The water status of plant leaves depends on the efficiency of the water supply, from the vasculature to inner tissues. This process is under hormonal and environmental regulation and involves aquaporin water channels. In *Arabidopsis thaliana*, the rosette hydraulic conductivity ($K_{\text{rosp}}$) is higher in darkness than it is during the day. Knockout plants showed that three plasma membrane intrinsic proteins (PIPs) sharing expression in veins (PIP1;2, PIP2;1, and PIP2;6) contribute to rosette water transport, and PIP2;1 can fully account for $K_{\text{rosp}}$ responsiveness to darkness. Directed expression of PIP2;1 in veins of a pip2;1 mutant was sufficient to restore $K_{\text{rosp}}$. In addition, a positive correlation, in both wild-type and PIP2;1-overexpressing plants, was found between $K_{\text{rosp}}$ and the osmotic water permeability of protoplasts from the veins but not from the mesophyll. Thus, living cells in veins form a major hydraulic resistance in leaves. Quantitative proteomic analyses showed that light-dependent regulation of $K_{\text{rosp}}$ is linked to diphosphorylation of PIP2;1 at Ser-280 and Ser-283. Expression in pip2;1 of phosphomimetic and phosphorylation-deficient forms of PIP2;1 demonstrated that phosphorylation at these two sites is necessary for $K_{\text{rosp}}$ enhancement under darkness. These findings establish how regulation of a single aquaporin isoform in leaf veins critically determines leaf hydraulics.

INTRODUCTION

Plant water status critically affects two major functions of plant leaves: stomata-mediated photosynthetic gas exchange and expansion growth, which both rely on the proper maintenance of leaf cell turgor and are regulated by the water stress–induced hormone abscisic acid (ABA) (Parent et al., 2009; Kim et al., 2010). Leaf water status itself is determined by the balance between the supply of water from the root into the leaf lamina and its loss by transpiration. Plant leaves can be represented as a complex network of water transport paths whereby sap is first transported through the vasculature, with veins of different orders, and then flows through living tissues, including xylem parenchyma, bundle sheath, and mesophyll, to ultimately evaporate in air spaces and substomatal chambers (Sack and Holbrook, 2006). In living tissues, water can flow along the cell wall continuum (apoplasma) or from cell to cell where it may pass cell membranes (Maurel et al., 2008; Heinen et al., 2009). Important questions remain about the sites or tissues that are limiting for liquid water transport in the leaf lamina and about the mechanisms that determine their water permeability (hydraulic conductivity) (Sack and Holbrook, 2006; Heinen et al., 2009).

Previous studies have shown that the pattern of leaf veins and xylem morphology allow a broad range of leaf hydraulic differentiations between species or during development (Sack and Holbrook, 2006; Sack et al., 2012). In addition, living tissues and associated membrane water transport can provide rapid and reversible regulation of leaf water transport capacity (hydraulic conductance) by environmental or physiological cues. For instance, the effects of light (Cochard et al., 2007; Postaire et al., 2010), air humidity (Levin et al., 2007), drought and ABA (Shatil-Cohen et al., 2011; Pou et al., 2013), or the circadian clock (Nardini et al., 2005) on leaf hydraulic conductance have been observed in numerous species. The nature of the living cells that constitute the major hydraulic resistance in leaves and therefore drive these physiological regulations are still under debate (Cochard et al., 2004; Sack and Holbrook, 2006; Voicu et al., 2008). To date, approaches have been mostly correlative. A parallel between the effects of ABA on leaf hydraulic conductance and water permeability of protoplasts from the bundle sheath but not from mesophyll suggested that the former tissue may represent one major hydraulic...
barrier in Arabidopsis thaliana leaves (Shatil-Cohen et al., 2011). By contrast, the hydraulic conductivities of whole leaves and bundle sheath cells were not positively correlated in tobacco (Nicotiana tabacum) under varying irradiance levels (Lee et al., 2009).

Plant aquaporins form a multigenic family of water channel proteins, with >30 members in each species (Maurel et al., 2008; Heinen et al., 2009). Plasma membrane intrinsic proteins (PIPs) represent the most abundant aquaporins in the plant plasma membrane and show marked cell-specific expression patterns in the leaves of several plant species (Hachez et al., 2008; Besse et al., 2011). They likely represent a major path for cell-to-cell water transport in these organs. Light-dependent hydraulic conductance of leaves has tentatively been associated with the transcriptional regulation of two PIP homologs in walnut (Juglans regia) (Cochard et al., 2007). However, no such regulation was found in bur oak (Quercus macrocarpa) (Voicu et al., 2009), and an aquaporin-independent response of shoot hydraulics to irradiance has been reported in laurel (Laurus nobilis) (Nardini et al., 2010). Combined physiological and genetic studies can be used to dissect the role of individual aquaporin genes in plant water transport (Javot et al., 2003; Da Ines et al., 2010; Postaire et al., 2010; Péret et al., 2012). We previously showed that the hydraulic conductivity of excised Arabidopsis rosettes (K_{ros}) varies diurnally and is the highest in plants at night due to enhanced aquaporin-mediated transport (Postaire et al., 2010). K_{ros} was further increased upon prolonging plant exposure to darkness (D) by 5 to 15 h. Knockout mutant analysis revealed that one aquaporin isoform, PIP1;2, contributes to ~20% of K_{ros} in these D conditions (Postaire et al., 2010).

