# Plasma Membrane Aquaporins Play a Significant Role during Recovery from Water Deficit[1]

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The role of plasma membrane aquaporins (PIPs) in water relations of Arabidopsis was studied by examining plants with reduced expression of PIP1 and PIP2 aquaporins, produced by crossing two different antisense lines. Compared with controls, the double antisense (dAS) plants had reduced amounts of PIP1 and PIP2 aquaporins, and the osmotic hydraulic conductivity of isolated root and leaf protoplasts was reduced 5- to 30-fold. The dAS plants had a 3-fold decrease in the root hydraulic conductivity expressed on a root dry mass basis, but a compensating 2.5-fold increase in the root to leaf dry mass ratio. The leaf hydraulic conductance expressed on a leaf area basis was similar for the dAS compared with the control plants. As a result, the hydraulic conductance of the whole plant was unchanged. Under sufficient and under water-deficient conditions, stomatal conductance, transpiration rate, plant hydraulic conductance, leaf water potential, osmotic pressure, and turgor pressure were similar for the dAS compared with the control plants. However, after 4 d of rewatering following 8 d of drying, the control plants recovered their hydraulic conductance and their transpiration rates faster than the dAS plants. Moreover, after rewatering, the leaf water potential was significantly higher for the control than for the dAS plants. From these results, we conclude that the PIPs play an important role in the recovery of Arabidopsis from the water-deficient condition.

Water transport through cellular membranes is facilitated by aquaporins, proteins that form water-selective channels. The presence of aquaporins in a membrane can increase the osmotic hydraulic conductivity of the membrane ($L_P$, meters per second per megapascal) by 10- to 20-fold (Preston et al., 1992). In plants, the physiological importance of aquaporins is currently mainly inferred from their widespread occurrence (Johansson et al., 2000) and the use of $HgCl_2$, a nonspecific inhibitor (Tyerman et al., 2002). Aquaporins, which are found in almost all types of tissues (Maurel, 1997), have changed the way we think about plant water relations (Maurel and Chrispeels, 2001).

Water movement through a living organ such as a root or a leaf can take an apoplastic route, which has a low resistance to flow, or a transcellular route, which has a higher resistance because water has to move through lipid bilayer membranes (Steudle, 2000). Bulk water flow associated with the transpiration stream is mostly apoplastic, except in the root exo- and endodermis (Zimmermann et al., 2000) and in the leaf bundle sheath (Koroleva et al., 2002), where apoplastic barriers (Casparian band, suberin lamellae, and secondary cell wall thickening) restrict the apoplastic path. Other important processes such as cell enlargement, refilling of embolized vessels, and movement of guard cells and pulvini may require rapid transport of water across membranes. Furthermore, the considerable growth-associated water potential difference (0.1–0.3 MPa) found in most growing organs of herbaceous plants (e.g. Nonami and Boyer, 1993; Fricke et al., 1997; Martre et al., 1999) suggests that the rate of water transport limits cell expansion. Thus, the rate of water transport along the transcellular pathway may be controlled by changing the abundance and/or the activity of aquaporins in the membranes through which this water flows and thus may influence several important processes, such as movement of guard cells or cell expansion.

Experiments in which the osmotic hydraulic conductivity ($L_P$) of isolated plasma membrane and tonoplast vesicles (Maurel et al., 1997; Niemietz and Tyerman, 1997) or isolated vacuoles and protoplasts (Morillon and Lassalles, 1999; Ramahaleo et al., 1999)

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was measured showed that most of the time, the tonoplast is more permeable to water than the plasma membrane. Moreover, $L_P$ values of isolated protoplasts have a much broader range (typically from 1 to 400 $\times$ $10^{-8}$ m $s^{-1}$ $MPa^{-1}$) than the values for isolated vacuoles (150 to 700 $\times$ $10^{-8}$ m $s^{-1}$ $MPa^{-1}$), indicating that the control of transcellular water movement may reside in the plasma membrane.

In Arabidopsis, the aquaporin family has at least 35 members (Johanson et al., 2001) and some of these proteins may also transport small neutral solutes such as glycerol or urea (Biela et al., 1999; Gerbeau et al., 1999; Weig and Jakob, 2000). In a cladogram, four major subfamilies of aquaporins can be identified, and the subfamily of the plasma membrane aquaporins (PIPs) is divided into two groups, PIP1 and PIP2. For Arabidopsis, the PIP1 group has five members (PIP1;1–PIP1;5), and the PIP2 group has eight members (PIP2;1–PIP2;8; Chaumont et al., 2000a, 2001; Johanson et al., 2001).

