The Role of Plasma Membrane Aquaporins in Regulating the Bundle Sheath-Mesophyll Continuum and Leaf Hydraulics

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Our understanding of the cellular role of aquaporins (AQP s) in the regulation of whole-plant hydraulics, in general, and extravascular, radial hydraulic conductance in leaves (Kleaf), in particular, is still fairly limited. We hypothesized that the AQP s of the vascular bundle sheath (BS) cells regulate Kleaf. To examine this hypothesis, AQP genes were silenced using artificial microRNAs that were expressed constitutively or specifically targeted to the BS. MicroRNA sequences were designed to target all five AQP genes from the PLASMA MEMBRANE-INTRINSIC PROTEIN1 (PIP1) subfamily. Our results show that the constitutively silenced PIP1 (35S promoter) plants had decreased PIP1 transcript and protein levels and decreased mesophyll and BS osmotic water permeability (Pf), mesophyll conductance of CO2, photosynthesis, Kleaf, transpiration, and shoot biomass. Plants in which the PIP1 subfamily was silenced only in the BS (SCARECROW:microRNA plants) exhibited decreased mesophyll and BS Pf, and decreased Kleaf but no decreases in the rest of the parameters listed above, with the net result of increased shoot biomass. We excluded the possibility of SCARECROW promoter activity in the mesophyll. Hence, the fact that SCARECROW:microRNA mesophyll exhibited reduced Pf but not reduced mesophyll conductance of CO2 suggests that the BS-mesophyll hydraulic continuum acts as a feed-forward control signal. The role of AQP s in the hierarchy of the hydraulic signal pathway controlling leaf water status under normal and limited-water conditions is discussed.

The flow of water through the leaf is one of the most important, but least understood, components of the whole-plant hydraulic system (Sack and Holbrook, 2006; Scoffoni et al., 2012). Water is transported axially from the roots to and through the petiole and veins and radially from the laminar xylem across the xylem parenchyma and bundle sheath (BS) cells to the mesophyll. Once it exits the xylem vessel, water can flow through or around mesophyll cells before evaporating from the cell walls and diffusing out of the stomata (Esau, 1965). The efficiency of water transport through the leaf is determined as the hydraulic conductance (Kleaf [the ratio of the water flow rate to the driving force of the flow]; for review, see Sack and Holbrook, 2006; Prado and Maurel, 2013), which is the sum of the vascular-axial and extravascular-radial conductance. Leaf vascular anatomy (vein density and the distance between the vein endings and the stomata) has a strong influence on Kleaf (Sack and Frole, 2006; Brodribb et al., 2007; Sack and Scoffoni, 2013). Nevertheless, Kleaf is not constant, and changes in this value reveal highly dynamic behavior, which is associated with changes in ambient conditions (i.e. temperature and irradiance) and abiotic stress (Martre et al., 2002; Sack and Holbrook, 2006; Cochard et al., 2007; Levin et al., 2007).

As the axial hydraulic structure of the mature leaf is fixed, the assumption is that in nonembolized or undamaged xylem, a substantial part of the dynamic hydraulic regulation is controlled by the radial movement of water through the parenchymal tissue surrounding the xylem elements (Sack and Holbrook, 2006). Recent studies...
have suggested that, in Arabidopsis (Arabidopsis thaliana), the leaf radial in-flow rate is controlled by the vascular BS, which is a bottleneck for leaf hydraulic conductance (Ache et al., 2010). These cells may be involved in regulating the radial transport activity of the xylem system (Kinsman and Pyke, 1998; Leegood, 2008), partially due to the extremely low apoplastic flow through the BS (Shatil-Cohen and Moshelion, 2012).

Recently, new evidence has emerged to support the hypothesized role of the BS, as well as xylem parenchymal cells, in the regulation of radial Kleaf possibly via the regulation of water channels (i.e. aquaporins [AQPs]; for review, see Moshelion et al., 2014). AQPs are considered to be key channel proteins for the transport of water, small and uncharged solutes, and CO2 through plant cell membranes (Tyrerman et al., 2002; Maurel et al., 2008). A number of previous studies have reported on the regulatory role of AQPs in cellular water transport (Maurel, 1997; Kaldenhoff et al., 2007). Arabidopsis has 35 AQPs (Johanson et al., 2001), which have been categorized into four major subfamilies. The PLASMA MEMBRANE-INTRINSIC PROTEIN (PIP) subfamily includes 13 members and is divided into two subgroups, AtPIP1 and AtPIP2. The AtPIP1 group includes five proteins (AtPIP1;1–AtPIP1;5), and the AtPIP2 group includes eight proteins (AtPIP2;1–AtPIP2;8).

It has been suggested that AQPs play a role in regulating Kleaf (for review, see Sack and Holbrook, 2006). More recent reports have noted a correlation between PIP AQP expression and dynamic leaf hydraulics under different irradiance conditions (Coëchard et al., 2007) as well as the role of BS AQPs in the regulation of Kleaf under stressful conditions (Shatil-Cohen et al., 2011; Pantin et al., 2013). Recently, it was demonstrated that the activity of the AtPIP2;1 isoform in veins can account for the responses of rosette hydraulic conductivity (Krc) under prolonged dark conditions (Prado et al., 2013). Nevertheless, the identification of the set of AQPs expressed in vessels and their respective roles in Kleaf is still incomplete, and the direct, quantitative effect of BS AQPs on leaf hydraulicity is not fully understood.

Many of the studies that have investigated the role of AQPs in regulating plant hydraulic conductance were performed using classical reverse-genetic approaches. For instance, transfer DNA mutant insertion lines lacking the PIP1,2, PIP2;1, or PIP2;6 isoform, which are abundant in leaf tissue (Alexander et al., 2005), exhibited a 20% decrease in whole-rosette Kros (Postaire et al., 2010; Prado et al., 2013), and antisense lines targeting PIP AQPs in Arabidopsis and tobacco (Nicotiana tabacum) exhibited decreased whole-plant hydraulic conductivity (Martre et al., 2002; Siefritz et al., 2002). However, these tools are of limited use when it comes to studying the role of gene-tissue interactions or redundancy caused by a high number of targeted isoforms (Ossowski et al., 2008).