The sensitivity of Arabidopsis K_{ros} to plant light regimes offers, with respect to the molecular and genetic resources developed in this species, a favorable context in which to investigate the function and regulation of leaf aquaporins. In this work, we used Arabidopsis plants grown in light (L) and D conditions to perform a comprehensive genetic dissection of the role of aquaporins in leaf hydraulics. We considered four of the most highly expressed PIP isoforms and determined their site of action. We also investigated the mode of aquaporin regulation and present proteomic and genetic evidence showing that phosphorylation of a single aquaporin isoform critically determines the response of leaf hydraulics to the light regime.

RESULTS

Contributions of Individual PIP Genes to Rosette Water Transport

PIPs occur as 13 closely related isoforms in Arabidopsis. Expression profiling with macroarrays carrying gene-specific tags (see Supplemental Figure 1 online) identified four PIP genes (PIP1;2, PIP2;1, PIP2;6, and PIP2;7) as highly expressed in the Arabidopsis rosette. Each of these PIPs ranks among the three most highly expressed isoforms in at least one previous gene or protein expression study of Arabidopsis leaves (Alexandersson et al., 2005; Monneuse et al., 2011). T-DNA insertion lines corresponding to knockouts for each of these PIPs in a Columbia-0 (Col-0) (PIP1;2, PIP2;1, and PIP2;6) or Landsberg erecta (Ler) (PIP2;7) background were available from previous studies (Da Ines et al., 2010; Postaire et al., 2010; Péret et al., 2012) or were molecularly characterized in this work (see Supplemental Figure 2 online). To evaluate Arabidopsis rosette aquaporin functionality, we previously established a method for measuring the K_{ros} of internal rosette tissues. In brief, an excised rosette is inserted into a pressure chamber containing a bathing solution. A flow of liquid water is pressed across the whole rosette (Postaire et al., 2010) and is monitored as it is released at the hypocotyl section. The pressure dependence of the flow allows us to assess K_{ros}, independent of any stomatal limitation (Postaire et al., 2010). In this work, measurement of K_{ros} in rosettes excised from plants grown in L or D conditions was used to evaluate the respective contribution of each of the four PIPs to rosette water transport (Figure 1A). Although a tendency for lower values was observed, none of the four classes of knockouts showed, with respect to their corresponding wild type, consistent alteration of K_{ros} in L conditions. In agreement with previous work (Postaire et al., 2010), Col-0 and Ler plants exhibited a higher K_{ros} (by 25 and 45%, respectively) in D than in L conditions (Figure 1A). When grown under D conditions, all mutant alleles except pip2;7-1 showed, with respect to the wild type, a significant reduction of K_{ros} by 16 to 35% (P < 0.004). Two independent isolates of a triple pip1;2 pip2;1 pip2;6 mutant displayed with respect to Col-0 a 31 to 39% reduction of K_{ros} in L and D conditions (see Supplemental Figure 3 online), similar to those observed in some single pip mutants (Figure 1A). The overall data identify PIP1;2, PIP2;1, and PIP2;6 as important contributors of aquaporin-mediated rosette water transport.

Expression Patterns of Individual PIP Genes

The expression patterns of PIP1;2, PIP2;1, PIP2;6, and PIP2;7 may provide useful indications about their function. To evaluate these patterns, we used transgenic plants carrying a β-glucuronidase (GUS) gene under the control of PIP promoter (ProPIP) sequences (Figure 1B). Whereas ProPIP1;2:GUS induced strong staining in essentially all leaf cell types (Postaire et al., 2010), ProPIP2;7:GUS showed patchy expression, including expression in the mesophyll (Figure 1B). The ProPIP2;1:GUS and ProPIP2;6:GUS reporters showed, by contrast, dominant (PIP2;1) or exclusive (PIP2;6) expression in the veins (Figure 1B). A close-up view of the vascular bundle showed even staining in the xylem parenchyma and bundle sheath (Figure 1B). The finding that all PIP genes that, when mutated, produce altered K_{ros} values share a common expression pattern in vascular tissues suggests that these tissues create a significant hydraulic barrier in the rosette.

