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Regulation of *Arabidopsis* Leaf Hydraulics Involves Light-Dependent Phosphorylation of Aquaporins in Veins[C][W]

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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_{ros}) 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_{ros} responsiveness to darkness. Directed expression of *PIP2;1* in veins of a *pip2;1* mutant was sufficient to restore K_{ros}. In addition, a positive correlation, in both wild-type and *PIP2;1*-overexpressing plants, was found between K_{ros} 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_{ros} 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_{ros} 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 (apoplasm) 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

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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* (L*er*) (*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 L*er* 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

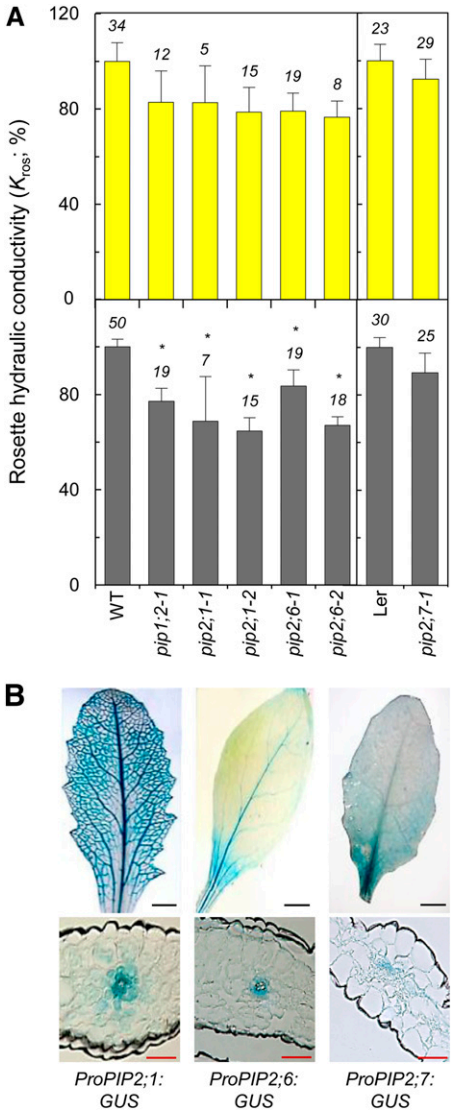

Figure 1. Contribution to Rosette Water Transport and Expression Patterns of Individual *PIP* Genes.

(A) K_{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_{ros} = 221 ± 17 μL s^{-1} MPa^{-1} m^{-2}; D, K_{ros} = 275 ± 9 μL s^{-1} MPa^{-1} m^{-2}; $P < 0.05$) (*Ler*, L, K_{ros} = 201.6 ± 13.9 μL s^{-1} MPa^{-1} m^{-2}; D, K_{ros} = 292.0 ± 11.9 μL s^{-1} MPa^{-1} m^{-2}; $P < 0.05$). Averaged data (±SE) 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).

L, two independently transformed *d35S:PIP2;1* lines showed similar K_{ros} under L conditions but an enhanced responsiveness (87 to 99%) of K_{ros} to D conditions (Figure 2A). With respect to *pip2;1-2*, *d35S:PIP2;1ko* also showed an enhancement of K_{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_{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_{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_{ros}.

Effects of Light Regime on the P_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_f) 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_f values (see Supplemental Figure 4A online) with a lower mean P_f in the latter condition (L, P_f = 43.7 ± 4.7 μm s^{-1}; D, P_f = 26.1 ± 6.0 μm s^{-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_f (13.2 ± 1.0 μm s^{-1}) in plants grown under L and a 39% higher P_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 (Ø) distribution and chloroplast number (Figure 3C; see Supplemental Figure 5A online). A tendency for higher P_f in D conditions ($P = 0.07$) was observed in small sized (mean Ø = 12.7 ± 0.4 μm), translucent (with approximately two chloroplasts) protoplasts (Class I) that possibly originate from xylem parenchyma (Figure 3D).