In an attempt to cope with these challenges, we employed methodology involving artificial microRNA (amiRNA), which exploits endogenous microRNA (miRNA) precursors to generate small RNA sequences that direct gene silencing in the cell. amiRNA-mediated gene silencing is a useful method for inducible and partial gene inactivation as well as the simultaneous, specific targeting of several related genes (Ossowski et al., 2008). Moreover, the effect of the construct is autonomous, and it can be used for studies of tissue-specific expression (Alvarez et al., 2006).

In this study, we used an amiRNA reverse-genetic approach to specifically down-regulate multiple PIPs. We hypothesized that this silencing would lead to decreased permeability of the cells to water and decreased Kleaf. We examined this hypothesis using specific down-regulation of multiple AQP genes under the control of both constitutive and BS-specific promoters and tested the effects of this down-regulation on the flow of water into the leaf.

RESULTS
Using amiRNA for the Down-Regulation of PIP1 Homologs

Several studies have reported the involvement of the PIP subfamily (both PIP1 and PIP2) in the regulation of hydraulic conductivity (Kaldenhoff et al., 1998; Martre et al., 2002; Siefritz et al., 2002; Postaire et al., 2010; Prado et al., 2013). Therefore, we chose to silence the PIP1 subfamily using amiRNA. To that end, we designed a single sequence of synthetic miRNA containing a consensus sequence of 21 nucleotides targeted at the entire Arabidopsis PIP1 subfamily (AtPIP1;1–AtPIP1;5; Fig. 1A).

This 21-nucleotide sequence met the criteria for allowable mismatches in number and position as well as the low free-energy characteristic of endogenous plant miRNAs (Schwab et al., 2005). It was introduced into the miR164b backbone (Alvarez et al., 2006) under the control of the 35S constitutive promoter to generate amiRNAPip1, which was used to transform Arabidopsis plants. Quantitative real-time (q-RT)-PCR was used to identify the transgenic expression of premiR-PIP1 (Fig. 1B). The plants that overexpressed 35S:amiRNAPip1 (35S:mir1) were somewhat smaller than the wild-type plants, depending on the level of 35Smir1 expression and the physiological age of the plant (Fig. 1, C–E).

To examine amiRNA activity, we assayed the relative abundance of the full-length PIP1 transcripts using RNA northern-blot analysis. For all five PIP1s, a significant reduction in RNA level was observed in the transgenic plants (Supplemental Fig. S1A). In addition, we detected the presence of amiRNAPip1 cleavage products of three candidate PIP1 genes using 5’ RNA ligase-mediated (RLM)-RACE (Kasschau et al., 2003; Supplemental Fig. S1B).

Our q-RT-PCR analysis identified a down-regulation of all PIP1 genes in the leaves from two independent 35S:mir1 lines (35S:mir1-3 and 35S:mir1-8) as compared with the control (Fig. 2). The 35S:mir1-8 line had 2-fold higher expression of the synthetic amiRNAPip1 and lower PIP1 transcript levels (70%–98% reduction).
as compared with the control and the 35S:mir1-3 line, in which there was a 52% to 94% reduction in PIP1 expression. Both lines displayed an approximately 50% reduction in the expression of the PIP2;1 gene, and the 35S:mir1-8 line had 37% less expression of the PIP2;6 gene (Fig. 2). In addition, most of the PIP2 genes were expressed at lower levels in the 35S:mir1 lines.

Western-blot analysis with specific PIP1 and PIP2 antibodies was used to detect the abundance of PIP1 and PIP2 proteins in the 35S:mir1 plants (Santoni et al., 2003). Reduced levels of PIP1 proteins were observed in both 35S:mir1 lines, although (and in accordance with the observed RNA expression levels) a greater decrease was observed in the 35S:mir1-8 line (Supplemental Fig. S2).
The expression of PIP2 proteins was slightly reduced in the 35S:mir1-8 plants, whereas more significant reduction was observed in the 35S:mir1-3 line (Supplemental Fig. S2).

**Physiological Characteristics of the 35S:mir1 Plants**

To compare AQP functioning in the different lines, we measured the cellular osmotic water permeability coefficient (Pf) of mesophyll and BS protoplasts isolated from wild-type plants and the two independent 35S:mir1 lines. Significantly lower Pf values were observed in the 35S:mir1 mesophyll and BS cells as compared with control cells (Fig. 3), indicating dominant PIP1 activity in the cells of wild-type plants.

We examined the whole-rosette Kros of the 35S:mir1 plants using a previously described pressure-chamber technique (Postaire et al., 2010; Prado et al., 2013). Plants were transferred to an extended-night (13–21 h of darkness) environment prior to hydraulic measurements, a condition previously shown to enhance Kros and its AQP-mediated component (Postaire et al., 2010; Prado et al., 2013). The 35S:mir1-8 line showed significantly lower Kros than the control, whereas the Kros of 35S:mir1-3 was similar to that of the control (Fig. 4A). The rosette surfaces of the two lines were similar to that of the control (Fig. 4B). Analysis of the PIP transcript levels in the enriched BS tissue (the leaf midveins) showed that, in both lines, all of the PIP1 genes except for PIP1:3 were down-regulated. However, the decrease observed in the 35S:mir1-8 line was more substantial than that observed in the 35S:mir1-3 line (Fig. 5).

Kros was measured using a detached-leaf approach (Shatil-Cohen et al., 2011) based on the determination of transpiration rate (E) and leaf water potential (Ψleaf, see “Materials and Methods”), to yield a calculated Kleaf (ratio of E to Ψleaf, Fig. 6). Consistent with the Kros results and the relative reduction in the transcript level, the Kleaf of the 35S:mir1-8 line (but not of the 35S:mir1-3 line) was significantly reduced as compared with the control (Fig. 6A). Nevertheless, examination of the morphological structure of the vascular systems of the different lines revealed no differences that could impact hydraulic conductivity (e.g. abnormal structure or size or leakage [Supplemental Fig. S3]; for a more detailed explanation of the relation of these measurements to BS apoplastic activity, see Shatil-Cohen et al., 2011).

