux associated with the transpiration stream follows an apoplastic pathway, which does not involve PIPs. The notion that PIPs might contribute to leaf hydraulic conductance was raised from some physiological studies. For instance, $HgCl_2$, which blocks the activity of some plasma membrane aquaporins (Javot and Maurel, 2002; Moshelion et al., 2002), has been shown to reduce leaf hydraulic conductance in sunflower (*Helianthus annuus*), suggesting that these proteins may contribute to modulating K_{leaf} (Nardini et al., 2005). Stimulation of leaf hydraulic conductance in response to light (Sack et al., 2004) was not due to a stomatal aperture because no difference was found between abscisic acid-treated and -untreated leaves in walnut (*Juglans regia*; Tyree et al., 2005) and because theoretical calculations excluded a role for

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stomatal limitation in whole-leaf conductance. This light effect on leaf conductance may be comparable to that already reported in the literature for roots and leaf pulvinus (Henzler et al., 1999; Moshelion et al., 2002; Lopez et al., 2003), indicating that intensity of water transport is related to aquaporin abundance. Tyree et al. (2005) hypothesized that light-mediated stimulation of leaf conductance may reflect an up-regulation of aquaporin abundance and/or activity required for increased water flow through leaf tissues, but this hypothesis has not been tested.

In the recent literature, many physiological and molecular data dealing with the importance of PIPs in the regulation of root conductance to water have been published (Javot and Maurel, 2002; Tyerman et al., 2002). For instance, $HgCl_2$ reduced root hydraulic conductance in some species, such as aspen (*Populus tremuloides*; Wan and Zwiazek, 1999); tomato (*Lycopersicon esculentum*; Maggio and Joly, 1995), and melon (*Cucumis melo*; Caravajal et al., 2000). Such studies indicate that the mercury-sensitive aquaporin pathway can account for up to 85% of root water transport (Tazawa et al., 1997; Amodeo et al., 1999; Caravajal et al., 1999; Barrowclough et al., 2000). The ability of the root to modulate its hydraulic conductance in response to a variety of stimuli has been shown to be correlated with the accumulation pattern of PIPs (Henzler et al., 1999; Lopez et al., 2003; Martínez-Ballesta et al., 2003). Plants with down-regulated PIP1 (Kaldenhoff et al., 1998; Siefritz et al., 2002) or PIP2 (Javot et al., 2003) were found to exhibit reduced root hydraulic conductance. These findings support the idea that PIP1 and PIP2, two subgroups of PIPs, play a central physiological role in root water movement and that a substantial part of this water movement occurs via the cell-to-cell route.

In contrast to living tissues, hydraulic conductance of xylem conduits (dead cells) is entirely governed by physical processes. For instance, hydraulic conductance is determined by the dimensions of the conduits and the structure of their walls according to the Poiseuille-Hagen law (Sperry et al., 2005). Similarly, variations of hydraulic conductance in the short term are explained by physicochemical processes, such as cavitation (Salleo et al., 2001), wall collapse (Cochard et al., 2004), changes in water viscosity with temperature (Cochard et al., 2000), or changes of wall permeability with sap chemical composition (Zwieniecki et al., 2001).

The objective of this study was to investigate the relative importance of plasma membrane aquaporins in leaf hydraulic conductance by combining physiological and molecular approaches. This study was focused on leaves of the walnut tree, where leaf hydraulic conductance has been shown to increase in response to light (Sack et al., 2002; Tyree et al., 2005) and was not associated with stomatal opening (Tyree et al., 2005). Here, we report that a light-induced increase of leaf conductance to water was significantly altered by temperature and completely inhibited by a physiological concentration of cycloheximide (CHX). The implication of stomata in this response was ruled out. Furthermore, analysis of expression patterns of two PIP2 aquaporin isoforms (*JrPIP2,1* and *JrPIP2,2* for walnut PIP2), which were found to be strongly expressed in walnut leaves (Sakr et al., 2003), showed a positive correlation between the value of leaf hydraulic conductance and abundance of walnut PIP2. Our data suggest that water flow across walnut leaves under high illumination preferentially follows a cell-to-cell pathway and hence is sensitive to an increase in aquaporin abundance.

RESULTS

Light and Temperature Effects on Leaf Hydraulic Conductance

To understand the light and temperature effects on leaf hydraulic conductance, the experimental setup in Figure 1 was used (see ''Materials and Methods''). The results of a typical experiment are shown in Figure 2 for a control leaf at 25°C (statistics for these treatments are shown in Fig. 8). In the dark condition, walnut leaves exhibited a small and constant conductance value ($K_{leaf} < 3$ mmol s^{-1} m^{-2} MPa^{-1}). When leaves were exposed to light, their hydraulic conductance showed a dramatic 400% increase. K_{leaf} increase was

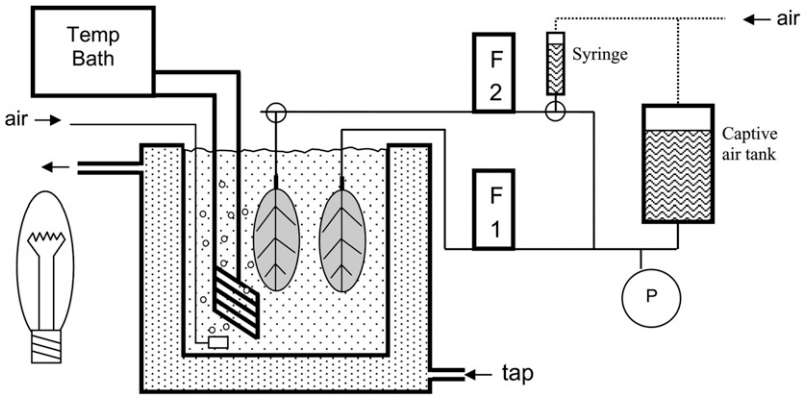

Figure 1. Experimental setup used to determine the light and temperature dependence of walnut leaf hydraulic conductance. Two leaves are submerged in a temperature-controlled bath with transparent walls. The leaves are perfused with water from a captive air tank at pressure P. The water flow entering each leaf is measured with two flow meters, F1 and F2. The leaf hydraulic conductance is then computed as F/P. A syringe enables the perfusion of leaf 2 with a chemical substance during the experiment.

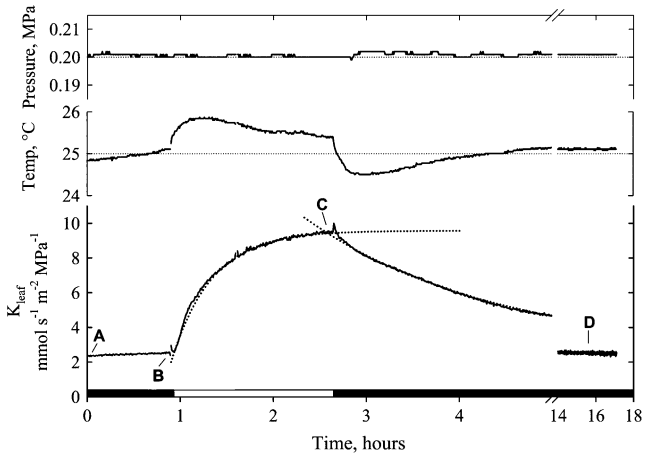

Figure 2. Typical time courses of leaf hydraulic conductivity (K_{leaf}, mmol s^{-1} m^{-2} MPa^{-1}) perfusion pressure and bath temperature for a control walnut leaf exposed to a dark-light-dark cycle. At the onset of the experiment (A), the leaf was in full darkness. After 1 h (B), the K_{leaf} value stabilized to a low value and the light was switched on, which provoked an immediate and dramatic K_{leaf} increase. After 1.5 h, the K_{leaf} value leveled off and the light was switched off (C), which caused the K_{leaf} value to return slowly to its initial dark value (D). The dotted lines represent exponential fits to the data.

exponential to a maximal value reached after approximately 1 h with a half-time $T_{½}$ = 19.8 min (SE = 1.87; n = 17). When the light was turned off again, K_{leaf} returned to its initial value more slowly ($T_{½}$ = 63.6 min; SE = 10.4; n = 8).