Inhibition of aquaporin water transport by sulfhydryl reagents, such as $HgCl_2$, and subsequent use of 2-mercaptoethanol to reverse this inhibition has permitted measurement of the proportion of water transported by mercury-sensitive aquaporins in a whole-root system under water-sufficient (Maggio and Joly, 1995; Wan and Zwiazek, 1999; Barrowclough et al., 2000) and water-deficient (North and Nobel, 2000; Martre et al., 2001b) soil conditions. Such studies indicate that aquaporins can account for 35% to 80% of root hydraulic conductance under wet conditions, and for 60% to 80% of root hydraulic conductance in drying or rewetted soil. However, because $HgCl_2$ rapidly depolarizes the plasma membrane of cells and may have other effects in addition to the direct inhibition of aquaporin activity (Zhang and Tyerman, 1999), such experimental data need to be confirmed by other approaches.

Another way to find out whether aquaporins play a role in water transport in the plant is to down- or up-regulate the expression of aquaporin genes. Plants of Arabidopsis (Kaldenhoff et al., 1998) and tobacco (*Nicotiana tabacum*; Siefritz et al., 2002) with down-regulated PIP1 aquaporins were shown to have a reduced osmotic water permeability for leaf and root protoplasts, respectively, compared with wild-type (WT) plants. Moreover, PIP1 antisense plants of Arabidopsis had a root to leaf dry mass ratio 5-fold higher than WT plants (Kaldenhoff et al., 1998). In the reverse approach, overexpression of a PIP2 cDNA of Arabidopsis in tobacco resulted in a reduced $t_{1/2}$ for water exchange through the plasma membrane, suggesting a higher hydraulic conductance of the plasma membrane for the transformed plants compared with the WT (Shukla and Chrispeels, 1998).

To assess in planta the function of PIP aquaporins at the cellular and whole-plant level, mutant lines of Arabidopsis that express a PIP2 cDNA (PIP2;3) in the antisense orientation were constructed, and one line was then crossed with the already described PIP1;2 antisense line, as23 (Kaldenhoff et al., 1998; this line hereafter termed PIP1AS), to obtain a line that expressed both PIP1 and PIP2 in the antisense orientation (double antisense [dAS] plants).

We found that under water-sufficient conditions, the antisense plants compensated for the lower hydraulic conductance of the roots by investing more carbon in root production, and that during recovery from water deficit, the recovery was impaired in the dAS plants. This points to a significant role for aquaporins during recovery from water deficit.

## RESULTS

### Characterization of the dAS Lines

The dAS lines were obtained by crossing an antisense PIP1 C24 line (Kaldenhoff et al., 1998) with an antisense PIP2 Columbia line produced in our laboratory. The control for all these experiments was a Columbia $\times$ C24 cross, hereafter referred to as double WT (dWT). Abundance of PIP1 and PIP2 proteins

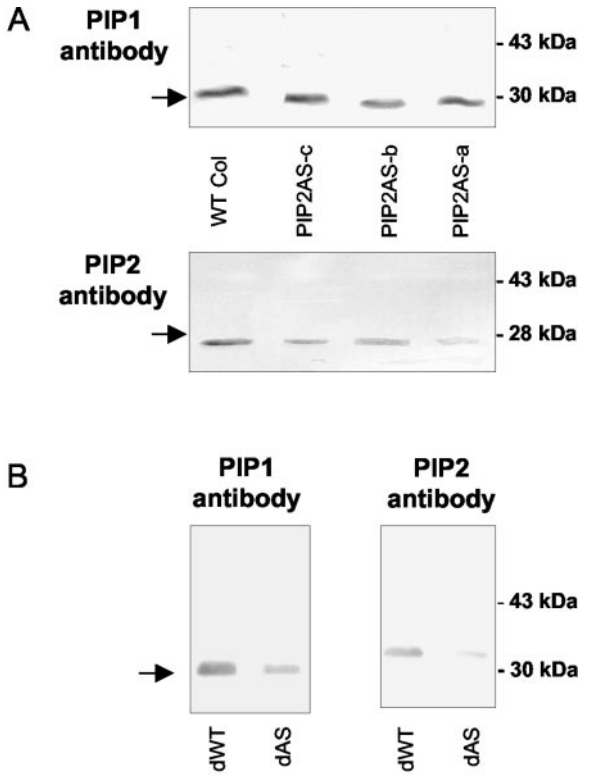


**Figure 1.** Immunoblot analysis of microsomal extracts for leaves of WT and AS lines of Arabidopsis using anti-PIP1 and anti-PIP2 antibodies for WT (WT Col) and PIP2 antisense (PIP2AS-a, PIP2AS-b, and PIP2AS-c) lines (A) and for double WT (dWT) and antisense (dAS) lines (B). Microsomal extracts were obtained from 4-week-old plants grown in soil under well-watered conditions. Fifty micrograms of microsomal protein were loaded in each lane. Arrows indicate the position of the monomer, and the numbers on the right indicate the $M_r$ standards.

was measured with specific antisera using immunoblots (Fig. 1). In the PIP2 antisense the abundance of PIP2 was significantly decreased for the three lines examined compared with its control (WT Col), but the abundance of PIP1 was not affected (Fig. 1A). In the dAS line, the abundance of both groups of aquaporins was much lower than in its control line (dWT; Fig. 1B).