Effects of Deregulated Expression of PIP2;1 on K_{ros}

PIP2;1, which is one of most highly expressed leaf isoforms and can individually account for most light-dependent K_{ros} (Figure 1A), was chosen as a prototypic isoform for further studies. Ectopic expression of PIP2;1 in Col-0 or pip2;1-2 leaves was obtained by placing PIP2;1 under the control of a double 35S cauliflower mosaic virus promoter, yielding d35S:PIP2;1 (Péret et al., 2012) and d35S:PIP2;1ko lines, respectively. By comparison to Col-0 grown in
Contribution to Rosette Water Transport and Expression Patterns of Individual ProPIP constructs (black bars = 2.5 mm; red bars = 0.1 mm). GUS sections show intense staining in the veins of plants expressing ProPIP (B). Asterisks indicate significant difference from the wild type (WT; Col-0, L, d35S::PIP2;1) grown in L conditions but an enhanced responsiveness (87 to 99%) of K\textsubscript{ros} to D conditions (Figure 2A). With respect to pip2;1-2, d35S::PIP2;1ko also showed an enhancement of K\textsubscript{ros} specifically under D conditions. The overall genetic data support the idea that, in Col-0 plants, expression of PIP2;1 is limiting for K\textsubscript{ros} enhancement in D conditions.

To address the function of PIP2;1 in veins, we used the 1565-bp promoter sequence of the P-subunit of a gly decarboxylase gene of Flaveria trinervia (ProGLDPA) that drives green fluorescent protein (GFP) expression in the vascular tissues and surrounding bundle sheath of Arabidopsis leaves, with no detectable expression in the mesophyll (Engelmann et al., 2008) (Figure 2B). When introduced into transgenic pip2;1-2, a ProGLDPA::PIP2;1 construct conferred a low overall expression level of PIP2;1, as revealed by immunoblot analysis, but a significant increase in K\textsubscript{ros} (36 to 40%) under D versus L conditions (Figure 2C), similar to that induced by native PIP2;1 (46%). The data show that expression of PIP2;1 in veins is sufficient to confer light dependence on K\textsubscript{ros}.

Effects of Light Regime on the P\textsubscript{f} of Tissue-Specific Protoplasts

Unlike other species with leaves that are amenable to the cell pressure probe technique (Kim and Steudle, 2009), the small size of Arabidopsis leaf cells precludes in situ measurements of cell hydraulic conductivity. By contrast, the osmotic water permeability (P) of individual leaf protoplasts can be accessed using an osmotic swelling assay in hypotonic conditions. Mesophyll protoplasts from Col-0 plants grown under L or D showed a large scattering of P\textsubscript{f} values (see Supplemental Figure 4A online) with a lower mean P\textsubscript{f} in the latter condition (L, P\textsubscript{f} = 43.7 ± 4.7 µm s\textsuperscript{-1}; D, P\textsubscript{f} = 26.1 ± 6.0 µm s\textsuperscript{-1}; P < 0.001) (Figure 3A). Leaf vein protoplasts were purified by manual sorting of fluorescent protoplasts in a leaf digest from Col-0 plants containing a ProGLDPA::GFP construct. These protoplasts showed a low mean P\textsubscript{f} (13.2 ± 1.0 µm s\textsuperscript{-1}) in plants grown under L and a 39% higher P\textsubscript{f} (P < 0.02) when plants were grown under D conditions (Figure 3B; see Supplemental Figure 4C online). The ProGLDPA::GFP-labeled protoplasts fell in three distinct classes (I to III) based on diameter (D) distribution and chloroplast number (Figure 3C; see Supplemental Figure 5A online). A tendency for higher P\textsubscript{f} in D conditions (P = 0.07) was observed in small sized (mean D = 12.7 ± 0.4 µm) transcluent (with approximately two chloroplasts) protoplasts (Class I) that possibly originate from xylem parenchyma (Figure 3D).

Under L conditions, the P\textsubscript{f} of d35S::PIP2;1 mesophyll protoplasts was 2.1- to 2.3-fold higher than that of Col-0 protoplasts (Figure 3A). Since the two genotypes exhibited similar K\textsubscript{ros} (Figure 2A), the cell water permeability of Col-0 mesophyll seemed not to be limiting K\textsubscript{ros} in L conditions. Under D conditions, the P\textsubscript{f} of d35S::PIP2;1 mesophyll protoplasts was decreased by approximately twofold (P < 0.005) with respect to protoplasts from L-grown plants (Figure 3A; see Supplemental Figure 4B online). This suggests that mesophyll cells did not contribute to the marked K\textsubscript{ros} increase induced by D in d35S::PIP2;1 plants (Figure 2A). Leaf vein protoplasts were investigated in d35S::PIP2;1 plants expressing the ProGLDPA::GFP construct (Figures 3B and 3D; see Supplemental Figure 4D online).