Response curves obtained at different temperatures were qualitatively similar, but differed quantitatively (Fig. 3). K_{leaf} values in the dark increased slightly with temperatures below 35°C (Fig. 3A). When K_{leaf} was corrected for changes in water viscosity with temperature (Fig. 3B), the dependence of K_{leaf} in full darkness with temperature was nearly absent. A significant increase remained, however, between 25°C and 35°C. K_{leaf} values in full light increased considerably and linearly with temperature (Fig. 3B). This response was not attributable to a change in water viscosity (Fig. 3B). Finally, $T_{½}$ values strongly decreased with temperature both for the dark-to-light and light-to-dark phases (Fig. 3C). At 5°C, the light response was absent; hence, it was not possible to compute $T_{½}$.

Stomatal Movements

Length and width of stomatal pores were constant whatever the treatments (Fig. 4, A and B), with the exception of control illuminated leaves that tended to exhibit more open stomata (Fig. 4, D and E). Hydraulic conductance on a surface area basis of stomatal pores was neither qualitatively nor quantitatively related to K_{leaf} values measured in this study (Fig. 4C). Indeed, the discrepancy between the two parameters was huge, stomatal K_{leaf} values being 5 to 7 orders of magnitude higher.

Light Response of K_{leaf} and Aquaporin Transcripts

To obtain consistent comparison between the pattern of K_{leaf} during the light phase and that of *JrPIP2,1* and *JrPIP2,2* abundance, real-time PCR was performed over a time course of 120 min (Fig. 5). Five time points were selected: 0, 15, 30, 60, and 120 min, which were representative of K_{leaf} dynamics. As was shown in Figure 5, the time course of K_{leaf} was well

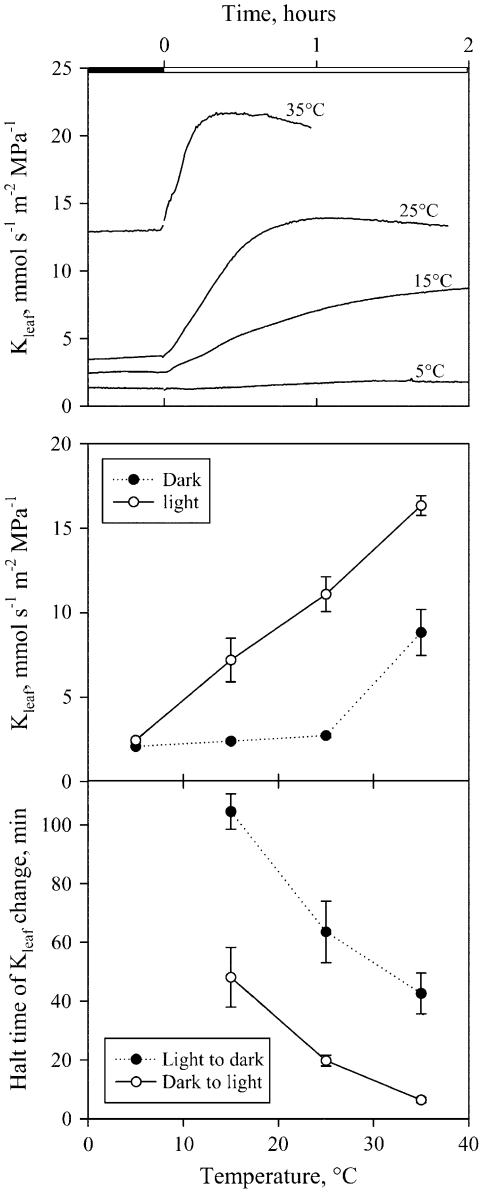

Figure 3. Temperature dependence of the K_{leaf} light response. A, Typical K_{leaf} time courses are shown for four different leaves at four different bath temperatures. At time 0, the light was switched on for all experiments. B, Mean steady-state K_{leaf} values under dark and light conditions are expressed versus the bath temperature. K_{leaf} values in B were corrected to account for the temperature dependence of water viscosity. C, Half time (min) of the dark-to-light and light-to-dark K_{leaf} responses versus temperature. Error bars = 1 SE.

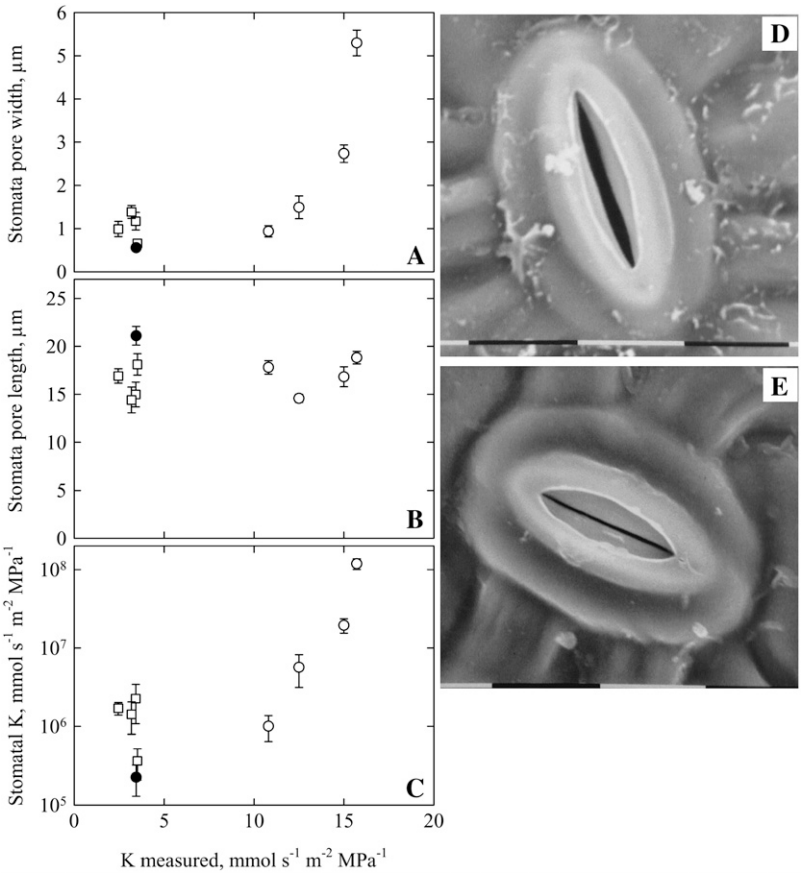

Figure 4. Stomatal movement during K_{leaf} light response. Pore major (A) and minor (B) axes were observed with a Cryo-SEM on control leaves under dark (black circles) and light (white circles) conditions and on illuminated leaves treated with CHX (white squares). Results are expressed as a function of the measured K_{leaf} value. C, Computed leaf area specific hydraulic conductance of all the stomatal pores is shown. D and E, Stomata under light and dark conditions, respectively. Black bars = 10 μm; error bars = 1 SE.

correlated with the time course of *JrPIP2* abundance. Indeed, *JrPIP2* transcripts were increased during the first 15 min after the light came on and continued to increase with increasing K_{leaf} through the time course. They exhibited strong abundance at the end of the time course, when K_{leaf} reached its maximal value.