In a parallel experiment, we measured the mesophyll CO2 conductance and gas-exchange characteristics of 35S:mir1 silenced lines grown in soil. Here again, only the 35S:mir1-8 line exhibited a significant reduction in photosynthesis and mesophyll conductance of CO2 (g35S:mir1) as compared with the wild-type plants (Table I). This experiment was performed under saturated light conditions (Flexas et al., 2007). Under these conditions, lower stomatal conductance (g) was observed (g and E in both 35S:mir1-8 and 35S:mir1-3; Table I) as compared with the wild type.

**Physiological Characteristics of the SCARECROW:microRNA Plants**

As a complementary approach to test the hypothesis that Kleaf is determined by AQP activity in the BS, we examined plants in which PIPs are specifically silenced in the BS cells by the expression of mir1 under the control of the SCARECROW (SCR) promoter (SCR: mir1). As described previously, prior to the physiological Kleaf measurements, we followed the expression patterns of all of the PIP genes in the enriched vein
tissue of SCR:mir1 plants and compared them with those of control plants. Moreover, the morphological structure of the SCR:mir1 vascular system exhibited no differences that could impact the hydraulic conductivity (e.g. abnormal structure or size or leakage; Supplemental Fig. S3). An analysis of RNA expression revealed down-regulation of all of the _PIP1_ genes (except for _AtPIP1:1_) and of four _AtPIP2_ genes (_AtPIP2:5–AtPIP2:8_; Fig. 7).

Next, we measured the _P_ of the BS cells. The mean _P_ of the SCR:mir1 BS cells was significantly lower than that of the control cells (Fig. 8A). Surprisingly, the _P_ of the mesophyll cells was also significantly lower in the SCR:mir1 line (Fig. 8B; see “Discussion”).

Following the molecular and cell _P_ analyses of the SCR:mir1 plants, we measured _E_ and _Ψ_ in order to calculate _K_ (Fig. 9). We found that _K_ was significantly reduced in this line as compared with the control (Fig. 9). This significant reduction in _K_ was associated with significantly lower _Ψ_. Interestingly, and similar to the 35S:mir1 plants, although a visible trend was observed, there was no significant reduction in _E_ (relative to the control) in the detached leaves (Fig. 9). These results were further confirmed using intact leaves (Supplemental Fig. S4). Examinations of the mesophyll _CO_ conductance and gas-exchange characteristics of SCR:mir1 plants grown in soil revealed no reductions in any of the parameters as compared with the wild type (Table II), and these plants were slightly larger than the wild-type plants (Supplemental Fig. S5, A and B). The fact that these plants did not show any reductions in _g_ or _E_ or any increase in their abscisic acid (ABA) biosynthetic gene transcript levels (9-cis-epoxycarotenoid dioxygenase; Supplemental Fig. S5C) but did exhibit reduced mesophyll _P_ suggests that hydraulic and biochemical (photosynthesis) processes proceed separately.

**DISCUSSION**

AQP s play a major role in mediating the transmembrane transport of water in plant cells (Maurel et al., 2008). It has been suggested that the BS acts as a xylem-mesophyll hydraulic barrier (Heinen et al., 2009; Ache et al., 2010; Shatil-Cohen and Moshelion, 2012), directing the apoplastic xylem flow into the leaf via the BS transmembrane pathway, which is controlled by AQP s. Indeed, vascular parenchymal tissue-specific _PIP2_ s were recently shown to control _K_ (Prado et al., 2013). Here, we demonstrate that BS AQP s play a role in the direct control of _K_ and also indirectly control mesophyll hydraulic conductance.

**amiRNA as a Tool for Down-Regulating AQP Expression**

amiRNA silencing is expected to be proportional to its expression level. That is, if more miRNA molecules
are present, they will silence more transcripts (Alvarez et al., 2006). Indeed, the line with the higher level of amiRNApip1 expression had a lower level of PIP expression (RNA and proteins) and a stronger phenotype (Figs. 1, 2, 4, and 5; Supplemental Fig. S2), confirming the quantitative nature of amiRNA activity.

In contrast to other reverse-genetic approaches, amiRNA can silence multiple AQP isoforms simultaneously with minimal risk of off-target effects (Ossowski et al., 2008). Moreover, amiRNA can be targeted (promoter related) to a specific tissue, since the miRNA silencing is local and not systemic (Alvarez et al., 2006).

Monogenic homozygous AQP knockout lines can be combined to eliminate several isoforms, and this approach can be used to exclude direct side effects on other related genes. However, there are limitations to this strategy, including gametophytic or early sporophytic lethality as well as the difficulty of combining mutant alleles on closely linked (tandem duplicated) genes. Moreover, targeting more than four AQP isoforms (e.g. the five PIP1 isoforms or eight PIP2 isoforms) using this approach is challenging.

RNA interference (RNAi) or antisense strategies can be used to silence several isoforms at once. However, the possibility of side effects, such as the systemic expression of the transformed construct and/or effects on other unrelated genes, cannot be completely excluded (Ossowski et al., 2008). For example, in previous studies, using RNAi to silence AQPs resulted in the unintentional silencing of unrelated genes, yielding an artificial phenotype (Ma et al., 2004; Schüssler et al., 2008). Since amiRNA expression results in a single silencing molecule (as opposed to RNAi, which creates a large number of small interfering RNA molecules), it is possible to avoid such phenomena in the amiRNA method (Ossowski et al., 2008). Taking into account sequence homology and empirical parameters for amiRNA (Schwab et al., 2005), we constructed an amiRNA sequence that targets all members of the AtPIP1 subfamily with an acceptable level of efficiency (Fig. 1A).