In L conditions, all classes of vein protoplasts (Figure 3D; see

![Figure 1](image.png)

**Figure 1.** Contribution to Rosette Water Transport and Expression Patterns of Individual PIP Genes. **(A)** K\textsubscript{ros} of single pip knockout mutants (Da Ines et al., 2010; Postaire et al., 2010; Péret et al., 2012) (see Supplemental Figure 2 online) grown in L (yellow bars) or D (gray bars) conditions. For comparison of mutants in various accessions, values were normalized to the corresponding wild type (WT; Col-0, L, K\textsubscript{ros} = 221 ± 17 µL s\textsuperscript{-1} MPa\textsuperscript{-1} m\textsuperscript{2}; D, K\textsubscript{ros} = 275 ± 9 µL s\textsuperscript{-1} MPa\textsuperscript{-1} m\textsuperscript{2}; P < 0.05) (Ler, L, K\textsubscript{ros} = 201.6 ± 13.9 µL s\textsuperscript{-1} MPa\textsuperscript{-1} m\textsuperscript{2}; D, K\textsubscript{ros} = 292.0 ± 11.9 µL s\textsuperscript{-1} MPa\textsuperscript{-1} m\textsuperscript{2}; P < 0.05). Averaged data (±sd) are from five independent cultures and the indicated number of plants. Asterisks indicate significant difference from the wild type (P < 0.004). **(B)** ProPIP::GUS reporter gene expression in transgenic plants. Cross sections show intense staining in the veins of plants expressing ProPIP2;1: GUS or ProPIP2;6::GUS constructs (black bars = 2.5 mm; red bars = 0.1 mm).
Supplemental Figure 5B online) exhibited $P_f$ values similar to those of their Col-0 counterparts. However, the $d35S$:PIP2;1 leaf vein protoplasts showed 2.1-fold higher $P_f$ ($P < 0.001$) under D than under L conditions (Figure 3B), with similar $P_f$ stimulation profiles in protoplasts possibly originating from the xylem parenchyma (Class I) or bundle sheath (Class III) (Figure 3D). The overall data show that, in contrast with mesophyll protoplasts, the $P_f$ of vein protoplasts is positively correlated to $K_{\text{ros}}$ in Col-0 and $d35S$:PIP2;1 plants grown under L and D conditions.

**Evidence for Posttranslational Regulation of Aquaporins**

To investigate the mechanisms of $K_{\text{ros}}$ regulation by the light regime, we profiled PIP expression in rosettes of L- and D-grown plants using multiple reaction monitoring (MRM) of prototypic aquaporin peptides in microsomal extracts (Monneuse et al., 2011). This proteomic approach is based on a comparison of the native aquaporin peptides to chemically synthesized and heavy isotope-labeled reference peptides. This approach allowed an absolute quantification under a high dynamic range of nine out of 13 PIPs. Most importantly, it showed that the light regime did not have any effect on their abundance (Figure 4). The stability of PIP2;1 abundance was confirmed by ELISA assays of rosette extracts from Col-0 and $d35S$:PIP2;1 (see Supplemental Figure 6 online). Thus, posttranslational mechanisms appear to dominate light-dependent regulation of $K_{\text{ros}}$ in both Col-0 and $d35S$:PIP2;1 plants.

**Functional Role of PIP2;1 Phosphorylation**

Liquid chromatography–tandem mass spectrometry analysis of protein digests from rosette membranes revealed adjacent phosphorylations in the cytosolic C-terminal tail of PIP2;1, at the penultimate (Ser-280) and ultimate (Ser-283) Ser residues. These two sites have already been observed in roots or seedlings in previous phosphoproteomics studies (Nühse et al., 2004; Nittylä et al., 2007; Prak et al., 2008; Kline et al., 2010). Although PIP2;2 and PIP2;3 are not abundant in the rosette (Figure 4), the two phosphorylations can also be attributed to these isoforms. In rosettes of plants grown under L or D, the two sites occur in three states, either nonphosphorylated (0P), monophosphorylated (1P; exclusively as pS280), or diphosphorylated (2P; pS280-pS283) (Figure 5). Because of the presence of an additional phosphorylatable neighboring Ser residue (Ser-277), it was crucial to carefully check by tandem mass spectrometry sequencing that the 1P and 2P forms are identical in plants grown under L or D conditions (see Supplemental Figure 7 online). The absolute abundance of 0P, 1P, and 2P forms (peptides) was then quantified by MRM using three transitions per peptide (see Supplemental Table 1 online) in leaves of Col-0 and pip2;1-2 plants grown under L or D conditions (see Supplemental Figure 7 online). The absolute abundance of 0P, 1P, and 2P forms (peptides) was then quantified by MRM using three transitions per peptide (see Supplemental Table 1 online) in leaves of Col-0 and pip2;1-2 plants grown under L or D conditions (Figure 5). The 0P and 1P peptides showed, with respect to Col-0, a dramatically reduced abundance in pip2;1-2. The 2P form was not even detectable in Col-0 was increased by 2.2-fold in response to D. The data support the notion that $K_{\text{ros}}$ is positively linked to PIP2;1 diphosphorylation.