To gain further insight into the link between K_{leaf} dynamics and the relative abundance of *JrPIP2* transcripts, these latter were investigated together under light and dark conditions (Fig. 6). Leaves under the dark condition (dark) exhibited low K_{leaf} values, which was associated with relatively low transcript abundance of both *JrPIP2,1* and *JrPIP2,2* (Fig. 6). On the contrary, leaves exposed for 90-min light treatment (light) displayed a higher value of K_{leaf} and a strong increase in transcript level of *JrPIP2,1* and *JrPIP2,2* (>27-fold). Interestingly, this situation was almost completely reversed when the light was turned off again (back to dark). In this condition, K_{leaf} value dropped to its initial value and abundance of *JrPIP2* decreased significantly as well. In summary, K_{leaf} values and transcript abundance of *JrPIP2,1* and *JrPIP2,2* appeared to be positively correlated.

CHX Effects on K_{leaf} and Aquaporin Transcripts

Typical results with leaves perfused under positive pressure with CHX during dark and light phases are shown in Figure 7 (statistics for these treatments are shown in Fig. 8A). A 100 μM CHX perfusion during the dark phase had no effect on K_{leaf} (top section). When such leaves were illuminated, their K_{leaf} value was not significantly different from that in the dark phase. When CHX was supplied during the light phase, K_{leaf} values rapidly returned ($T_{½}$ = 26.3 min; SE = 2.5; n = 7)

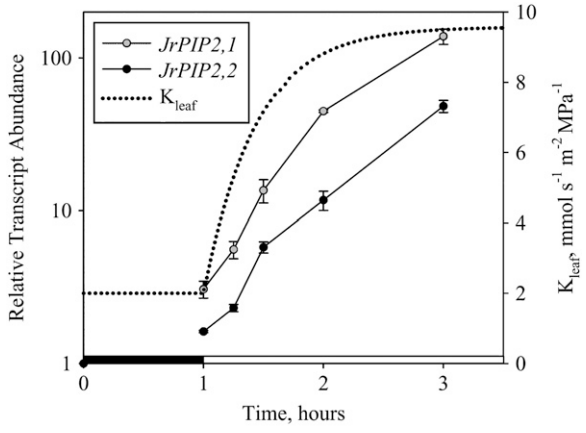

Figure 5. Time courses of relative transcript abundance of *JrPIP2,1* and *JrPIP2,2* and K_{leaf} values under the light condition. Light was switched on at time 1 h. Relative transcript abundance of the indicated genes was determined by real-time PCR. All data are presented as means of at least three independent experiments. Error bars = 1 SE. The dotted line represents a typical time course of K_{leaf} values.

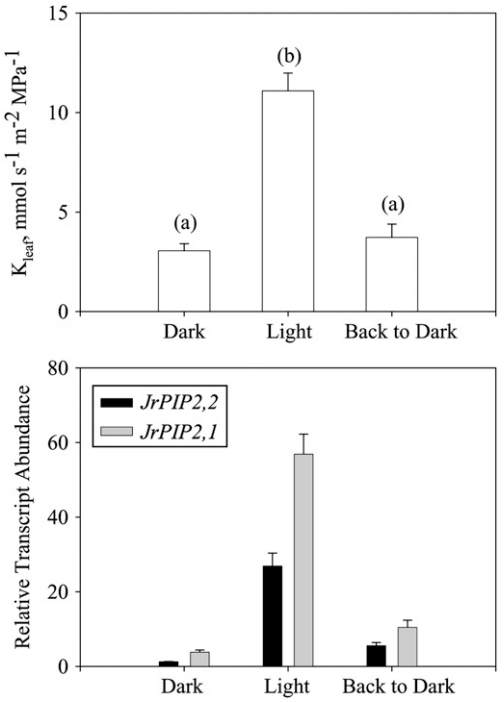

Figure 6. Mean K_{leaf} values (a) and relative transcript abundance of *JrPIP2,1* and *JrPIP2,2* (b) under dark conditions (dark), light conditions (light), and when the light was turned off again (back to dark). Relative transcript abundance of the indicated genes was determined by real-time PCR and corresponds to the means of three independent experiments. Error bars = 1 SE. Treatments having a letter in common are not significantly different at $P = 0.01$.

to values not significantly different from those observed during the dark phase (bottom section).

Because light-induced stimulation of K_{leaf} was markedly inhibited by a 100 μM CHX perfusion (Fig. 7) and K_{leaf} was found to be linked to *JrPIP2* abundance (Figs. 5 and 6), we wanted to determine whether this inhibition can be explained by potential modification in *JrPIP2* abundance. When CHX solution was perfused in the dark period, leaves that showed no K_{leaf} response to light consistently exhibited a small accumulation of *JrPIP2,1* and *JrPIP2,2* transcripts compared to the CHX-untreated leaves (control-light). A similar pattern was found when CHX was supplied during the light phase, when K_{leaf} was at its maximal value (CHX-light 2; Fig. 8). Indeed, within less than 1 h after CHX application, both K_{leaf} values and accumulation of *JrPIP2,1* and *JrPIP2,2* strongly decreased and reached the level under dark conditions (control-dark).

CHX Effects on K_{leaf} of Transpiring Leaves

To get insight into the functional significance of leaf aquaporins on leaf-water relations, experiments were conducted on transpiring leaves and treated with 100 μM CHX under dark and light conditions as above. Results were similar to those obtained with leaves perfused under positive pressure (Fig. 9). Indeed, both CHX treatments significantly reduced K_{leaf} under light conditions (Fig. 9C). This reduction resulted from significantly more negative leaf water potential values (Fig. 9B), whereas leaf absorption remained unchanged (Fig. 9A). Leaves treated with CHX remained green and healthy looking.

DISCUSSION

This study demonstrates that leaf hydraulic conductance (K_{leaf}) in walnut leaves is variable and can respond to some environmental stimuli (light and temperature) or CHX treatment (an inhibitor of protein biosynthesis). Stomatal observations and direct leaf evaporation rate measurements confirmed that stomatal movements did not explain the variations in K_{leaf} values. In addition, the magnitude of K_{leaf} was shown to be closely linked to the abundance of *PIP2* transcripts.