Under L conditions, the P_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_{ros} (Figure 2A), the cell water permeability of Col-0 mesophyll seemed not to be limiting K_{ros} in L conditions. Under D conditions, the P_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_{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

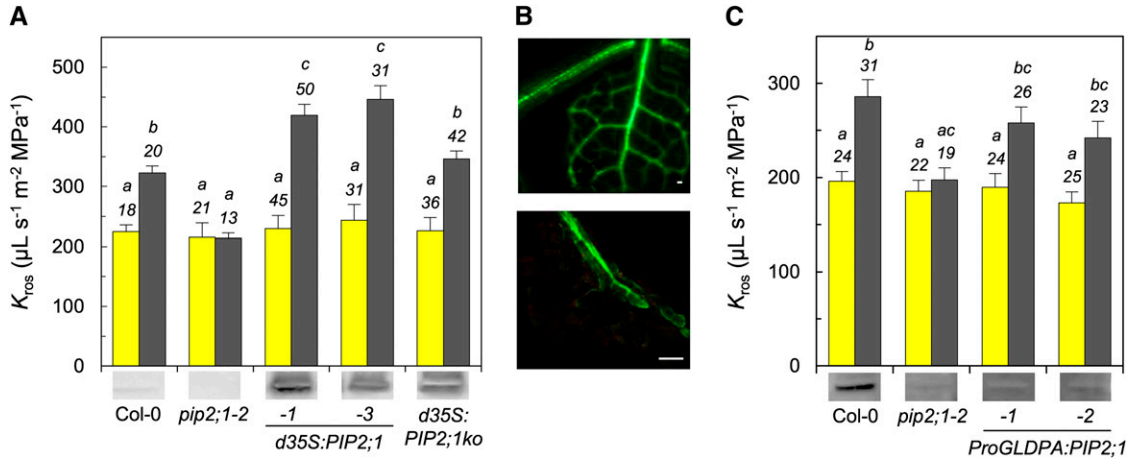

Figure 2. Effects of Deregulated Expression of *PIP2;1* on K_{ros}.

(A) K_{ros} and PIP2;1 expression in two *d35S:PIP2;1* lines (-1 and -3), one *d35S:PIP2;1ko* line, and their untransformed controls (Col-0 and *pip2;1-2*, respectively). The top panel shows averaged K_{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 epifluorescence and biphoton 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)**.

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_{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_{ros} regulation by the light regime, we profiled PIP expression in rosettes of L- and D-grown plants using multiple reaction monitoring (MRM) of proteotypic 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_{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; Niittylä 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 and 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 the latter genotype. 0P and 1P abundance was independent of plant light regime in *pip2;1-2* and was slightly reduced under D conditions in Col-0 plants. By contrast, 2P abundance in Col-0 was increased by 2.2-fold in response to D. The data support the notion that K_{ros} is positively linked to PIP2;1 diphosphorylation.

In the search for a causal link between PIP diphosphorylation and K_{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

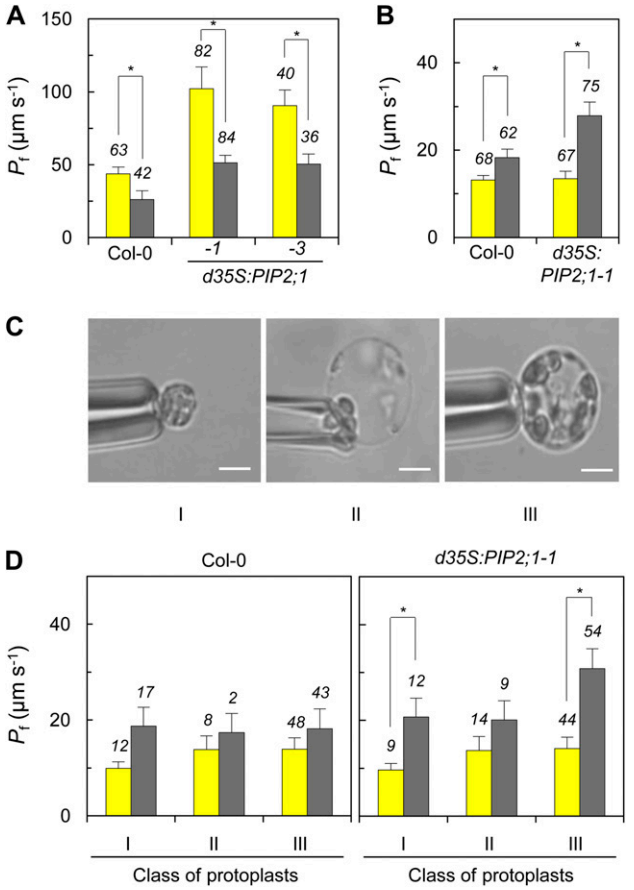

Figure 3. Effects of Light Regime on P_f Coefficients of Mesophyll or Vein-Specific Protoplasts.