In the search for a causal link between PIP diphosphorylation and $K_{\text{ros}}$, we assayed the function of PIP2;1 phosphorylation mutants in planta (Figure 6). A S280A/S283A double mutant of PIP2;1 (PIP2;1-AA) was designed to mimic a constitutive phosphorylation deficiency at these two sites, whereas a double

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**Figure 2. Effects of Deregulated Expression of PIP2;1 on $K_{\text{ros}}$.**

**(A)** $K_{\text{ros}}$ and PIP2;1 expression in two $d35S$:PIP2;1 lines (-1 and -3), one $d35S$:PIP2;1$k$ line, and their untransformed controls (Col-0 and pip2;1-2, respectively). The top panel shows averaged $K_{\text{ros}}$ values (± se) from three independent cultures, with the indicated number of plants. Plant were grown in L (yellow bars) or D (gray bars) conditions. Letters indicate statistically different values at $P < 0.05$. The bottom panel shows immunodetection of PIP2 in leaf extracts of the indicated lines grown in L.

**(B)** Imaging of an entire leaf (top) and secondary vein region (bottom) of Col-0 plants expressing a ProGLDPA:GFP construct, using epi-fluorescence and biphoto fluorescence microscopy, respectively (bars = 50 μm).

**(C)** Characterization of Col-0, pip2;1-2, and two independent pip2;1-2 lines expressing a ProGLDPA:PIP2;1 construct. Same conventions as in (A).
S280D/S283D mutant (PIP2;1-DD) is expected to act as a constitutive phosphomimetic form. When expressed under control of ProPIP2;1 in a pip2;1-2 background, and at levels comparable to those seen in Col-0, PIP2;1-AA showed $K_{\text{ros}}$ values that were independent of the plant light regime and similar to those of wild-type or pip2;1-2 plants grown under L conditions (Figure 6; see Supplemental Figure 8 online). By contrast, a lower level expression of PIP2;1-DD was sufficient to reproduce the $K_{\text{ros}}$ profile seen in wild-type plants or pip2;1-2 plants expressing a wild-type PIP2;1-SS form (Figure 6; see Supplemental Figure 8 online). The ability of PIP2;1-DD, but not of PIP2;1-AA, to confer light dependence on $K_{\text{ros}}$ indicates that diphosphorylation of PIP2;1 is necessary for $K_{\text{ros}}$ activation under D conditions, thereby establishing direct genetic evidence for a role of aquaporin phosphorylation in regulating plant water transport.

DISCUSSION

Three PIP Isoforms Contribute to Water Transport in the Arabidopsis Rosette

Very little is known about the molecular and genetic bases of plant leaf hydraulics. Although Arabidopsis differs from most other investigated species, with enhanced leaf hydraulics at night and not during the day (Sack and Holbrook, 2006; Postaire et al., 2010), the Arabidopsis rosette proved to be an interesting system to investigate fundamental aspects of leaf water transport. Based on previous and our own expression surveys, we identified four PIP isoforms (PIP1;2, PIP2;1, PIP2;6, and PIP2;7) that are among the most highly expressed in the Arabidopsis rosette and investigated corresponding individual loss-of-function mutant plants. For this, we used a type of high-pressure technique (Postaire et al., 2010) that is comparable to two other methods.
the vacuum pump and the evaporative flux methods, alternatively used for characterizing leaf hydraulics (Sack et al., 2002). In the latter method, leaf water transport is monitored in freely transpiring plants or excised leaves, with subsequent determination of leaf water potential using a pressure chamber. The method used in this work also relies on a pressure chamber, but to press a flow of water across the inner leaf tissues. In support for the equivalence of these two methods, the leaf hydraulic conductance ($K_{\text{leaf}}$) values that we recorded were similar to those previously described in works using the evaporative flux method (Martre et al., 2002; Shatil-Cohen et al., 2011). Our approach did not reveal major $K_{\text{leaf}}$ phenotypes when plants were grown in L conditions. This is consistent with the importance of vascular (xylem vessel) resistance in our pressure chamber assay. The predominance of xylem resistance on leaf hydraulics has been observed in many other plant species (Sack and Holbrook, 2006). In the case of Arabidopsis grown in L, previous pharmacological evidence indicates that aquaporins have a low contribution (13%) to $K_{\text{leaf}}$ (Postaire et al., 2010). More extensive measurements would possibly be required to detect a significant aquaporin phenotype in plants grown in these conditions. In agreement with an enhancement of $K_{\text{leaf}}$ and its aquaporin component under D, three of the four investigated PIP isoforms (PIP1;2, PIP2;1, and PIP2;6) were found to contribute to rosette water transport in these conditions. The phenotypic effects of individual gene disruption were similar in amplitude and close to the maximum reduction in $K_{\text{leaf}}$ seen after pharmacological inhibition (25%) (Postaire et al., 2010) or in two independent isolates of a triple PIP mutant (Figure 1A; see Supplemental Figure 3 online). The similar phenotypes of the single and triple PIP mutant plants indicate that, rather than phenotypic compensation or functional redundancy, the three PIP isoforms would show cooperation to confer light-dependent hydraulic properties on rosette leaves. The molecular interactions or cellular mechanisms involving these three aquaporins remain to be determined.