### Morphological Traits

Values for the number of leaves, median leaf area, total leaf area per plant, leaf and root dry mass, and root to leaf dry mass ratio are given in Table I. The number of leaves per plant and the leaf areas per plant were not significantly different for the dWT and dAS plants; however, the number of leaf per plant tended to by higher for the dWT than for the dAS, whereas the leaf area per plant tended to by higher for the dAS than for the dWT, which resulted in a higher (65%) median leaf area for the dAS than for the dWT. The leaf dry mass per plant was similar for the simple and double as when compared with their respective controls. The root dry mass, on the other hand, was 1.8 times greater for the simple antisense plants compared with their respective controls, although not significantly for PIP1AS, and 2.7 time greater for the dAS compared with the dWT. Thus, down-regulation of plasma membrane aquaporins increased the root to leaf dry mass ratio by 43%, 108%, and 154% for PIP1AS, PIP2AS-a, and dAS, respectively, as compared with their respective controls.

### Osmotic Hydraulic Conductivity of Leaf and Root Protoplasts

To find out whether the observed decrease in aquaporin abundance was correlated with a change in $L_p$ of isolated protoplasts, we measured $L_p$ values on root and leaf protoplasts. An example of the results obtained is shown in the histograms of Figure 2. In these histograms, protoplasts in specific $L_p$ intervals are grouped. The data show that in WT Col plants, most of the root protoplasts had $L_p$ values greater than $64 \times 10^{-8}$ m s$^{-1}$ MPa$^{-1}$ (Fig. 2A), whereas in PIP2AS-a plants all the $L_p$ values were smaller than $32 \times 10^{-8}$ m s$^{-1}$ MPa$^{-1}$ (Fig. 2C). Similar but slightly different results were obtained for leaf protoplasts (Fig. 2, B and D).

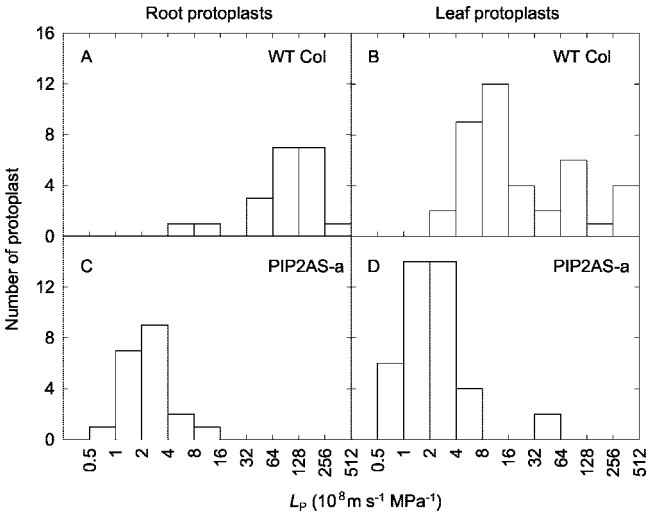


**Figure 2.** Frequency distribution of $L_P$ for root and leaf protoplasts of WT Col and PIP2AS-a lines of Arabidopsis. Root protoplasts were obtained from 15-d-old plants grown in vermiculite and leaf protoplasts from 4-week-old plants grown in soil ($n$ = 20 root protoplasts from three plants and 40 leaf protoplasts from four to six plants).

Mean $L_P$ values for these plants and for all the other lines are shown in Table II. The most striking result here is the low $L_P$ for all the AS lines, whether single or double, compared with their respective controls. This result is surprising because it appears as if the group of aquaporins that is not down-regulated (whether PIP1 or PIP2) is inactive when the other group is down-regulated. $L_P$ values below 10 suggest inactive aquaporins. Experiments where $L_P$ and the Arrhenius energy of activation ($E_a$, kilojoules per mole) were measured indicate that $L_P$ values for plant plasma membrane of 4 to 11 $\times 10^{-8}$ m s$^{-1}$ MPa$^{-1}$ correspond to values of $E_a$ of 50 to 70 kJ mol$^{-1}$ (Maurel et al., 1997; Niemietz and Tyerman, 1997; Ramahaleo et al., 1999). Such high values of $E_a$ correspond to situations where water moves through