Bioinformatic analysis (weigelworld.com) suggested that some PIP2 AQP genes (AtPIP2;1, AtPIP2;2, AtPIP2;3, and AtPIP2;7) might serve as possible targets for our amiRNApip1 (Supplemental Fig. S6). Nevertheless, in contrast to the steady and constitutive down-regulation of PIP1 isoforms, PIP2 down-regulation varied between tissues, conditions, and genotypes (Figs. 2, 5, and 7; Supplemental Fig. S2), suggesting that PIP2 genes are most likely not targeted by the amiRNA. This might be related to the fact that these putative targets have somewhat higher rates of sequence mismatch (four to five mismatches; Supplemental Fig. S6). From the physiological point of view, the fact that PIP2 genes were down-regulated does not interfere with the notion that AQPs (either PIP1s or others) can determine the membrane $P_f$ and $K_{leaf}$

**Whole-Plant Constitutive Silencing of PIP AQPs**

The reduction in $P_f$ observed in the constitutively silenced plants (Fig. 3) was expected, as decreased mesophyll $P_f$ has been observed previously in AQP knockout and antisense lines (Martre et al., 2002; Postaire et al., 2010). The role of PIPs in controlling $K_{leaf}$ and radial influx, in particular, is an interesting question. Research in this area has shown that PIP1 and PIP2 antisense lines do not exhibit reduced leaf and plant hydraulic conductivity under normal moisture conditions. However, the lack of functional PIPs results in the significantly slower recovery of plant hydraulic conductivity following exposure to drought (Martre et al., 2002; Secchi and Zwieniecki, 2014). Additional studies have described the

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**Table 1.** Gas-exchange characteristics of 35S:mir1 and control plants (determined at 1,200 $\mu$mol $m^{-2} s^{-1}$, approximately 25˚C, and 400 $\mu$mol mol$^{-1}$ CO$_2$, as described by Flexas et al., 2007).

<table>
<thead>
<tr>
<th>Plant</th>
<th>$A_n$</th>
<th>$C_i$</th>
<th>$g_s$</th>
<th>$g_m$</th>
<th>$E$</th>
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<tr>
<td></td>
<td>$\mu$mol CO$_2$ $m^{-2} s^{-1}$</td>
<td>$\mu$mol CO$_2$ mol$^{-1}$</td>
<td>mol water $m^{-2} s^{-1}$</td>
<td>mol CO$_2$ $m^{-2} s^{-1}$</td>
<td>mmol water $m^{-2} s^{-1}$</td>
</tr>
<tr>
<td>Wild type (n)</td>
<td>$6.9 \pm 0.31$ (33)</td>
<td>$301.63 \pm 3.01$ (33)</td>
<td>$0.145 \pm 0.007$ (33)</td>
<td>$0.035 \pm 0.002$ (33)</td>
<td>$2.085 \pm 0.1$ (33)</td>
</tr>
<tr>
<td>35S:mir1-8 (n)</td>
<td>$5.65 \pm 0.37$ (22)*</td>
<td>$304.95 \pm 3.37$ (22)</td>
<td>$0.115 \pm 0.007$ (22)*</td>
<td>$0.028 \pm 0.002$ (22)*</td>
<td>$1.716 \pm 0.09$ (22)*</td>
</tr>
<tr>
<td>35S:mir1-3 (n)</td>
<td>$6.10 \pm 0.24$ (22)</td>
<td>$296.06 \pm 3.99$ (22)</td>
<td>$0.118 \pm 0.008$ (22)*</td>
<td>$0.031 \pm 0.001$ (22)</td>
<td>$1.755 \pm 0.095$ (22)*</td>
</tr>
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Data are given as means $\pm$ SE (n, number of independent biological repetitions). Asterisks indicate significant differences ($P < 0.05$) between a genotype and the wild type, as calculated using Dunnett’s method. $A_n$, Photosynthesis; $C_i$, substomatal CO$_2$ concentration.
The role of Arabidopsis PIP1s and PIP2s in maintaining Kvos under extended-night conditions (Postaire et al., 2010; Prado et al., 2013). Recently, PIPs in walnut (Juglans nigra) were shown to control stem hydraulic conductivity (Steppe et al., 2012).

Our data confirm these findings, with a significant reduction in Pf, as well as Kvos and Kleaf in 35Smir1 plants (Figs. 3, 4, and 6). The reduction in Kvos might be the result of the reduced Pf in the living cells of the vascular system, including the BS cells, as demonstrated recently by Shatil-Cohen et al. (2011), Pantin et al. (2013), and Prado et al. (2013). Thus, our reverse-genetic approach effectively reduced PIP expression and cellular activity, and as a result, leaf tissue hydraulic conductivity decreased. Moreover, using the detached-leaf method, we were able to measure leaf water efflux (E) and, separately, estimate the ratio between influx and outflux through \( \Psi_{\text{leaf}} \). These measurements revealed that the reduction in Kleaf was reflected by a decreased \( \Psi_{\text{leaf}} \) and was not due to a decrease in E (i.e. a decrease in water influx but not \( g_m \); Figure 6, B and C). A \( \Psi_{\text{leaf}} \) reduction with no significant change in transpiration was also observed in intact leaves (from plants grown in soil, as opposed to excised leaves with submerged petioles and an unlimited water supply; Supplemental Fig. S7).