A Major Site of Hydraulic Resistance in Inner Leaf Tissues

Several lines of evidence establish that veins form the major hydraulic resistance among living tissues present in the inner leaf. First, all three PIP genes, the disruption of which had marked effects on $K_{\text{leaf}}$, were consistently expressed in veins, suggesting that the function of several aquaporin isoforms in this tissue is necessary for proper leaf hydraulics (Figure 1). The common expression territory of these isoforms includes bundle sheath and vascular parenchyma cells. Second, the directed expression of PIP2;1 in veins of a pip2;1 mutant was sufficient to restore $K_{\text{leaf}}$ responsiveness to light regime (Figure 2C). For this, we used ProGLDPA, which drives strong expression in all cell types of Arabidopsis leaf veins (Engelmann et al., 2008; Wiludda et al., 2012). In other words, the results show that the function of PIP2;1 in the mesophyll is not required for light-dependent regulation of $K_{\text{leaf}}$. Third, in line with the approach developed by Shatil-Cohen et al. (2011) in ABA-treated leaves, we established a strong positive correlation, in both Col-0 and PIP2;1-overexpressing (dp3S:PPIP2;1) plants, between $K_{\text{leaf}}$ and the $P_i$ of vein protoplasts (Figure 3B). Comparison of the two genotypes demonstrated that, under L conditions, mesophyll cell water permeability was, by contrast, not limiting for $K_{\text{leaf}}$ in Col-0 plants. With respect to previous studies that focused on bundle sheath cells exclusively (Ache et al., 2010; Shatil-Cohen et al., 2011), our protoplast approach points to the additional role of small cells lacking chloroplasts, which we tentatively identify as the xylem parenchyma (Figure 3C). This is consistent with the strong expression of PIPs in these cells (Figure 1B) and with geometrical constraints imposed by centripetal efflux of sap from the xylem into the leaf. The water transport measurements in isolated protoplasts also indicated that all vein cell types would undergo a similar activation of $K_{\text{leaf}}$ in D conditions. It is not yet known whether such coordinated hydraulic response occurs in response to other stimuli such as ABA (Galvez-Valdivieso et al., 2009; Shatil-Cohen et al., 2011; Pantin et al., 2013). Finally, the protoplast

Figure 5. Effects of Light Regime on PIP2;1/PIP2;2 C-Terminal Phosphorylation.

All studies were performed in rosettes from Col-0 or pip2;1-1 plants grown under L (yellow bars) or D (gray bars) conditions. Phosphoproteomics analysis reveals the existence of three unmodified or phosphorylated forms for a C-terminal tryptic peptide (Ser-277 to Val-287) of PIP2;1/PIP2;2 (top). Absolute abundance of each form ($\pm$SE) was determined with three technical replicates from three (Col-0) or two (pip2;1-2) independent plant cultures. Letters indicate statistically different values at $P < 0.05$. [See online article for color version of this figure.]
data indicated light-activated $P_1$ in the mesophyll. This response is at variance with the inverse relationship between mesophyll protoplast water permeability and transpiration previously reported by Morillon and Chrispeels (2001). Nevertheless, this study of the mesophyll and our study of veins indicate that, in Arabidopsis and in contrast with other species, such as grapevine (Vitis vinifera) (Pou et al., 2013), the transpiration demand does not necessarily drive an increase in leaf cell water permeability. Along these lines, remote regulation of stomatal conductance by predominant hydraulic resistance in leaf veins was recently proposed as a protective mechanism against excessive leaf dehydration (Ache et al., 2010; Shatil-Cohen et al., 2011; Pantin et al., 2013).

In summary, the overall data of our work point to distinct tissue-specific regulation and roles for leaf aquaporins. In veins, aquaporins would contribute to whole rosette water transport. Because the $K_{ros}$ assay specifically reports on PIP2;1 activity in leaf veins, our phosphorylation mutant analysis also points to the functional importance of aquaporin regulation in a well-defined plant territory. However, this analysis revealed an apparent lack of activity of the PIP2;1-DD form for $K_{ros}$ under L conditions. This suggests that PIP2;1 phosphorylation at Ser-280 and Ser-283 is not necessary for PIP2;1 activation under D conditions. For unknown reasons, we could only select transgenic lines expressing PIP2;1-DD at lower levels than its wild-type (PIP2;1-SS) counterpart (see Supplemental Figure 8 online). Yet, the amplitude of light-dependent $K_{ros}$ variation was similar in the two types of lines, pointing to the high physiological activity of PIP2;1-DD. The role of plant aquaporin phosphorylation has previously been addressed in vitro and at the subcellular level (Guenther et al., 2003; Prak et al., 2008). Here, we provided genetic evidence that aquaporin phosphorylation plays a role in regulating an important aspect of whole-plant water transport. Because the $K_{ros}$ assay specifically reports on PIP2;1 activity in leaf veins, our phosphorylation mutant analysis also points to the functional importance of aquaporin regulation in a well-defined plant territory. However, this analysis revealed an apparent lack of activity of the PIP2;1-DD form for $K_{ros}$ under L conditions. This suggests that PIP2;1 phosphorylation at Ser-280 and Ser-283 was not sufficient for PIP2;1 activation. We speculate that subsidiary PIP2;1 posttranslational modification(s) or activator(s) are specifically present in D conditions and required for PIP2;1-dependent activation.