**Table I.** *Leaf number, median leaf area, leaf area per plant ($A_L$), leaf ($M_L$) and root ($M_R$) dry mass per plant, and root to leaf dry mass ratio ($M_R$:$M_L$) for simple WT (WT C24 and WT Col), and PIP1 (PIP1AS) and PIP2 (PIP2AS-a) antisense, and dWT and dAS lines of Arabidopsis grown in soil under well-watered conditions*

Plants were 5 weeks old. Data are means ± 1 SE ($n$ = 10 for WT C24 and PIP1AS, and 6 for WT Col, PIP2AS-a, dWT, and dAS). Different letters within a row indicate a statistically significant difference ($P < 0.05$) using one-way ANOVA followed by a Tukey's test.

| Morphological Traits | Lines | | | | | |
|---|---|---|---|---|---|---|
| | WT C24 | PIP1AS | WT Col | PIP2AS-a | dWT | dAS |
| Leaves (no. per plant) | 28.9 ± 2.5c | 37.7 ± 4.1b | 52.3 ± 4.5a | 46.0 ± 4.0ab | 25.3 ± 1.7c | 23.5 ± 2.8c |
| Median leaf area (cm$^2$) | 0.77 ± 0.10b | 0.48 ± 0.09a | 0.34 ± 0.05a | 0.41 ± 0.08ab | 0.73 ± 0.17ab | 1.20 ± 0.19c |
| $A_L$ (cm$^2$) | 22.8 ± 0.9c | 25.3 ± 1.4c | 47.7 ± 1.6a | 45.3 ± 2.5ab | 39.1 ± 0.6b | 41.3 ± 2.2ab |
| $M_L$ (mg) | 33.2 ± 1.8b | 39.5 ± 2.9b | 112.6 ± 8.1a | 101.8 ± 5.7a | 61.8 ± 2.3c | 66.1 ± 3.4c |
| $M_R$ (mg) | 4.7 ± 0.8d | 8.0 ± 1.3bd | 13.5 ± 1.4ab | 25.9 ± 4.4c | 8.2 ± 1.0bd | 21.8 ± 1.1ac |
| $M_R$:$M_L$ | 0.14 ± 0.02a | 0.20 ± 0.02ab | 0.12 ± 0.01a | 0.25 ± 0.03bc | 0.13 ± 0.01a | 0.33 ± 0.02c |

**Table II.** *$L_P$ for leaf and root protoplasts for simple WT (WT C24 and WT Col), and PIP1 (PIP1AS) and PIP2 (PIP2AS-a, PIP2AS-b, and PIP2AS-c) antisense, and double WT (dWT) and antisense (dAS) lines of Arabidopsis grown under well-watered conditions*

Root protoplasts were obtained from 15-d-old plants grown in vermiculite and leaf protoplasts from 4-week-old plants grown in soil. Data are means ± SE ($n$ = 20 root protoplasts from three plants and 40 leaf protoplasts from four or six plants). Different letters within a row indicate a statistically significant difference ($P < 0.05$) using one-way ANOVA followed by a Dunn's test.

| $L_P$ ($10^8$ m $s^{-1}$ $MPa^{-1}$) | Lines | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | WT C24 | PIP1AS | WT Col | PIP2AS-a | PIP2AS-b | PIP2AS-c | dWT | dAS |
| Leaves | 49 ± 10a | 10 ± 3b | 60 ± 16a | 5 ± 2b | 5 ± 1b | 5 ± 2b | 46 ± 11a | 10 ± 3b |
| Roots | 106 ± 13a | 6 ± 3b | 117 ± 17a | 4 ± 1b | – | – | – | – |

the plasma membrane by diffusion and thus where there are no active water channels (Haines, 1994).

### Hydraulic Conductances

There was no effect of PIP aquaporin down-regulation on either whole-plant ($K^{A_L}_{plant}$) or leaf ($K^{A_L}_{leaf}$) hydraulic conductance (Table III). However, the root hydraulic conductance, based on the root dry mass per plant ($K^{M_R}_{root}$), was reduced by 60%, 47%, and 68% for PIP1AS, PIP2AS-a, and dAS compared with their respective controls, respectively. With the exception of PIP1AS, the higher root to leaf dry mass ratio fully compensated for the lower $K^{M_R}_{root}$ in AS plants, and the root hydraulic conductance based on the leaf area per plant ($K^{A_L}_{root}$) was normal in PIP2AS-a and dAS. The decrease in $K^{M_R}_{root}$ in the single as plants compared with there respective controls is correlated with the decrease in $L_P$ for root protoplasts, but this is not seen in the leaves where $K^{A_L}_{leaf}$ was not affected by the down-regulation of PIP aquaporins.