(A) Mesophyll protoplasts were purified from Col-0 or *d35S:PIP2;1* (lines -1 and -3) plants grown under L (yellow bars) or D (gray bars) conditions. P_f was characterized using an osmotic swelling assay as described in the text. Averaged data (±SE) from the indicated number of protoplasts are obtained from at least 12 independent preparations and three plant cultures. Asterisks indicate significant effect of light on a genotype ($P < 0.05$).
(B) Fluorescent protoplasts were sorted out using a micropipette from a leaf digest of transgenic Col-0 or *d35S:PIP2;1-1* plants expressing a *ProGLDPA:GFP* construct and grown under L (yellow bars) or D (gray bars) conditions. Experimental conditions for measuring P_f and conventions are as in **(A)**.
(C) Leaf fluorescent protoplasts purified from transgenic Col-0 expressing a *ProGLDPA:GFP* construct were characterized under transmission light microscopy according to their diameter (Ø) and chloroplast content (see Supplemental Figure 5A online). The figure shows protoplast images representative of three distinct classes: (I) small sized (mean Ø = 12.7 ± 0.4 μm), translucent (with approximately two chloroplasts) protoplasts, (II) large (mean Ø = 17.3 ± 1.0 μm) protoplasts containing at most three chloroplasts, (III) large protoplasts (mean Ø = 22.0 ± 0.5 μm) with more than three chloroplasts. The same three subclasses could also be observed in *d35S:PIP2;1-1* plants expressing the same *ProGLDPA:GFP* construct (see Supplemental Figure 5B online). Bars = 8 μm.
(D) Effects of light on the P_f of the three subclasses of leaf vein protoplasts. Protoplasts were isolated from Col-0 (left panel) and *d35S:PIP2;1-1* plants (right panel) expressing *ProGLDPA:GFP* and grown under L (yellow bars) or D (black bars) conditions. Experimental conditions for measuring P_f and conventions are as in **(A)**.
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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_{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_{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_{ros} indicates that diphosphorylation of PIP2;1 is necessary for K_{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,

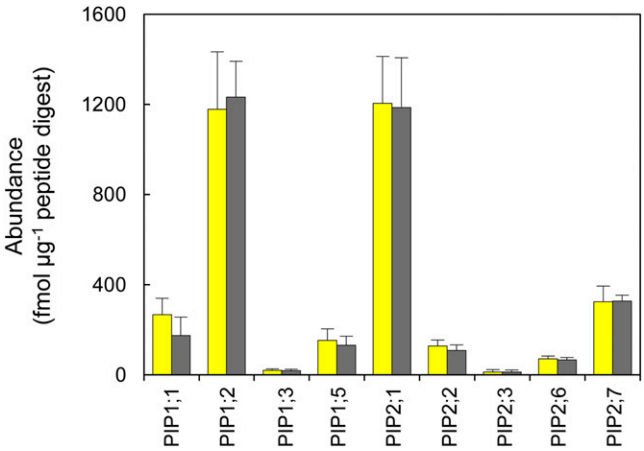

Figure 4. Effects of Light Regime on PIP Abundance.

The figure shows a quantitative proteomic profiling of PIPs in rosettes of Col-0 plants grown under L (yellow bars) or D (gray bars) conditions. Absolute abundance of each isoform (±SE) was determined with three technical replicates.
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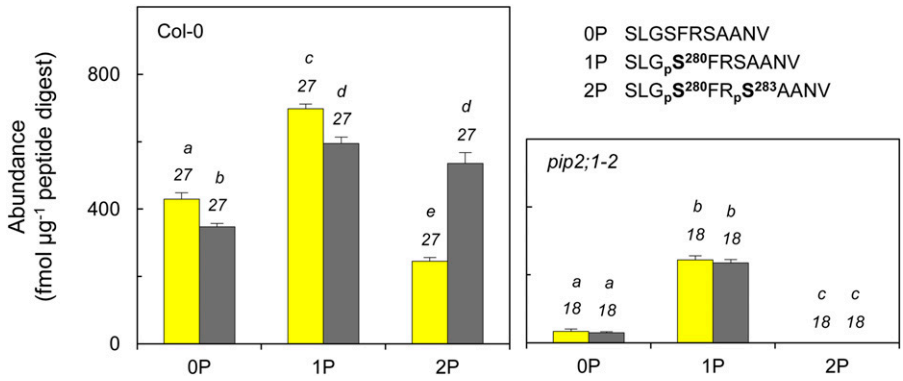

Figure 5. Effects of Light Regime on PIP2;1/PIP2;2 C-Terminal Phosphorylation.