Reliability of Leaf K_{leaf} Values

In this study, most leaf hydraulic conductance values were obtained by perfusing submerged leaves

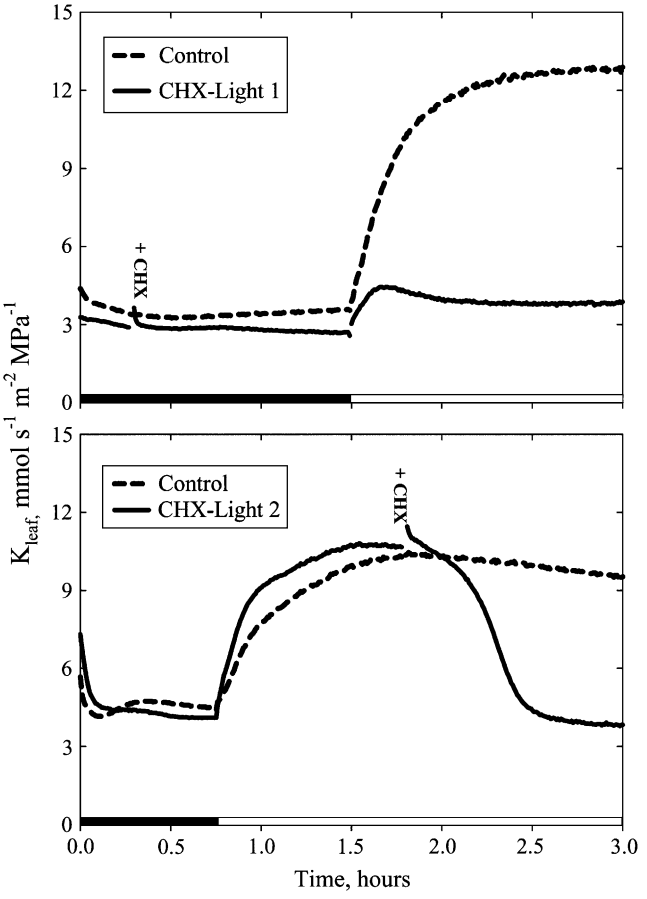

Figure 7. Typical time courses of K_{leaf} light response for control (dotted lines) and experimental (solid line) leaves treated with CHX. CHX was either supplied during the dark phase (top) or during the light phase (bottom). Black bars on the *x* axis correspond to the dark period.

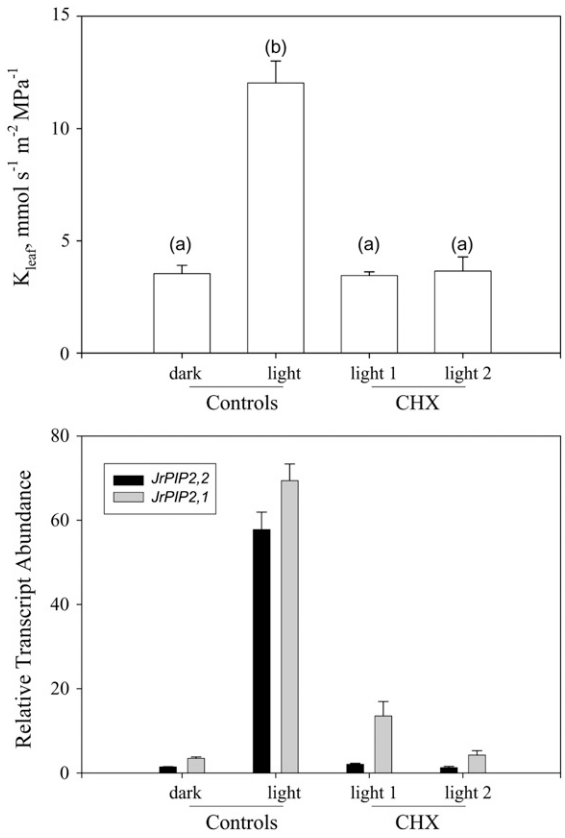

Figure 8. Mean K_{leaf} values (a) and relative transcript abundance of *JrPIP2,1* and *JrPIP2,2* (b) in CHX-untreated (control) and CHX-treated leaves (CHX). Apart from dark conditions (dark control), K_{leaf} values and relative abundance of JrPIP2 were determined under light conditions. CHX was applied either 1 h before (light 1) or 1 h after lights were switched on. Relative transcript abundance of the indicated genes was determined by real-time PCR and corresponds to the means of three independent replicates. Error bars = 1 SE. Treatments having a letter in common are not significantly different at $P = 0.01$.

with water under high pressure. This technique saturates the intercellular spaces in the leaves with water, including the stomatal chambers. Two possible drawbacks might be associated with the technique. First, as liquid water flows through the stomata, the hydraulic conductance of the stomatal pore is measured in a series with the leaf hydraulic conductance (Tyree et al., 2005). Although significant stomatal movements were observed in this study, our computation suggests that the stomatal pore's conductance is several orders of magnitude higher than the total measured leaf conductance (Fig. 4C). Therefore, the variation in K_{leaf} values did not reflect stomatal movement in this study. This is also confirmed by our data on transpiring leaves, where variations in K_{leaf} values were not correlated with fluctuations in leaf transpiration (Fig. 9). A second drawback of the perfusion technique might arise from an alteration of the water pathway in the leaf (Mencuccini and Magnani, 2000), which would result in overestimation of leaf conductance. Leaves treated with CHX showed similar K_{leaf} values with the perfusion and evaporation techniques, but the K_{leaf} of illuminated leaves was higher with the perfusion technique. However, the evaporation technique may have some drawbacks as well, caused by heterogeneous transpiration rates or inaccurate estimates of water potential gradients. We are therefore confident that light produced variable hydraulic conductance in walnut leaf, but the magnitude of the light and dark values of K_{leaf} might be slightly overestimated with our perfusion technique.

Light-Mediated Up-Regulation of *JrPIP2*

The water channel activity of *JrPIP2,1* and *JrPIP2,2* was previously demonstrated by expression in *Xenopus laevis* oocytes (Sakr et al., 2003). *JrPIP2,1* and

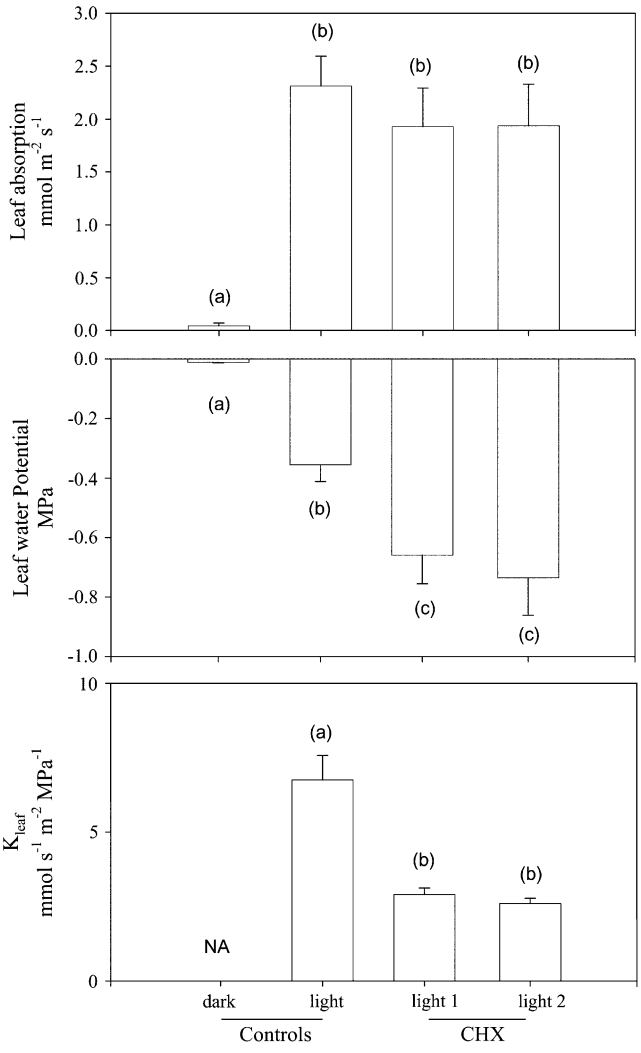

Figure 9. Absorption rate (a) and bulk leaf water potential (b) of transpiring leaves under dark and light conditions and after exposure to CHX. CHX was supplied either in the dark (CHX-light 1) or light (CHX-light 2) phases. Whole-leaf K_{leaf} values were computed as the ratio of leaf absorption to leaf water potential. Error bars = 1 SE. Treatments having a letter in common are not significantly different at $P = 0.01$.