### Response of the dWT and dAS Plants to Soil Drying and Rewatering

To understand the effect of aquaporin down-regulation on water relations under conditions of water deficit and recovery, we examined a series of plants during 8 d when water was withheld and then after rewatering for another 4-d period. We measured the following parameters: soil water potential ($\Psi_{soil}$), stomatal conductance ($g_s$), leaf transpiration rate ($E$), $K^{A_L}_{plant}$, leaf water potential ($\Psi_{leaf}$), leaf osmotic pressure, and turgor pressure. Under all soil moisture conditions, $\Psi_{soil}$ was similar ($P = 0.74$) for the dWT and dAS plants, and averaged $-0.04 \pm 0.01$ MPa. $\Psi_{soil}$ decreased slowly during the first 6 d of soil drying, reaching $-0.5 \pm 0.0$ MPa after 6 d of soil drying; it then decreased much faster and reached $-2.8 \pm 0.5$ MPa at 8 d of soil drying. Figure 3 shows the diurnal pattern of $g_s$ for the dWT and dAS plants during the gradual drying out period (Fig. 3A) and after rewatering (Fig. 3B). Under well-watered conditions, $g_s$ increased from a low value in the morning and peaked at 4 PM, to decrease again thereafter. As a result of soil drying, $g_s$ gradually decreased as the soil dried out, but there was no significant difference between the dWT and dAS plants. After 4 d of rewatering, $g_s$ at midday had recovered to 84% and 59% of its initial value for the dWT and dAS, respectively, but these values were not significantly different.

Other aspects of the physiology of these plants during the same period are shown in Figure 4. Under wet conditions, $\Psi_{leaf}$ was similar for the dWT and dAS plants (Fig. 4A). $\Psi_{leaf}$ for the dWT and dAS did not decrease significantly during the first 6 d of soil drying, but decreased by 60% and 71% at 8 d of soil drying for the dWT and dAS, respectively, and was significantly more negative for the dAS than for the dWT at 4, 6, and 8 d of soil drying. Rewatering for 0.5 d caused $\Psi_{leaf}$ to increase to 86% and 55% of its initial value under wet conditions for the dWT and dAS, respectively, with no further increase 4 d after rewatering. Similar results were observed for the leaf turgor pressure (Fig. 4B). The converse was observed for changes in osmotic pressure (Fig. 4B). Under wet conditions, the osmotic pressure for the dWT and dAS was similar and did not change during the first 6 d of soil drying, but increased by 48% and 87% at 8 d of soil drying for the dWT and dAS, respectively, and was then 23% lower for the dWT than for the

**Table III.** *$K^{A_L}_{plant}$, $K^{A_L}_{leaf}$, $K^{A_L}_{root}$, and $K^{M_R}_{root}$ for simple WT (WT C24 and WT Col), and PIP1 (PIP1AS) and PIP2 (PIP2AS-a) antisense, and dWT and dAS lines of Arabidopsis grown in soil under well-watered conditions*

Plants were 5 weeks old. Data are means ± 1 SE ($n$ = 10 for WT C24 and PIP1AS, and 6 for WT Col, PIP2AS-a, dWT, and dAS). Different letters within a row indicate a statistically significant difference ($P < 0.05$) using one-way ANOVA followed by a Tukey's test.

| Hydraulic Conductances | Lines | | | | | |
|---|---|---|---|---|---|---|
| | WT C24 | PIP1AS | WT Col | PIP2AS-a | dWT | dAS |
| $K^{A_L}_{plant}$ (mmol $s^{-1}$ $m^{-2}$ $MPa^{-1}$) | 6.6 ± 0.4b | 6.1 ± 0.4b | 4.4 ± 0.5a | 4.7 ± 0.3a | 6.5 ± 0.5b | 5.6 ± 0.5ab |
| $K^{A_L}_{leaf}$ (mmol $s^{-1}$ $m^{-2}$ $MPa^{-1}$) | 13.4 ± 2.1abc | 15.8 ± 1.6c | 9.6 ± 1.3a | 9.9 ± 0.7ab | 14.1 ± 1.6bc | 12.1 ± 0.8abc |
| $K^{A_L}_{root}$ (mmol $s^{-1}$ $m^{-2}$ $MPa^{-1}$) | 16.6 ± 1.9c | 10.8 ± 1.0ab | 8.9 ± 0.9a | 9.4 ± 0.9ab | 13.0 ± 1.5bc | 11.2 ± 1.7ab |
| $K^{M_R}_{root}$ (mmol $s^{-1}$ $g^{-1}$ $MPa^{-1}$) | 9.3 ± 1.0a | 3.7 ± 0.6b | 3.6 ± 0.6b | 1.9 ± 0.3c | 6.6 ± 1.1a | 2.1 ± 0.3cb |

dAS. Rewatering for 0.5 d caused the osmotic pressure to decrease to 111% and 135% of its initial value for the dWT and dAS, respectively, with no further decrease 4 d after rewatering.