These results support the hypothesized role of BS AQPs in regulating the movement of water into BS cells and Kleaf (or xylem efflux), as suggested by the hydraulic g feedback theory (i.e. down-regulation of AQPs reduces Kleaf which reduces \( \Psi_{\text{leaf}} \) and serves as a feed-forward signal for stomatal closure; Shatil-Cohen et al., 2011; Pantin et al., 2013). It has been suggested that AtPIP1;2 may transport CO\(_2\) and regulate mesophyll CO\(_2\) conductance (Heckwolf et al., 2011; Uehlein et al., 2012) in addition to its ability to control a cell’s hydraulic conductivity (Postaire et al., 2010). Those observations of PIP1s affecting both hydraulic conductivity and mesophyll CO\(_2\) conductance are not restricted to Arabidopsis; similar effects have also been observed in tobacco (Nicotiana tabacum) and poplar (Populus tremuloides; Siefritz et al., 2002; Flexas et al., 2006; Secchi and Zwieniecki, 2013, 2014). Indeed, gas-exchange measurements of our 35Smir1 silenced lines, taken under saturated light, revealed reduced photosynthesis and reduced \( g_m \) (in 35Smir1-8), \( g_m \) and E (in both 35Smir1-8 and 35Smir1-3) in the silenced plants as compared with the wild type (Table I). Recently, Atpip1;2 mutants were reported to have lower Kvos (Postaire et al., 2010), less photosynthesis, and lower \( g_m \) (Heckwolf et al., 2011). However, in both studies (Postaire et al., 2010; Heckwolf et al., 2011), the effect was restricted to one substance (water or CO\(_2\)), whereas we have shown that both water and CO\(_2\) are affected in...
our 35S:mir1 plants (Table I). The fact that SCR:mir1 lines exhibited no reduction in any of the gas-exchange parameters as compared with the control plants (Table II), yet did exhibit reduced mesophyll $P_f$, suggest the possibility that a BS-mesophyll hydraulic signal controls $K_{leaf}$ yet further research is needed to elucidate this signal.

### BS-Mesophyll Hydraulic Feed-Forward Signal

Down-regulation of BS AQPs (SCR:mir1 plants) was found to be effective at the transcript level (Fig. 7) and at the functional level, as demonstrated by the lower $P_f$ of the silenced BS cells of those plants (Fig. 8A). Unexpectedly, the mesophyll $P_f$ of those plants was reduced as well (Fig. 8B), which raises questions about how it is controlled.

We excluded the possibility of SCR promoter activity in the mesophyll, as this promoter is known to be expressed specifically in the BS and not at all in the mesophyll (Wysocka-Diller et al., 2000; Shatil-Cohen et al., 2011; Sade et al., 2014). In addition, it is not likely that the silencing agent moves to the mesophyll because (1) miRNA is known to be cell autonomous (Alvarez et al., 2006), (2) the relative expression of the silencing agent was 5 times higher in the midribs than along the leaf margins (Supplemental Fig. S8), and (3) unlike the effectiveness of the miRNA agent against PIP1 members in midvein tissue (Figs. 5 and 7), here the relative expression of three of the five PIP1s in the leaf margin area (i.e. enriched mesophyll) was similar to that of the control (Supplemental Fig. S9). Since the silencing of the miRNA agent in a cell should not vary between the different PIPs, we ruled out the possibility of its presence in the mesophyll cells.

In fact, SCR:mir plants showed mild stress symptoms, in the form of reductions in their $K_{leaf}$, $\Psi_{leaf}$ and BS and mesophyll $P_f$, but they did not exhibit other stress symptoms of reduced transpiration, $g_{m'}$ or reduced photosynthesis (nor did they exhibit increased ABA biosynthetic gene transcript levels; Supplemental Fig. S5). And unlike the 35S:mir1 plants, the SCR:mir plants were not smaller than the wild-type plants. In fact, they were a bit larger (Supplemental Fig. S5). And unlike the 35S:mir1 plants, the SCR:mir plants were a bit larger (Supplemental Fig. S5).

This physiological phenotype suggests that the $P_f$ and $K_{leaf}$ are very sensitive and the first to change once conditions become less than optimal (before any reduction in $g_s$ or increase in ABA levels). This raises a question regarding the hierarchy of the hydraulic signal cascade controlling leaf water status (for review, see Chaumont and Tyerman, 2014).

Previous studies addressing this question suggested the existence of a generalized sensitivity cascade of a plant’s physiological responses to water stress. Accordingly, the first symptoms to appear at the onset of stress are turgor loss and the inhibition of cell growth, followed by gradual increases in ABA levels, the initiation of stomatal closure, and a reduction in the rate of photosynthesis (for review, see Hsiao and Acevedo, 1974; Taiz and Zeiger, 2010).

Our results in SCR:mir plants suggest that the imposed reduction in $K_{leaf}$ might serve as a hydraulic feed-forward signal that regulates the mesophyll $P_f$ prior to the appearance of secondary and more severe signals, such as increased ABA levels and reduced $g_s$. Two more pieces of information support this hydraulic stress signal cascade hypothesis. First, almost no reduction in the expression levels of the PIP2s was measured in the SCR:mir1 mesophyll. Reduced expression of PIP2s under stress is a well-known symptom of stress (Jang et al., 2004; Alexandersson et al., 2005; Boursiac et al., 2005). Second, $g_{m'}$, which was reduced under the constitutive 35S:mir promoter (probably due to the reduced expression of PIP1 AQPs; Uehlein et al., 2003, 2008; Flexas et al., 2006; Heckwolf et al., 2011; Sade et al., 2014), was not affected by the expression of SCR:mir1 (Tables I and II). The fact that BS cells are tightly linked with the mesophyll via many plasmodesmata connections (Ache et al., 2010) serves as additional evidence supporting the existence of the hypothesized BS-mesophyll hydraulic regulation signal.