JrPIP2,2 were initially identified in walnut xylem tissues as involved in local recovery of winter embolism by mediating water transport between xylem parenchyma cells and embolized vessels (Sakr et al., 2003). Because their transcripts have a large expression spectrum, it can be expected that *JrPIP2* can play key roles in the overall water transport in plants.

Transcript abundance of these two aquaporins (*JrPIP2,1* and *JrPIP2,2*) was substantially up-regulated by light in walnut leaves (Fig. 5), which is in accordance with the capacity of aquaporin gene expression to change in response to a variety of environmental stimuli, such as salt stress (Yamada et al., 1995; Martínez-Ballesta et al., 2003; Boursiac et al., 2005), drought (Kawasaki et al., 2001; Smart et al., 2001), circadian rhythms (Henzler et al., 1999; Moshelion et al., 2002), light (Siefritz et al., 2004), and cold (Wei et al., 2005). This light effect occurred in the short term (within 30 min) and was reversible in that it was abolished when the light was turned off again (Fig. 6B). Because the light-mediated transcriptional up-regulation of *JrPIP2,1* and *JrPIP2,2* was almost prevented in CHX-treated leaves under dark conditions (CHX-light 1; Fig. 8B), this up-regulation apparently depends on de novo protein synthesis, as is the case for other genes (Agrawal et al., 2003; Hauschild and Schaewen, 2003; Hirasawa et al., 2003). Under our physiological condition, synthesis of a positive regulator may be required for light-mediated *JrPIP2* up-regulation.

The precise mechanism by which light modulates the accumulation of *JrPIP2* transcripts is unclear at this moment and several hypotheses must be considered. For instance, it is well known that the transcriptional regulation of many light-regulated genes involves specific cis-elements in their promoter region that are recognized by light-specific trans-acting factors (Green et al., 1988; Ngai et al., 1997; Shen et al., 2005). Such light-regulation sites were found on the promoter sequence of the tobacco (*Nicotiana tabacum*) plasma membrane aquaporin gene (*NtAQP1*; Siefritz et al., 2004) and this may also be true for walnut *JrPIP2*. Experiments are under way in our laboratory to verify this hypothesis.

Two Distinct Water Pathways in Walnut Leaves

Like root, leaf hydraulic conductance has been reported to respond to different environmental factors (Nardini et al., 2003, 2005; Sack et al., 2003; Lo Gullo et al., 2005; Tyree et al., 2005). Here, we have clearly identified two distinct water pathways in walnut leaves, as occurs in roots. The first pathway was measured under dark conditions and was found to correspond to a low conductance value (Fig. 2). Temperature and CHX treatments did not have any effect on the conductance of this pathway, which was always associated with relatively low abundance of PIP2 transcripts. Temperature treatment has been reported to alter root hydraulic conductivity (Wan and Zwiazek, 1999; Aroca et al., 2004) and this effect can be attributed to a change in abundance and/or activity of aquaporins (Aroca et al., 2004). In our experimental conditions, the apparent temperature dependence of this dark pathway can simply be due to a change in water viscosity (Fig. 3), except for 35°C when membranes probably begin to lose integrity. A similar finding was reported in oak (*Quersus robur*) leaves (Cochard et al., 2000), where a dramatic and irreversible K_{leaf} increase was observed for temperatures above 35°C. Because CHX has already been reported to inhibit the osmotic water permeability in some plant species (Moshelion et al., 2002; Voicu and Zwiazek, 2004), we also investigated the effect of short CHX treatment (within 1 h) on the conductance of this dark water pathway. Like temperature, 0.1 mM CHX perfusion was found to have no significant effect on the dark conductance (Fig. 7A), suggesting that water transport through this pathway did not require any protein biosynthesis under dark conditions. Interestingly, expression analysis of two walnut aquaporins (*JrPIP2,1* and *JrPIP2,2*) showed that these aquaporins were weakly accumulated under dark conditions (control-dark), compared to light conditions (control-light; Fig. 8B). Plasma membrane aquaporins are generally localized wherever hydraulic conductivity is relatively high (Chrispeels and Agre, 1994; Javot and Maurel, 2002) and their activity has been shown in many examples to be correlated with RNA accumulation (Moshelion et al., 2002; Siefritz et al., 2004; Luu and Maurel, 2005). Therefore, this relatively low abundance of *JrPIP2* transcripts in walnut leaves can mean a minor contribution of these proteins in the dark water pathway. From the above combination of facts, we propose that most water transport through leaves might follow an apoplastic route under dark conditions.

The second pathway was observed in response to light and corresponded to a high conductance value (Fig. 2). In contrast to the dark route, the conductance of the light-induced pathway was proven to be highly modulated by both temperature and CHX and closely related to strong accumulation of *PIP2* aquaporin transcripts. The temperature effect on the conductance of the light pathway was not explained by changes in water viscosity (Fig. 3) and rather can reflect modifications in the properties of the lipid matrix and/or the activity of the membrane protein (Sack et al., 2004). The light-induced route was sensitive to CHX because CHX-treated leaves under light conditions (CHX-light 2) showed K_{leaf} values similar to those of untreated leaves under dark conditions (control-dark; Figs. 7B and 8A). Similar short effects of CHX on hydraulic conductivity have been reported in rain tree (*Samanea saman*) motor cells (Moshelion et al., 2002) and in aspen seedling roots (Voicu and Zwiazek, 2004) and are indicative of the importance of the protein-mediated water transport through these organs.

We demonstrated that conductance of the light-induced water pathway was positively related to light-mediated up-regulation of walnut *JrPIP2*: (1) leaves

that exhibited a high K_{leaf} value contained a higher abundance of *JrPIP2,1* and *JrPIP2,2* transcripts (Fig. 6); (2) *JrPIP2* transcripts and K_{leaf} value share similar time courses (Fig. 5); and (3) this response to light was reversible because K_{leaf} value and relative abundance of *JrPIP2* transcripts dropped together when the light was turned off again (Fig. 6). Although our data showed a close relation between transcript abundance and activity of aquaporins (K_{leaf}), we cannot exclude the notion that light might regulate plasma membrane aquaporins at multiple levels (transcriptional and posttranslational levels), as is the case for other proteins (Lillo and Appenroth, 2001). This could explain the slight delay between the response of K_{leaf} to light and *JrPIP2* transcript accumulation within the first 15 min. In conclusion, our data support the fact that the water pathway under light conditions is closely linked to membrane aquaporins (*JrPIP2*) and that their transcriptional regulation by light can lead to activation of a cell-to-cell route and, consequently, to higher K_{leaf} values.