Many physiological and environmental stimuli, in addition to changes in irradiance, are known to alter PIP phosphorylation in...
shoots and roots (Johansson et al., 1996; Niittylä et al., 2007; Prak et al., 2008; Hsu et al., 2009; Kline et al., 2010; Engelsberger and Schulze, 2012). Of particular relevance to this work are the effects of ABA, which reduced PIP2;1 phosphorylation (Kline et al., 2010) and downregulated Arabidopsis leaf hydraulic conductance through a mechanism that involves bundle sheath cells (Shatil-Cohen et al., 2011; Pantin et al., 2013). By contrast, phosphorylation of PIP2;6 was transiently enhanced under osmotic stress (Niittylä et al., 2007). Thus, sequential events, including multiple phosphorylation and additional activation steps, may ultimately act on both aquaporin trafficking and gating (Tönroth-Horsefield et al., 2006; Prak et al., 2008; Eto et al., 2010) to constantly adjust leaf hydraulics to rapid and composite environmental variations.

In conclusion, this work has uncovered the light-dependent molecular mechanisms that target a specific PIP aquaporin in leaf veins. More generally, it stresses the importance of exploring in future studies the tissue-specific regulation and in vivo functions of leaves. More generally, it stresses the importance of exploring in future studies the tissue-specific regulation and in vivo functions of leaves. In conclusion, this work has uncovered the light-dependent molecular mechanisms that target a specific PIP aquaporin in leaf veins. More generally, it stresses the importance of exploring in future studies the tissue-specific regulation and in vivo functions of leaves.

METHODS

Transgenic Plant Materials

The Arabidopsis thaliana pip1;2-1 (Postaire et al., 2010), pip2;1-1 (Pérét et al., 2012), and pip2;1-2 (Da Ines et al., 2010) Col-0 mutants were described elsewhere. The SALK_92140c (pip2;6-1) and SALK_118213c (pip2;6-2) Col-0 T-DNA insertion lines and the CSHL_GT19652 (pip2;7-1) Ler transposon insertion lines were obtained from the Nottingham Arabidopsis Stock Center and the Cold Spring Harbor Laboratory, respectively. Homozygous mutations and gene inactivation were verified by genotyping and RT-PCR (see Supplemental Figures 2 and 3 online) (Da Ines et al., 2010; Postaire et al., 2010; Pérét et al., 2012) using the primers indicated in Supplemental Table 2 online. All procedures for plant genotyping were as described (Postaire et al., 2010). Plants overexpressing a PIP2;1 cDNA under the control of a double enhanced cauliflower mosaic virus 35S promoter (35S:PIP2;1) were as described (Pérét et al., 2012). The ProPIP2;1:2:GUS and ProPip2;1:1:GUS lines have been described elsewhere (Postaire et al., 2010; Pérét et al., 2012). Fragments comprising 2559 and 2577 bp upstream of the start codon of PIP2;6 and PIP2;7, respectively, were cloned into pBGWFS7 to generate transcriptional ProPip2;6:GUS and ProPip2;7:GUS constructs, which were introduced into transgenic Arabidopsis by the floral dip method (Clough and Bent, 1998). Col-0 plants expressing a ProGLDPA:GFP (GLDPA-F:mGFP5-ER) construct were as described (Engelmann et al., 2008), The same construct was introduced into 35S:PIP2;1-1 plants via Agrobacterium tumefaciens GV3101 by the floral dip method (Clough and Bent, 1998), and transgenic lines were selected according to both kanamycin resistance and GFP fluorescence levels.