These results as well as the literature concerning the generalized sensitivity hierarchy cascade of plant physiological characteristics to water stress led us to the following hypothetical model of action; plants exhibit a rapid decrease in the turgor of their leaf cells in response to a root-evoked drop in $\Psi_w$ (Christmann et al., 2013). Under water stress, local changes in $\Psi_w$ are quickly relayed throughout the plant xylem, thanks to the cohesion and tension properties of water. A turgor decrease below the plant’s specific threshold value in the leaf lamina leads to increased ABA production (Liu et al., 1978; Pierce and Raschke, 1981; Lee et al., 2006; Tuteja, 2007; Ache et al., 2010), probably at its synthesis sites in shoot vascular parenchyma tissues and guard cells (Boursiac et al., 2013). The increase in the concentration of ABA in the xylem leads to reduced $\Psi_{leaf}$ and $K_{leaf}$ in the BS, possibly due to reduced AQP expression and/or activity (Martre et al., 2002;
Kleaf is regulated via PIP AQPs in BS cells. Moreover, this supports the hypothesized role of PIP AQPs in this process. Thus, the cells that surround the xylem. Our results suggest that radial unloading of water from the plant has been considered to be passively driven by transpiration and limited by the vascular structure. While this still holds true for the axial movement of water through dead xylem vessels, several recent studies have suggested that radial unloading of water from the xylem to the leaf is tightly regulated by the membranes of the cells that surround the xylem. Our results support the hypothesized role of PIP AQPs in this process. Thus, Kleaf is regulated via PIP AQPs in BS cells. Moreover, this control of BS AQP activity and thereby Kleaf can explain the dynamic Kleaf response to changing ambient conditions, including stress, or via changes in the turgor of the BS (e.g. in response to light or temperature conditions; Brodribb and Holbrook, 2006; Sack and Holbrook, 2006; Shatil Cohen et al., 2011). The fact that BS hydraulic control the permeability of the mesophyll to water under initial stress conditions suggests the existence of a tight hydraulic connection between the BS and the mesophyll as well as feed-forward regulation. Further research is needed to elucidate the interaction between the BS and the mesophyll with respect to their hydraulic functions and the specific roles of stress and AQP in this interaction. In addition, it would be interesting to use the miRNA approach to target the PIP2 subfamily and the entire PIP family.

CONCLUSION

For years, the extravascular flow of water through the plant has been considered to be passively driven by transpiration and limited by the vascular structure. While this still holds true for the axial movement of water through dead xylem vessels, several recent studies have suggested that radial unloading of water from the xylem to the leaf is tightly regulated by the membranes of the cells that surround the xylem. Our results support the hypothesized role of PIP AQPs in this process. Thus, Kleaf is regulated via PIP AQPs in BS cells. Moreover, this control of BS AQP activity and thereby Kleaf can explain the dynamic Kleaf response to changing ambient conditions, including stress, or via changes in the turgor of the BS (e.g. in response to light or temperature conditions; Brodribb and Holbrook, 2006; Sack and Holbrook, 2006; Shatil Cohen et al., 2011). The fact that BS hydraulic control the permeability of the mesophyll to water under initial stress conditions suggests the existence of a tight hydraulic connection between the BS and the mesophyll as well as feed-forward regulation. Further research is needed to elucidate the interaction between the BS and the mesophyll with respect to their hydraulic functions and the specific roles of stress and AQP in this interaction. In addition, it would be interesting to use the miRNA approach to target the PIP2 subfamily and the entire PIP family.

MATERIALS AND METHODS

Growth Conditions

All plants were grown in potting mix containing 30% (w/w) vermiculite, 30% (w/w) peat, 20% (w/w) tuff, and 20% (w/w) perlite (Shacham). The plants were kept in a growth chamber under long-day conditions (14 h of light) at a controlled temperature of 20°C to 22°C with 70% humidity and light intensity of 75 to 150 μmol m⁻² s⁻¹. For Kgrow measurements, plants were grown in hydroponic solution for 21 d and transferred under extended-night conditions, prior to the measurements, as described (Postaire et al., 2010).

Plant Material

35S:mir1 Plants

The premiR-PIP1 and synthetic genes were synthesized by DNA 2.0, based on a premiR164 backbone (Alvarez et al., 2006). We used the Web-based mfold program (http://mfold.rna.albany.edu) to produce premiRNA stem-loop representations (Zuker, 2003). After sequence verification, the premiR-PIP1 was cloned behind the 35S promoter (ART7), transferred into the binary pMLBART vector (Eshed et al., 2001), and transformed to Arabidopsis (Arabidopsis thaliana) ecotype Columbia using the floral dip method (Clough and Bent, 1998).

SCR:mir1 Plants

Arabidopsis (ecotype Landsberg) plants were used in the LHRG4-1OP two-component system, in which the synthetic transcription factor LHRG4 was expressed under the control of the promoter of interest. Transactivation lines were generated by transcriptional fusion of the SCR promoter (BS-specific promoter) in front of the chimeric LHRG4, and endodermal reticulum-GFP was subcloned behind an operator array in the BJ36 vector (Moore et al., 1998). The SCR:LHRG4 line and the 10OP:endodermal reticulum-GFP line were generous gifts from Yuval Eshed, Weizmann Institute of Science [Shatil-Cohen et al., 2011]). Those lines were crossed to generate plants in which GFP was expressed specifically in the endodermis and BS cells (Shatil Cohen et al., 2011). Those plants were further crossed with 10OP:mir1 lines (Columbia background) in which mir1 was specifically expressed under the control of the SCR promoter (SCR:mir1 plants). For control plants, a similar cross was made with wild-type Columbia plants. All Arabidopsis plants were genetically transformed using the floral dip method (Clough and Bent, 1998).

Protoplast Isolation

Protoplasts were isolated from 30- to 35-d-old plants. The lower leaf epidermis was peeled off at the leaf center (when extracting the midrib BS cells, the epidermis right above those cells was peeled off), and the peeled leaves were cut into small squares and incubated in enzyme solution (3.3% [w/w] of an enzyme mix containing the following enzymes in the given proportions: 0.55 g of cellulase [ Worthington], 0.1 g of pectolyase [Karlan], 0.33 g of polyvinyl pyrrolidone K 30 [Sigma-Aldrich], and 0.33 g of bovine serum albumin [Sigma-Aldrich]) containing 10 mM KCl, 1 mM CaCl₂, 560 mM D-sorbitol, and 8 mM MES, pH 5.7. After 20 min of incubation at 28°C, the leaf tissue was transferred to the same solution, without the enzymes, and agitated at 100 rpm for 3 min or until all of the protoplasts were released into the solution. The remaining tissue pieces were removed, and the remaining solution containing the protoplasts was collected into a 5-mL tube using a pipet tip. This protoplast isolation procedure resulted in a very high yield of protoplasts (20 million protoplasts per gram of leaf tissue). For detailed description (video article) of the protoplast isolation and Pf measurement, please see Shatil-Cohen et al. (2014).