Both K_{leaf} and *JrPIP2* transcripts decreased and returned to typical dark levels when CHX was supplied to leaves during the light period (CHX-light 2; Fig. 8B). This decrease is much faster in the presence of CHX than when the light was turned off (Fig. 6). These findings suggest that application of CHX under light conditions could act at the transcript level (by enhancing the instability of aquaporin transcript) and/or at the protein level (by decreasing the turnover of aquaporins and/or that of components essential to aquaporin activity), as already proposed in the literature (Bogre et al., 1997; Mitsui et al., 1999; Sieberer et al., 2000). This is consistent with the fact that regulation of plasma membrane aquaporins is complex and occurs at multiple levels (Luu and Maurel, 2005).

Composite Model for the Water Pathway in Walnut Leaves

Based on the conclusions of our experiments, we can propose a putative resistive model for water transport in walnut leaves under dark and light conditions (Fig. 10). We will present the most parsimonious model for clarity. In a leaf, water first takes a vascular

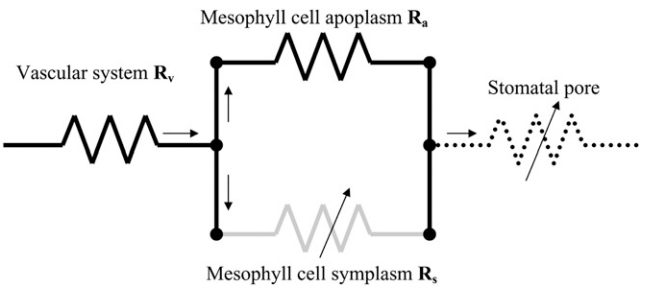

Figure 10. Putative resistance diagram for a composite sap pathway in walnut leaves. See text for details.

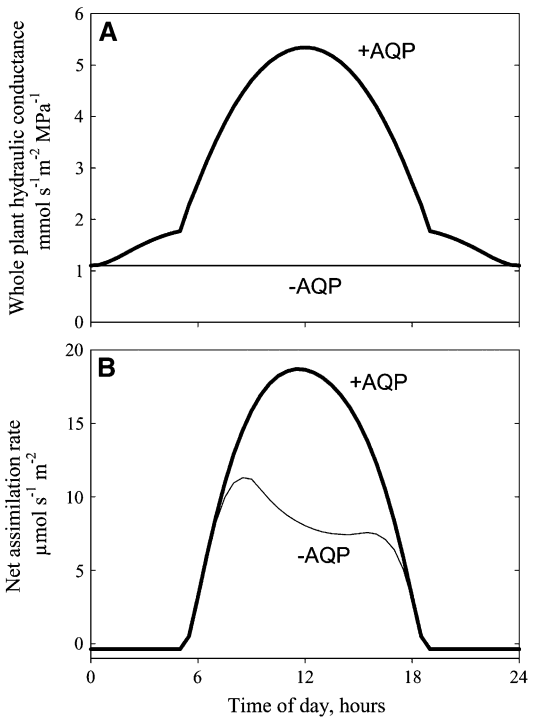

Figure 11. Theoretical output of a model comparing a plant that invests enough into roots, stems, and leaves to have low, constant, whole-plant conductance (top figure, -AQP) and an identical plant that augments root and leaf conductance through aquaporin activity (top figure, +AQP). The +AQP plant modulates its root hydraulic conductance like tobacco roots (figure 6.22 in Tyree and Zimmermann, 2002) and has an irradiance response in leaves as in this article. The resulting impact on net assimilation rate was computed (bottom figure) with a model to be published elsewhere. Briefly, the model computes net light interception of leaves on a typical sunny day, uses energy budget calculations and a stomatal model to compute leaf temperature and evaporative flux density of leaves, leaf water potential from the whole-plant hydraulic conductance (top figure), and stomatal conductance from a model similar to figure 6.15 in Tyree and Zimmermann (2002). Then the Farquhar-von Caemmerer model is used to compute the net assimilation rate. The model in the form of a Microsoft Excel spreadsheet can be obtained by e-mailing Melvin T. Tyree (mel.tyree@afhe.ualberta.ca).

route up to the terminal veins. The hydraulic resistance (R_v) of this vascular pathway is probably constant for well-watered leaves (Cochard et al., 2004). At the outlet of the vascular system, the water can take two routes in parallel: an apoplasmic route through cell walls (R_a) or a cell-to-cell route across one or more cell membranes (R_s), hence involving PIP aquaporins. Downstream of these two routes, water flow occurs in a gas phase.

The leaf hydraulic resistance ($R_{leaf} = 1/K_{leaf}$) is equal to:

$$R_{leaf} = R_v + \frac{R_a \times_s}{R_a + R_s}.$$

In the dark, water flows through the vascular system and then through the apoplasmic route, but because aquoporins are not active, R_s is high so flow through

the cell-to-cell route is reduced. In the light, there is more flow through R_s due to aquaporin accumulation. From this, it is clear that the share of vascular resistance in a leaf (R_v/R_{leaf}) will vary according to the light conditions.

What Is the Functional Significance of Aquaporin Regulation in Response to Light?

Our result suggests that aquaporin regulation may play a major role in the control of leaf water status and hence leaf physiology. Leaf water status is determined by hydraulic conductance of the water pathway and leaf transpiration rate. When aquaporin activity was inhibited in transpiring detached leaves, a substantial drop in bulk leaf water potential was observed (Fig. 9). This suggests that conductance upstream of the mesophyll cells was altered in this experiment. This plays in favor of aquaporin expression at the entry of the symplasm, potentially in leaf vein bundle sheaths. This expression would strongly be implicated in the regulation of leaf water status.

In planta, a drop in water loss and, hence, carbon uptake, would be predicted because walnut operates at a water potential very close to the point of xylem cavitation and cell turgor loss. Under these conditions, any decrease in tree hydraulic conductance could result in stomatal closure (Cochard et al., 2002). Indeed, whole-plant hydraulic conductance, K_{plant}, has an impact on gas exchange and net assimilation rate, wherein high K_{plant} improves net assimilation during times of high irradiance (Brodribb et al., 2005). As explained by Tyree (2003), high K_{plant} makes leaf water potential less negative, which increases leaf stomatal conductance (g_s), which increases the net assimilation rate according to the Farquhar-von Caemmerer model (A-Ci curve). Tyree (2003) has explained how g_s can be computed from K_{plant} given appropriate estimates of transpiration rates and measured relationships between g_s and leaf water potential. In general, plants have three options to regulate K_{plant}: (1) Invest enough carbon in roots, stems, and leaves to maintain high K_{plant} values to meet maximal hydraulic demands without assistance from aquaporins and, hence, have more hydraulic efficiency than needed most of the day and night; (2) invest less in roots, stems, and leaves, but boost K_{plant} by enhancing water transport though aquaporin activity 24 h a day; or (3) follow option 2, but express aquaporins only at times of high hydraulic demands (i.e. during high irradiance). Option 3 is likely to be the most energy-conservative paradigm; however, there may not be much of an energetic difference between options 2 and 3. The ability to express aquaporins in response to high irradiance might be most important in energy-starved seedlings released to rapid growth by gap formation in the forest understory. To test the hypothesis that option 3 makes a difference to net assimilation, we programmed an Excel spreadsheet to quantify the model proposed by Tyree (2003). The spreadsheet computes (1) incident solar radiation versus time of day on a cloudless day (using the methods in Y-plant [Pearcy and Yang, 1996]); (2) light interception by leaves and hence evaporation rates and leaf water potential from solar energy budgets (equations from Y-Plant); (3) stomatal conductance from a stomatal physiology model (e.g. figure 6.15 in Tyree and Zimmermann, 2002); and (4) internal CO_2 concentration net assimilation rate from typical A-Ci curves and parameters from the Farquhar-von Caemmerer model using g_s values from 3.