During the 8 d that water was withheld, $E$, integrated over 24 h (Fig. 4C), and $K^{A_L}_{plant}$ (Fig. 4D) decreased gradually and faster for the dWT than for the dAS plants; and after 8 d of soil drying, $E$ and $K^{A_L}_{plant}$ were 1.5-times higher for the dWT than for the dAS plants. After rewatering for 1 d, $E$ increased to 55% of its initial value under wet conditions for both the dWT and dAS; but after rewatering for 4 d, $E$ for the dWT increased further to 72% of its initial value, whereas for the dAS, $E$ did not increase further after 4 d of rewatering. $K^{A_L}_{plant}$ for the dWT and the dAS had a similar pattern of recovery to that of the integrated 24-h $E$ (Fig. 4D), and rewatering for 4 d caused $K^{A_L}_{plant}$ to increase to 60% of its initial value under wet soil conditions for the dWT but to only

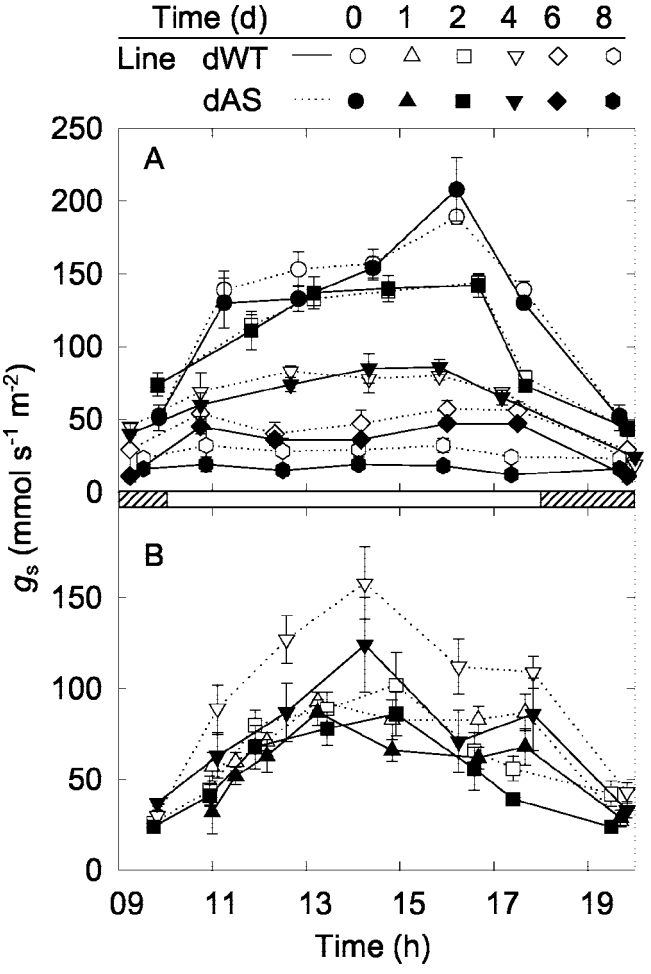


**Figure 3.** Diurnal variations of $g_s$ for plants of the dWT and dAS lines of Arabidopsis in wet soil and during soil drying (A) and after soil rewatering (B). Water was withheld for 8 d when the plants were 5 weeks old. The soil was then rewetted by immersing one-half of the height of the pots in the nutrient solution from 10 to 12 AM, and $\Psi_{soil}$ was then maintained above −0.1 MPa. Data are means ±1 SE for $n$ = 5 plants for each line.

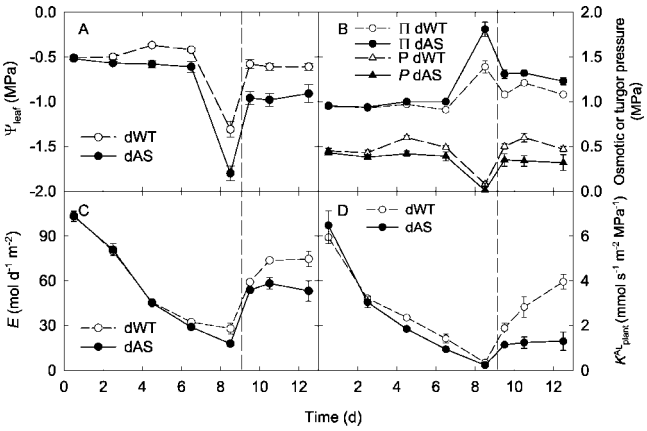


**Figure 4.** $\Psi_{leaf}$ (A), osmotic and turgor pressure (B), integrated 24-h transpiration rate ($E$; C), and $K^{A_L}_{plant}$ (D) for plants of the dWT and dAS lines of Arabidopsis in wet soil, during soil drying, and after soil rewatering. $\Psi_{leaf}$, leaf osmotic and turgor pressure, and $K_{plant}$ were determined between 1 and 2 PM. Soil drying and rewatering procedure was as for Figure 3. Data are means ± 1 SE for $n$ = 5 plants for each line.