Measurements of the Protoplast Pf

Pf was measured in single protoplasts based on the initial (recorded) rate of their increase in volume in response to hypotonic challenge (transfer from a 600-mosmol isotonic bath solution to a 500-mosmol hypotonic solution). Pf was determined using a numerical approach, an offline curve-fitting procedure using the PFFIT program, as described in detail previously (Moshelion et al., 2002, 2004; Volkov et al., 2007). To identify the BS protoplasts labeled with GFP and the unlabeled mesophyll cells, we screened the protoplast population as described by Shatil-Cohen et al. (2011).

Whole-Rosette Kros

Hydrostatic Kros was measured on 21-d-old plants as described by Postaire et al. (2010) by inserting an excised rosette into a pressure chamber containing a bathing solution. A flow of liquid water was pressed across the whole rosette and was monitored as it was released at the hypocotyl section. The flow-versus-pressure relationship was used to determine Kros (Supplemental Fig. S10), which was shown previously to be independent of any stomatal limitation (Postaire et al., 2010).

Measurements of Kleaf

Kleaf was measured using detached leaves (Shatil-Cohen et al., 2011). Fully expanded leaves were harvested from 32- to 45-d-old Arabidopsis plants in the dark and immediately immersed (petiole deep) in artificial xylem sap (AXS; containing 1 mM KH₂PO₄, 1 mM K₂HPO₄, 1 mM MgSO₄, 3 mM NO₃, and 0.1 mM MnSO₄ buffered to pH 5.8 with 1 M HCl or KOH; Wilkinson et al., 1998) while leaf blades were exposed to air. All of the leaves were similar in size and had similar vascular areas with no noticeable anomalies (Supplemental Fig. S3).
E was measured using a Li-Cor 6400 gas-exchange system (LI-COR) equipped with a 2- × 3-cm² aperture standard leaf cuvette (150 μm m⁻² s⁻¹ light with 400 μmol mol⁻¹ CO₂ surrounding the leaf (Cₑ), the leaf temperature was approximately 24°C, and the vapor pressure deficit was approximately 1.3 kPa). The measuring conditions were set to be similar to the growth chamber conditions. The measurement of E was followed immediately by the determination of Ψₛ using a pressure chamber (ARIMAD-3000; MRC) and a home-made silicon adapter especially designed for Arabidopsis petioles in order to fit the O-ring of the pressure chamber. After placing the leaf in the chamber and tightly closing it, pressure (Pₑ) was gradually applied until the appearance of water at the cut was observed with the help of a binocular microscope (SZ; Olympus) under the illumination of a cool light (Intralux 5000; Volpi) directed toward the petiole. K_leaf/Dry weight leaf (Marte et al., 2002; Sack and Holbrook, 2006) was calculated for each individual leaf by dividing the whole-leaf E by the Ψₛ. In our calculations, Ψₛ was calculated as the leaf petiole was dipped in A XS at a water potential of approximately 0) For the ABA experiment, petioles were left in A XS supplemented with 10 μM ABA for 1 h before measurement.

Gas-Exchange Measurements

Gas-exchange measurements were assayed using a Li-Cor 6400 portable gas-exchange system (LI-COR). Analysis was performed on fully expanded leaves from 32- to 45-d-old plants grown under favorable conditions. All measurements were conducted between 10 a.m. and 1 p.m. Photosynthesis was induced under saturating light (1,200 μmol m⁻² s⁻¹) with 400 μmol mol⁻¹ CO₂ surrounding the leaf (Cₑ), as described by Flexas et al. (2007). In addition, light curves were measured on wild-type plants (Supplemental Fig. S11). The amount of blue light was set to 10% of the photosynthetically active photon flux density to optimize stomatal aperture. Vapor pressure deficit was approximately 1.3 kPa, and the temperature was approximately 24°C. Chlorophyll fluorescence was measured using an open gas-exchange system with an integrated fluorescence chamber head (Li-Cor 6400; LI-COR). The actual photochemical efficiency of PSII was determined by measuring steady-state fluorescence and maximum fluorescence during a light-saturating pulse of approximately 8,000 μmol m⁻² s⁻¹, as described (Genty et al., 1989).

Measurements of gₘᵢ

gₘᵢ was calculated according to Harley et al. (1992), as described by Ribas-Carbo et al. (2007), based on coupled gas exchange and chlorophyll fluorescence measurements.

Quantitative Analysis of Gene Expression by Quantitative PCR

Vascular tissue is found throughout the leaf. Therefore, in order to have a relatively high number of BS cells in our tissue samples, we excised the midvein area (Hibberd and Quick, 2002; Brown et al., 2010), which has more BS cells than mesophyll cells, and used that excised tissue for our analysis. (Pieces from the edges of the leaves had more mesophyll cells than BS cells.) Tissue samples were taken from each plant and immediately frozen in liquid nitrogen.

Total RNA was extracted using Tri-Reagent (Molecular Research Center) and treated with RNase-free DNase (Fermentas). Complementary DNA (cDNA) was prepared using the EZ-First Strand cDNA synthesis kit (Biological Industries) according to the manufacturer's instructions. Quantitative PCR was performed in the presence of SYBR Green I (Takara) in a Corbett Research Rotor-Gene 6000 cycler. The reaction was as follows: 30 s at 94°C, followed by 40 cycles consisting of 10 s at 94°C, 30 s at 60°C, and 20 s at 72°C. A standard curve was generated for each gene using dilutions of cDNA samples, and data analysis was performed using Rotor-Gene 6000 series software 1.7. PCR primers used to amplify specific regions of the genome are listed in Supplemental Table S1. The specificity of primers was determined by melting-curve analysis; a single, sharp peak in the melting curve indicates that a single, specific DNA species was amplified. Arabidopsis xylanase 2 (AK128780) was used as a reference for the standardization of cDNA amounts.