Many different model calculations can be done based on various paradigms, but option 3 above compares the net assimilation rate of a plant that expresses aquaporins versus the same plant that does not express aquaporins. The purpose of this calculation was not to compare a plant that invests more carbon in roots and stems instead of expressing aquaporins. These computations revealed enhanced net carbon gain during daylight hours in plants that modulated root and leaf hydraulic conductance during times of high irradiance (Fig. 11). An added benefit of the third paradigm is that reduced carbon investment in roots will translate into reduced overall respiration. A high energetic cost associated with the increase in leaf conductance during the light phase may explain why the aquaporin-mediated water pathway is not activated when transpiration is low at night.

In conclusion, leaf hydraulic conductance seems tightly correlated to the environmental conditions with the effect of maximizing leaf gas exchange while buffering leaf water status. The ability to modulate K_{leaf} is consistent with the revised composite water transport model for leaves, which includes an aquaporin-mediated symplasmic and/or transcellular pathway.

MATERIALS AND METHODS

Plant Material

Experiments were conducted during the summers of 2004 and 2005 on a mature, 10-m-tall walnut (*Juglans regia*) tree growing in an orchard at the Institut National de la Recherche Agronomique site of Crouël (Clermont-Ferrand, France). Leafy shoots were randomly collected from the basal sun-exposed part of the tree and immediately recut under water. Shoots were enclosed in a black plastic bag and allowed to rehydrate in full darkness for at least 24 h. Leaves with typically five leaflets were then cut from the shoots and their hydraulic conductance determined as described below.

Leaf Hydraulic Conductance

Leaf hydraulic conductance on a surface area basis (K_{leaf}, mmol s^{-1} m^{-2} MPa^{-1}) was measured with XYL'EM apparatus (Bronkhorst). The principle was to measure the water flow (F; mmol s^{-1}) entering the petiole of a cut leaf when exposed to positive pressure ($+P$; MPa) or allowed to transpire and exposed to negative leaf pressure ($-P$) as determined by a Scholander chamber. Upon steady state, K_{leaf} was computed as:

$$K_{leaf} = F/(P \times LA)$$

where LA = the total leaf area (m^2).

Most experiments were performed in the positive pressure mode ($+P$). The evaporative method ($-P$) was used only with leaves treated with CHX. Our XYL'EM apparatus was equipped with a pressure transducer and two flow meters (Liquiflow; Bronkhorst; 15 and 20 g h^{-1} ranges). The XYL'EM was interfaced with a computer to log different data automatically. In essence, the

techniques were similar to the high-pressure flow meter and the evaporative/flux methods described by Tyree et al. (1995).

To determine the light dependence of K_{leaf}, the experimental setup represented in Figure 1 was used. A cylindrical container made of transparent Plexiglas with two compartments was designed. The outer compartment was 4 cm thick and contained tap water continuously renewed to prevent overheating when lamps were on. Light was provided by two 1,000-W iodine lamps (Philips) delivering approximately 1,400 μmol m^{-2} s^{-1} at leaf level as measured by a Quantum sensor (Licor 1600 porometer). When the positive pressure technique was used, two leaves were submerged in the inner compartment filled with 9 L of tap water. The water temperature in this compartment was adjusted with a regulated bath (Ministat; Huber) and continuously aerated. Preliminary experiments revealed that temperature was homogeneous in the inner compartment (<0.5°C variation) because of the stirring provided by aeration. When lights were turned on, the temperature rose, transiently, less than 1°C above the preset value (see Fig. 2). When the evaporative technique was used, the inner compartment was of course empty and air was stirred with a small fan. An approximately 5°C air temperature increase was measured upon exposure to light.

Petioles were coupled by compression fittings to Luer tubes and connected to the XYL'EM apparatus. The flow entering each leaf was measured independently and simultaneously by the two flow meters. Leaves were supplied with water from the XYL'EM 2-L captive air tank. For one leaf, it was possible to bypass this main reservoir and to supply the leaf with the solution contained in a 10-mL syringe positioned before the flow meter. The pressure in the syringe equaled the pressure in the main reservoir. A 3-way stopcock at the leaf entrance was used to purge the Luer tube and rapidly supply the leaf with the solution in the syringe. The leaf not connected to the syringe was used as a control.

We first filled the reservoirs with deionized, filtered (0.1 μm) water delivered by an ultrapure water system (Milli Qplus 185; Millipore). However, we were unable to obtain steady K_{leaf} values because conductance was slowly decreasing to zero with time. The cause of this plugging was not identified. When deionized water was further distilled with a classical water-distilling apparatus (Schott Geräte GmbH), the problem was solved and K_{leaf} values were no longer decreasing with time. Experiments with deionized water were not included in this analysis.

A typical experiment was as follows. The water temperature in the container was first adjusted to 25°C. Two leaves were detached from the shoot, rehydrated in full darkness, and immediately inserted in the container and connected to the XYL'EM apparatus with pressure in the reservoirs adjusted to, respectively, 0.2 MPa and 0 MPa in the $+P$ and $-P$ modes, respectively. The container was first entirely covered with a black plastic sheet to reduce light levels below 1 μmol m^{-2} s^{-1}. Typically, after one-half hour, the flow reached a stable value and the lights were switched on. The flow value increased to a new stable value after 1 to 2 h and the lights were switched off. Whereas the control leaf was always treated as described above, the experimental leaf was exposed to a CHX solution at a predefined time. At the end of the experiment, the projected leaf area (LA; m^2) was measured with a LI-3000A area meter and a LI-3050A belt conveyer (LI-COR). Water in the inner compartment was renewed between two experiments to avoid possible artifacts caused by the accumulation of CHX in the water bath.

Treatments

Temperature Dependence of Leaf K_{leaf} Light Response

The changes in K_{leaf} value caused by light exposition were evaluated at 5°C, 15°C, 25°C, and 35°C according to the procedure described above. To account for the change in K_{leaf} caused by the temperature dependence of water viscosity, K_{leaf} values were standardized at 20°C as follows:

$$K_{leaf\ 20°C} = K_{leaf\ T} \times \eta_{20°C}/\eta_T$$

where $K_{leaf\ 20°C}$ is the temperature-corrected K_{leaf}, $K_{leaf\ T}$ is the actual K_{leaf} measured at temperature T, and $\eta_{20°C}$ and η_T are water viscosity at 20°C and T. Viscosity values were from Lide (1996).