20% for dAS. After rewatering for 4 d, plants of the dWT and dAS lines had produced 2.5 ± 0.2 ($n$ = 8 plants) and 0.4 ± 0.4 ($n$ = 5 plants) new leaves per plant, respectively ($P < 0.001$). Together, these data show a greater water stress for the dAS than for the dWT plants during soil drying, and they show that the dAS plants recovered more slowly after rewatering and made significantly fewer leaves.

## DISCUSSION

The experiments reported here were designed to provide genetic evidence for a role of aquaporins in the water relations of plants. At the moment, the evidence linking aquaporins to plant water relations is still quite scanty and based largely on the inhibition of transmembrane water transport by $HgCl_2$, a nonspecific inhibitor. We generated plants in which two groups of PIPs were down-regulated by crossing an already established PIP1AS line with a new PIP2AS line. Because the two lines were made in different Arabidopsis ecotypes, comparisons were conducted with a cross of the WT plants (dWT). The results show (a) that down-regulation of PIP aquaporins and $L_P$ of root cortical protoplasts affects root hydraulic conductance, however the down-regulation of PIP aquaporins and $L_P$ of leaf mesophyll protoplasts does not affect $K^{A_L}_{leaf}$; (b) that plants compensate for the impairment of root hydraulic conductance by investing more energy in root growth; and (c) that plants with a low level of PIP aquaporins are at a disadvantage during rewatering and rehydration, when gas exchange and metabolism resume.

### Expression and Activity of Aquaporins

To determine whether the antisense plants had the expected phenotype with respect to the down-

regulation of aquaporin abundance and activity, we measured abundance with immunoblots and activity by determining the $L_P$ of leaf and root protoplasts. The two sera used here recognize some PIPs, but probably not all. The PIP2 antiserum made against a C-terminal peptide probably recognizes PIP2;1, PIP2;2, and PIP2;3 because of the sequence identity of their C termini, but not PIP2;4 through PIP2;8. Among the PIP2 genes, PIP2;1 is the most highly expressed, and except for PIP2;7, PIP2;4 through PIP2;8 are poorly expressed (R. Jung, personal communication). Similarly, the antibody made against the N terminus of PIP1;1 can be expected to recognize PIP1;2 and PIP1;3 and possibly PIP1;4 and PIP1;5. However, these last two are much more poorly represented in the expressed sequence tag databases (R. Jung, personal communication). Thus, we feel that the two antisera used recognized the major PIP aquaporins likely to be present in the cells.

Our results show that down-regulation of PIP2 did not affect the abundance of PIP1. In the dAS plants, both types of aquaporins were down-regulated. The results with the dAS plants are in agreement with those obtained by Kaldenhoff et al. (1998). A surprising result was that the $L_P$ of PIP1AS, PIP2AS-a, and dAS plants was always in the 5 to 15 $\times$ $10^{-8}$ m $s^{-1}$ $MPa^{-1}$ range. The results are surprising because they mean that, in the PIP1AS plants, the PIP2 aquaporins are inactive and conversely that in PIP2AS-a plants, the PIP1 aquaporins are inactive. PIP1 aquaporins are usually inactive in the *Xenopus* oocyte swelling assay; however, they can be activated by very small amounts of PIP2 aquaporins (Chaumont et al., 2000b), suggesting that these two classes of aquaporins may function cooperatively.

### Water Relations under Wet, Drying, and Rewatering Soil Conditions

$K^{M_R}{}_{root}$, which defines the intrinsic efficiency of the root hydraulic pathway, was similarly reduced by about 50% for the simple and dAS plants compared with their respective controls. This means that WT plants invest less carbon to provide adequate water transport pathways than do AS plants, with no additional effect for the dAS compared with the simple antisense. This reduction in $K^{M_R}{}_{root}$ is consistent with recent results obtained with PIP1 antisense plants of tobacco where the $K^{A_L}{}_{root}$ was reduced by 42% (Siefritz et al., 2002); it is also consistent with a 35% to 80% reduction of root hydraulic conductance for several species after treatment with $HgCl_2$ (Wan and Zwiazek, 1999; Barrowclough et al., 2000; Martre et al., 2001b).