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 (±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 $P < 0.05$.
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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_{ros}) 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_{ros} 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_{ros} (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_{ros} 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_{ros} 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_{ros}, 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_{ros} 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_{ros}. 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 (*d35S:PIP2;1*) plants, between K_{ros} and the P_f 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_{ros} 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_{ros} 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

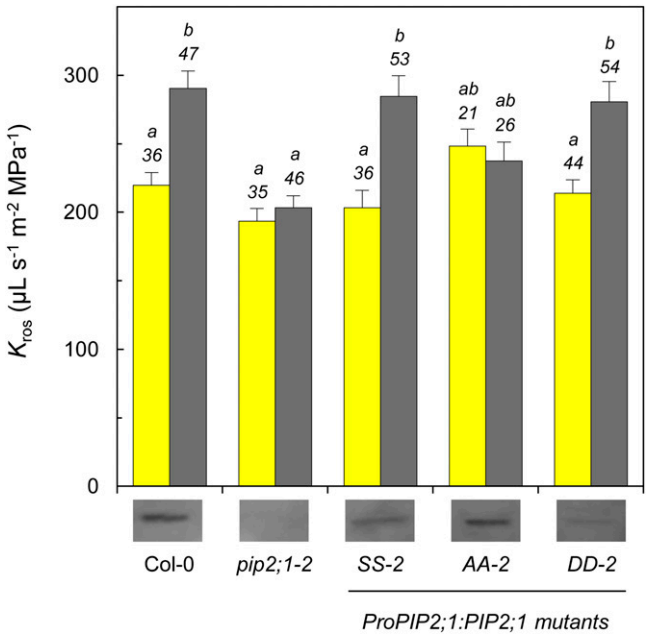

Figure 6. Effects of Light Regime on K_{ros} of *pip2;1-2* Plants Expressing Phosphorylation Mutants of PIP2;1.

Plants expressing the indicated PIP2;1 mutant forms were grown under L (yellow bars) or D (gray bars) conditions, and their K_{ros} was compared with values in the wild type (Col-0) and *pip2;1-2*. Averaged data (±SE) are from the indicated number of plants and three independent cultures. Letters indicate statistically different values at $P < 0.05$. The figure shows K_{ros} data obtained in one individual clone representative of each genotype, with corresponding immunoblot analysis of PIP2 expression in the bottom panel. Complementary analyses are shown in Supplemental Figure 8 online. [See online article for color version of this figure.]

data indicated light-activated P_f 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 hydraulics (K_{ros}) to facilitate leaf water supply and possibly optimize growth at night or dawn (Wiese et al., 2007; Postaire et al., 2010). Consistent with this, *Arabidopsis* leaves show, from 4 d after emergence, hydraulically limited expansion during the day (Pantin et al., 2011). Independent of hydraulic regulation in veins, enhanced mesophyll cell water permeability during the day could possibly secure the water status of tissues active in photosynthesis.

Diphosphorylation of PIP2;1 Mediates the Increase of K_{ros} in D

To further explore PIP functions in leaves, we dissected the molecular mechanisms of aquaporin regulation by the light regime. Quantitative proteomics was pivotal to showing that this regulation is essentially posttranslational. Despite varying transcriptional regulation profiles under L and D conditions (Postaire et al., 2010), none of the nine PIP isoforms detected in leaves showed light-dependent variation in protein abundance (Figure 4). We then focused on PIP2;1, which can account for most if not all of light-dependent K_{ros}. Transcriptional deregulation (under a *GLDPA* or a *d35S* promoter) of *PIP2;1* did not change the light-dependent K_{ros} regulation profile conferred by this aquaporin. Although Col-0, *ProGLDPA:PIP2;1* or *d35S:PIP2;1* plants showed contrasting differences in PIP2;1 accumulation, PIP2;1 contribution to K_{ros} was only present under D conditions. In line with a putative posttranslational regulation mechanism, we investigated PIP2;1 phosphorylation at two adjacent sites (Ser-280 and Ser-283). Similar sites have been described in the C-terminal tail of PIP2 homologs from various plant species (Johansson et al., 1998; Van Wilder et al., 2008; Whiteman et al., 2008). Although structure-function analysis of a tobacco PIP2 in yeast (Fischer and Kaldenhoff, 2008) seems to be at variance with reports in other models, the Ser-280 and Ser-283 sites are thought to act on stimulus-induced gating and on trafficking of PIP2;1, respectively (Törnroth-Horsefield et al., 2006; Prak et al., 2008).