Northern-Blot Analysis

Total RNA was extracted from Arabidopsis leaves using Tri-Reagent (Molecular Research Center). Ten micrograms of total RNA was separated according to size on a 1% (v/v) denaturing agarose-formaldehyde gel and transferred to a nylon membrane by capillary blotting. The nucleic acids were UV light cross linked and prehybridized in DIG Easy Hyb (Roche). Hybridization with digoxigenin (DIG)-labeled probes and washing were carried out at 42°C (very stringently) according to the manufacturer's instructions. The DIG system includes an anti-DIG antibody that is linked to alkaline phosphatase. Target AQP mRNA was detected via this enzymatic activity. DIG-labeled probes for specific AQPs were obtained by using qRT-PCR primers to amplify the respective sequence of the AQP.

Western Blotting

About 100 mg of fresh leaf tissue was ground to homogeneity in a 1.5-ml tube with a pestle in a grinding buffer of 500 mM Suc, 10% (v/v) glycerol, 50 mM NaF, 20 mM EDTA, 20 mM EGTA, 5 mM β-glycerophosphate, 1 mM phenanthroline, 0.6% (w/v) polyvinylpyrrolidone, 10 mM ascorbic acid, 0.5 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 1 mM sodium vanadate, and 50 mM Tris-HCl, pH 8. After centrifugation (5 min at 10,000 g), the supernatant was subjected to SDS-PAGE on 12% (v/v) acrylamide gels. After SDS-PAGE, separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore) according to the manufacturer's instructions.

The blot was blocked for 1 h in a modified phosphate-buffered saline containing 0.1% (v/v) Tween 20 and 1% (w/v) bovine serum albumin (PBSTB) and then incubated at room temperature (2 h) or at 4°C (overnight) in the presence of the primary antibodies, anti-PIP1 (AHP1;1–AHP1:4) and anti-PIP2 (AHP2:1–AHP2P2:3), diluted 1:5,000 (Santoni et al., 2003). After washing (2 × 10 min) in PBSTB, the blot was incubated for 1 h with a peroxidase-labeled secondary antibody at 1:20,000 dilution in PBSTB. Secondary anti-rabbit antibodies were used to detect the anti-PIP1 and anti-PIP2 primary antibodies. After washing (2 × 10 min) in phosphate-buffered saline, the signal was observed using a chemiluminescent substrate (Super Signal; Pierce).

RLM-RACE

RACE analysis of cleaved target-gene cleavage sites in the miRNA target genes was mapped using RLM-RACE, a modified 5' RACE procedure, as described (Kasschau et al., 2003), using the GeneRacer (Invitrogen) protocol coupled with nested gene-specific primers 200 to 400 nucleotides downstream of the predicted miRNA target site. The PCR products were purified and sequenced directly.

Leaf-Clearing Procedure

Leaves were harvested, immersed in 96% (v/v) ethanol, and incubated at 50°C for 3 h. The ethanol was replaced with fresh ethanol three times and then replaced with lactic acid for an overnight incubation.

Calcofluor White Staining and Imaging

Leaves were cut and immediately immersed (petiole deep) in concentrated (1 g L⁻¹) Calcofluor White, an apoplastic fluorescent marker that stains the cellulosic cell wall. Twenty-four hours after the staining, fluorescence was imaged by epifluorescence inverted microscopy (Olympus-IX8 Cell-R; 380-nm excitation, 475-nm emission) to observe the distribution of the dye in the leaf.

Imaging Leaf Veins and Calculating Their Areas

Following the clearing procedure, leaves were washed with water and put on microscope slides. Images were taken using NIS-Elements software and a Leica MZFLIII stereomicroscope on which a Nikon DS-Fil digital camera was mounted. Images were later analyzed to determine leaf vein area using ImageJ software (http://rsb.info.nih.gov/ij/). A fixed ellipse (6,000-μm length, 3,000-μm width) was used in all images, and the area of the veins was determined and calculated relative to the total (ellipse) leaf area.

Supplemental Data

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

Supplemental Figure S1. Northern-blot and RLM-RACE analyses of AHP1PI AQP in 35S:mir1 plants as compared with the wild type.

Supplemental Figure S2. The quantities of PIP1 and PIP2 proteins were lower in the 35S:mir1 plants.

Supplemental Figure S3. Comparison of the leaf morphology of the different genotypes.

Supplemental Figure S4. E and \( \Psi_{\text{soil}} \) of intact leaves from soil-grown, BS-silenced AtPIP1 plants.

Supplemental Figure S5. Plant morphological characteristics and relative expression profiles of ABA biosynthetic gene transcript levels (9-cis-epoxycarotenoid dioxygenase) in leaves of the SCrmir1 and control plants.

Supplemental Figure S6. Twenty-one base pairs of the consensus sequence alignment of amiRNApip1 and some possible targets from the AtPIP2 subfamily.

Supplemental Figure S7. E and \( \Psi_{\text{soil}} \) of intact leaves from the AQP-modified lines.

Supplemental Figure S8. Relative expression profile of the miRNA (mir164) in the margins (mesophyll-enriched tissue) and midveins of SCrmir1 leaves.

Supplemental Figure S9. Relative expression profile of the PIP genes in the margins (mesophyll-enriched tissue) of SCrmir1 leaves.

Supplemental Figure S10. Representative pressure-to-flow relationship measured in a rosette from an approximately 20-d-old 35S:mir1-3 plant exposed to prolonged night (11–21 h of darkness).

Supplemental Figure S11. Relationship between net photosynthesis, photosynthesis, and incident light intensity, photon flux density.

Supplemental Table S1. Primers used for q-RT-PCR gene expression analyses.

LITERATURE CITED


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