Expression of Wild-Type and Mutant PIP2;1 Forms in pip2;1-1

Promoter fragments from Arabidopsis PIP2;1 (ProPIP2;1) and Flaveria trinervia GLDPA (Engelmann et al., 2008) (ProGLDPA), and encompassing 2285 and 1565 bp, respectively, upstream of the start codon were PCR amplified and cloned into the HindIII-Sfi restriction sites of a pGWB501 Gateway destination vector (Nakagawa et al., 2007). The native PIP2;1 cDNA in a pDONR207 (Invitrogen) plasmid was inserted via Gateway recombination into the ProGLDPA destination vector. Mutant versions of PIP2;1 cDNA at Ser-280 and Ser-283 were generated by successive PCR using mutagenic primers (Prak et al., 2008) to contain the following C-extremity sequences with the desired mutations (AA or DD; bold letters): SS, 5′-GGATCCTGCAAGTTGCGCCACGTTGCTGA-3′; AA, 5′-GGAGCTGCGTTGGCTGCAAGTTGCGCCACGTTGCTGA-3′; and DD, 5′-GGATCCTGCAAGTTGCGCCACGTTGCTGA-3′. The mutant cDNAs were cloned in pDONR207 entry vector and further inserted into a ProPIP2;1 destination vector by Gateway recombination (Invitrogen). All constructs were introduced into an Agrobacterium GV3101 strain and used to transform pip2;1-2 by the floral dip method (Clough and Bent, 1998). Prior to any physiological characterization, transgenic lines of T2 and T3 progenies were selected in vitro for antibiotic resistance, and at least two independently transformed lines of each genotype were selected for highest PIP2;1 expression by protein gel blot analysis.

Plant Growth Conditions

For physiological or PIP expression analysis, plants were germinated in vitro and grown in hydroponic conditions with a 16-h-light (250 µmol photons m−2 s−1) at 22°C/8-h-dark at 21°C cycle (Postaire et al., 2010). All assays were performed in 21- to 25-d-old plants. Plants collected from 3 to 16 h after the onset of light were referred to as plants grown in L. For D treatments, plants were grown under normal light cycle and were maintained after night for five to 15 additional hours in the dark.

Macroarray Expression Analysis

Total RNA was extracted from 1 to 2 g fresh weight of rosette tissues as described by Boursiac et al. (2005) except that 0.15 M ammonium oxalate was substituted for 0.3 M sodium acetate during isopropanol precipitation. Total RNA (20 µg) was used for synthesis of labeled cDNA probes and hybridized to a macroarray carrying aquaporin gene-specific tags (Boursiac et al., 2005).

Protein Expression Analyses

For proteomic analyses, rosette tissues in 2 mL fresh weight 1 1 of the modified homogenizing buffer described by Gerbeau et al. (2002) was homogenized in a Waring blender (4 × 10 s). A microsomal fraction was isolated (Gerbeau et al., 2002), stripped from extrinsic membrane proteins (Santoni et al., 2003), and digested overnight with endolysin-C (Monneuse et al., 2011). MRM analyses (Monneuse et al., 2011) were performed on three transitions per peptide (see Supplemental Table 1 online). Corresponding heavy isotope labeled peptides were chemically synthesized and used for absolute quantification (Monneuse et al., 2011). The identification of phosphorylated forms of the C-terminal tail of PIP2;1 was performed with a quantitative time-of-flight mass spectrometer (Maxis; Bruker Daltonik), interfaced with a nano-HPLC Ultimate 3000 (Dionex). Samples were loaded onto the precolumn (C18 PepMap100, 300 µm × 5 mm, 5 µm, 100 A; Dionex) at a flow rate of 20 µL min−1 for 5 min with solvent A (0.1% formic acid and 2% acetonitrile in water, v/v/v). After preconcentration, peptides were separated on the reverse-phase column (C18 PepMap100, 75 µm × 250 mm, 3 µm, 100 A; Dionex) at a flow rate of 0.3 µL min−1 using a two-step linear gradient, from 7 to 25% solvent B (0.1% formic acid and 90% acetonitrile in water, v/v/v) from 0 to 57 min, and from 25 to 40% solvent B, from 57 to 68 min, and eluted into the mass spectrometer. The instrument was operated in the positive ion mode, and the nano-electrospray ionization source parameters were as follows: a capillary voltage of 5000 V, a nebulizer gas pressure of 0.4 bars, and a dry gas flow rate of 4 liters min−1 at 140°C. After an initial mass spectrometry scan at 2 Hz over the mass range of 50 to 2200 Th, the five most intense precursors were fragmented by collision-induced dissociation.
Expression of PIP2;1 in vitro or hydroponically grown plants was also probed by protein gel blotting of crude leaf protein extracts (Boursiac et al., 2005) using an anti-PIP2 antibody raised against a 17-aminoc acid C-terminal peptide of Arabidopsis PIP2;1 (Santoni et al., 2003). ELISA assays were performed in microsomal extracts of rosettes using the same antibody (Santoni et al., 2003). GUS staining was done as previously described (Postaire et al., 2010).

**Water Transport Assays**

Pressure chamber measurements of hydrostatic rosette \( K_\text{oos} \) were performed as described (Postaire et al., 2010). In brief, whole excised rosettes bathing to light. Plant Physiol. 16: 735–743.

**Supplemental Figure 8.** Expression and Function of Double Phosphorylation Mutants of PIP2;1 in Rosettes of pip2;1-2 Plants.

**Supplemental Table 1.** Selected Transitions for Monitoring Endogenous Nonmodified, Mono- and Diphosphorylated Forms of a C-Terminal PIP2;1 Peptide.

**Supplemental Table 2.** Nucleotide Sequences of Primers Used for Mutant Genotyping.

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**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