To quantify the contrasted time responses at different temperatures, we computed the half time ($T_{½}$; min) of the kinetics for both the dark-to-light and light-to-dark transitions. A negative exponential function was fitted to the data and the half time of the relation computed with Pfloek, version 1.06 (Matthias Knauer, Universität Bayreuth, Germany). An illustration of the fitting is given in Figure 2. The temperature dependence of the K_{leaf} light response was established with seven to nine different leaves for each temperature. All the following experiments were performed at 25°C.

Effect of CHX on K_{leaf} Light Response

A 100 μM CHX solution was obtained by dissolving in a 0.05% (v/v) aqueous dimethyl sulfoxide solution. At such a low concentration, we tested that dimethyl sulfoxide alone had no detectable effect on K_{leaf} light response (data not shown). We determined the effect of CHX with the $+P$ and $-P$ methods. With the $+P$ method, the experimental and control leaves were first perfused in full darkness at 25°C with distilled water at 0.2 MPa until steady state. For a first set of four experimental leaves, the CHX solution was perfused for 1 h before the light was turned on. For a second set of seven experimental leaves, the CHX solution was perfused for only about 1 h after the lights were turned on (i.e. when a new steady state was achieved). Eleven leaves were used as controls.

With the $-$P method, leaf water potential (=balance pressure) was estimated by the Scholander-Hammel pressure chamber. Because leaves were provided with distilled water, xylem pressure potential (as measured by the pressure chamber) should approximately equal the water potential (Tyree and Zimmermann, 2002). Water potential gradients that exist in transpiring leaves are dissipated when balance pressure is measured; hence, the leaf water potential measured in the pressure chamber probably underestimated the gradient from the base of the leaf to the site of evaporation in our experiments. Three leaves were inserted in the empty inner chamber perfused at 0 MPa with distilled water at 25°C and kept in full darkness for one half hour. The flow entering the leaves was then <3% of the flow in full light and leaf-balancing pressure close to 0 MPa as determined on one of the three leaves. As described above, two sets of four experiments each were performed with CHX perfused either in the dark or light phase. At least 1 h after the light was turned on, leaf absorption was steady and the balancing pressure of the terminal leaflet was determined for each leaf. It was not possible to measure leaf K_{leaf} under dark conditions with this technique because P and leaf absorption were both close to zero.

Stomatal Density and Aperture

When leaves were infiltrated under pressure, liquid water eventually passed through stomata. Stomata may therefore represent extra resistance in series with leaf mesophyll hydraulic resistance. To quantify the putative effect of stomatal openness on total leaf hydraulic conductance, we measured the dimensions of the stomata pore with a cryogenic scanning electron microscope (Cryo-SEM, model SEM 505; Philips). The observations were performed on nine selected control or experimental leaves from the different treatments described above. Treatments were selected to obtain high or low K_{leaf} values under high or low light conditions. After the K_{leaf} value of the selected leaves was determined, they were rapidly removed from the water bath and immediately submerged in a bath of liquid nitrogen and stored at -80°C until examination. A small leaf part (approximately 5×5 mm) from the middle part of a leaflet was detached under liquid nitrogen and rapidly transferred, still frozen, in the Cryo-SEM chamber. Stomata were observed at about -100°C and 2,500$\times$ magnification. For each sample, we measured the length and the width of 10 to 15 pores to the nearest 0.05 μm.

Following Tyree et al. (2005), the hydraulic conductance K of all stomatal pores in parallel on the leaf blade was computed assuming pores were cylinders having a depth of 15 μm.

$$K = \frac{\Pi}{64N\eta}\sum_{1}^{N}\frac{10^{15}D}{18e}\frac{L^3 \times l^3}{L^2 + l^2}$$

where N is the number of stomata measured, η is the water viscosity at 20°C (10^{-3} Pa s), L and l are the pore major and minor axes (m), e is the stomatal pore depth (15×10^{-6} m), D is stomatal density (m^{-2}), and $10^{15}/18$ is a conversion factor between m^3 s^{-1} Pa^{-1} m^{-2} to mmol s^{-1} MPa^{-1} m^{-2}. Stomatal density (2.30×10^8 m^{-2}; SE = 6.90×10^6; n = 30) was measured with a light microscope by counting the number of stomata in the field of the microscope (0.11 mm^2). Only e was not measured in this study and roughly estimated to be 15 μm. According to the equation above, it can be seen that K is inversely proportional to e. Doubling e will divide K by 2, which will not change our conclusions.

RNA Isolation and cDNA Synthesis

A subset of control and treated leaves from the experiments described above were sampled for an analysis of aquaporin transcript abundance by real-time quantitative reverse transcription (RT)-PCR. Leaves were rapidly disconnected from the XYL'EM apparatus and immediately immersed in liquid nitrogen. Samples were stored at -80°C until analysis.

Total RNA was extracted from about 500 mg of leaves as described by Chang et al. (1993) and then treated with RNase-free DNase I (Qiagen). RNA was quantified spectrophotomically and its intactness was checked out by visual inspection of rRNA banding following agarose gel electrophoresis.

For first-strand cDNA synthesis, 2 μg of total RNA were reverse transcribed using oligo(dT) and SuperScript III (Invitrogen) following the protocol of the supplier.

Real-Time Quantitative RT-PCR Experiments

Expression analysis of two aquaporin isoforms (JrPIP2,1 and JrPIP2,2) was done by real-time RT-PCR amplification, using an iCycler iQ (Bio-Rad Laboratories) and SYBR green as a fluorescent dye. Each PCR reaction (50 μL) contained the following: reverse-transcribed cDNA (1 μL of 1:5 dilution of the first cDNA strands); PCR buffer (1 ×), the corresponding primer (each primer 0.4 μM), dNTP mix (each 0.25 mM); $MgCl_2$ (2 mM), platinium Taq polymerase (1 unit; CLONTECH), and SYBR green I (1/1,000; Sigma). After a heat step at 94°C for 4 min, PCR cycling conditions were 35 cycles of denaturation (94°C, 15 s), annealing (58.5°C, 15 s), and elongation (72°C, 20 s). Because the primer pairs WC11/WC12 and WC21/WC22 were initially shown to be specific for JrPIP2,1 and JrPIP2,2, respectively (Sakr et al., 2003), they were used for this real-time PCR. Elongation factor-1α (EF-1α) was chosen for normalization of aquaporin transcript accumulation. Relative quantitative abundance (Qr) of each investigated gene transcript was calculated by comparison to the expression of reference gene (EF-1α) using the delta-delta method mathematical model (McMaugh and Lyon, 2003).

$$\mathrm{Qr} = \frac{2^{(\mathrm{Ccontrol} - \mathrm{Ctreated})_{\mathrm{target}}}}{2^{(\mathrm{Ccontrol} - \mathrm{Ctreated})_{\mathrm{reference}}}}$$

Here, *C* is the cycle number of PCR and the control corresponds to leaves directly harvested from 24-h dark-acclimated plants before the experiment.

Specificity of amplification was confirmed by determining the melt curves for the PCR products at the end of each run and by using a gel electrophoresis. Real-time PCR amplifications were done on at least three independent experiments and every run was carried out in triplicate. The walnut *EF-1α* transcripts were detected by amplifying 660 bp with the primers EF-1α forward, 5′-TGG(A/T/C)GG(A/T)ATTGACAAGCGTG-3′ and EF-1α reverse, 5′-CAAT(T/C)TTGTA(A/G)ACATCCTGAAG-3′.

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