The high quantitative resolution of MRM revealed that, in contrast with other phosphorylated forms, a twofold increase in PIP2;1 diphosphorylation is associated with a K_{ros} increase in D conditions. Interestingly, the diphosphorylated form was virtually absent in PIP2;2 and PIP2;3 (see *pip2;1-2* in Figure 5). Site-directed mutants of PIP2;1 proved crucial to establish, beyond correlative evidence, the activating role of Ser-280 and Ser-283 modifications. More specifically, the ability of the PIP2;1-DD, but not of the PIP2;1-AA form, to complement *pip2;1* demonstrated that phosphorylation at these two sites is 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 diphosphorylation 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 (Törnroth-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 machinery that governs aquaporin activity. Beyond the response of leaves, it will also be crucial to explore in other organs how a given PIP phosphorylation signature can result in cell- or stimulus-specific regulation profiles.

METHODS

Transgenic Plant Materials

The *Arabidopsis thaliana pip1;2-1* (Postaire et al., 2010), *pip2;1-1* (Péret 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*) L*er* 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; Péret 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 (*d35S:PIP2;1*) were as described (Péret et al., 2012). The *ProPIP1;2:GUS* and *ProPIP2;1:GUS* lines have been described elsewhere (Postaire et al., 2010; Péret 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-Ft:mGFP5-ER*) construct were as described (Engelmann et al., 2008). The same construct was introduced into *d35S: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 in the *Hin*dIII-*Sbf*I 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 characters): SS, 5′-GGATCATTCAGAAGTGCTGCCAACGTCTGA-3′; AA, 5′-GGA**G**CATTCAGA**GC**TGCTGCCAACGTCTGA-3′; and DD, 5′-GGA-**GAT**TTCAGA**GA**TGCTGCCAACGTCTGA-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 oxaloacetate 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 g fresh weight^{-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 nebulization 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–amino 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_{ros} were performed as described (Postaire et al., 2010). In brief, whole excised rosettes bathing in a liquid solution were inserted into a pressure chamber, the sectioned hypocotyl being tightly adjusted through the metal lid of the chamber. Pressurization of the chamber resulted in a flow of liquid entering through the leaf surface and exiting from the hypocotyl section. The flow rate (J_v) was proportional to the applied pressure (P). The slope of a J_v(P) plot was reported to the cumulated surface of the rosette leaves to calculate the K_{ros} of an individual rosette (in $\mu L\ s^{-1}\ m^{-2}\ MPa^{-1}$). For isolation of mesophyll or vein protoplasts, leaf tissues were incubated as described (Postaire et al., 2010) in the presence of 1.5% cellulase RS and 0.25% pectolyase Y23 for 45 and 90 min, respectively. In the latter case, Col-0 or *d35S:PIP2;1-1* plants expressing the *ProGLDPA:GFP* construct were used. Fluorescent protoplasts were visualized under a microscope with a 488-nm excitation light and selected manually with a micropipette for subsequent P_f assay. In all cases, P_f was measured as described (Postaire et al., 2010) by monitoring the osmotic swelling of individual protoplasts by video microscopy.

Statistics

The effects of genotype and light regime on physiological parameters were investigated by analysis of variance using R software, with a generalized linear model followed by a multiple testing procedure ($P < 0.05$). Pair comparisons were performed using the Wilcoxon's test at $P < 0.05$.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *PIP1;2* (At2g45960), *PIP2;1* (At3g53420), *PIP2;6* (At2g39010), and *PIP2;7* (At4g35100).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Profiling of *PIP* Gene Expression in the *Arabidopsis* Rosette.

Supplemental Figure 2. Molecular Characterization of *pip2;6* and *pip2;7* T-DNA Insertion Mutants.

Supplemental Figure 3. Molecular Characterization and Rosette Water Transport Phenotype of Triple *pip1;2 pip2;1 pip2;6* Knockout Mutants.

Supplemental Figure 4. Distribution of Osmotic Water Permeability Coefficients of Mesophyll or Vein-Specific Protoplasts from Col-0 or *d35S:PIP2;1-1* Plants Grown under L or D Conditions.

Supplemental Figure 5. Size Repartition of Subclasses of Leaf Vein Protoplasts.

Supplemental Figure 6. PIP2 Abundance in Col-0, *pip2;1-2*, and *d35S:PIP2;1-1* Plants Grown in L or D Conditions.

Supplemental Figure 7. MS/MS Sequencing of Mono- and Diphosphorylated Forms of PIP2;1 in Col-0 Plants Cultivated in L and D Conditions.

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

K.P., Y.B., C.T.-R., and C.M. designed the research. K.P., Y.B., C.T.-R., J.-M.M., and S.H. performed research. O.P. performed crucial preliminary experiments. O.D.I. and A.R.S contributed new transgenic plants. K.P., V.S., and C.M. analyzed data. The article was written by C.M. and read by all the authors.

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