The lower $K^{M_R}{}_{root}$ for antisense plants of Arabidopsis was fully compensated by a higher root to leaf dry mass ratio for dAS compared with dWT plants, so AS plants maintained homeostasis of the efficiency of the root system to supply leaves with water, as determined by $K^{A_L}{}_{root}$ (Tyree et al., 1998). Such scaling of the root to leaf dry mass or area ratio to the intrinsic hydraulic conductance of the root system has also been reported for WT plants of tall fescue (*Festuca arundinacea*), where $K_{root}$ is correlated to the leaf area per plant but not to the root dry mass per plant (Martre et al., 2001a). However, it was surprising that the higher root to leaf dry mass ratio for antisense plants of Arabidopsis was solely attributable to a higher root dry mass, because this means that the overall mass of antisense plants was higher than that of WT plants.

The reduction of $L_P$ for leaf mesophyll protoplasts was not correlated with a reduction of $K^{A_L}{}_{leaf}$, which may reflect the essentially apoplastic nature of the transpiration stream in leaves. Large differences in water oxygen and hydrogen isotopic composition were observed in leaf of cotton (*Gossypium hirsutum*), suggesting that only the water residing in the cell walls and the intercellular spaces interacts directly with the external environment and that the large symplastic pool of water responds to the external environment to a limited extent via its relatively slow exchange with water in the transpiration pool (Yakir et al., 1990). This may explain why decreasing the level of aquaporins in the leaf had no effect on the hydraulic conductance of the transpiration route in leaves of Arabidopsis. The activity and level of expression of PIPs in leaves of Arabidopsis appeared to be negatively correlated with the transpiration rate (Morillon and Chrispeels, 2001). The reduced activity of aquaporins under high transpiration conditions may help to isolate the symplast from the transpiration stream and may thereby help stabilize the water status of the mesophyll cells during the day.

In contrast with leaves, the decrease of $K^{M_R}{}_{root}$ was correlated with a decrease of $L_P$ for root protoplasts, which indicates that a significant part of the water movement through roots used the cell-to-cell pathway. Similar results for roots of PIP1 antisense plants of tobacco have been recently reported (Siefritz et al., 2002). The difference of water pathways between leaves and roots for Arabidopsis may be related to the need to control root uptake of nutrient ions transported axially by mass flow in the xylem, such as $NO_3^-$ or $Ca^{2+}$; whereas in leaves, the process of evaporation permits discrimination of what is actually lost out of the leaves.

Under wet conditions, values of $E$, $g_s$, and $\Psi_{leaf}$ agreed well with values previously reported for Arabidopsis (Assmann et al., 2000; Xing and Rajashekar, 2001). $E$ and $g_s$ for the dWT and dAS plants were similar, and because $K^{A_L}{}_{plant}$ was also similar for the dWT and dAS plants, $\Psi_{leaf}$ for the dAS plants was not modified by the reduced level of PIPs. This is consistent with measurements of root water uptake (using a potometer) and stem xylem pressure (using the xylem pressure probe) for PIP1AS and WT C24 plants of Arabidopsis (Kaldenhoff et al., 1998), and

with $E$, $\Psi_{leaf}$, and stem water potential ($\Psi_{stem}$) measurements for PIP1 antisense and WT plants of tobacco (Siefritz et al., 2002).

During soil drying, $K^{A_L}{}_{plant}$, $E$, and $\Psi_{leaf}$ decreased faster for the dAS than for the dWT plants, and the opposite was true for the osmotic pressure, all indicating greater water stress for the dAS than for the dWT. The expression of some aquaporins is induced under conditions of moderate water stress (e.g. Yamada et al., 1997; Kirch et al., 2000), whereas the expression of other aquaporins is down-regulated under mild drought conditions (Sarda et al., 1999; Smart et al., 2001). In leaves of spinach (*Spinacia oleracea*), the PIP2 aquaporin PM28A is dephosphorylated and possibly inactivated under conditions of low apoplastic water potential (Johansson et al., 1996, 1998), whereas recent results have demonstrated a positive (Quintero et al., 1999; Hose et al., 2000; Morillon and Chrispeels, 2001) and direct (Siefritz et al., 2001) effect of abscisic acid on aquaporin activity for several species. During soil drying, the deposition of suberin lamella in the root exo- and endodermis (North and Nobel, 1996, 2000) and in the leaf bundle sheaths (Canny, 1990) may increase the contribution of the cell-to-cell pathway to the overall water flow. Thus, the presence of functional aquaporin under conditions of mild water shortage may facilitate water uptake as indicated in the present study by the delay of the decrease of $E$, $K^{A_L}{}_{plant}$, and $\Psi_{leaf}$ for the dWT as compared to the dAS, at least during the first days of soil drying.

At 4 d of rewatering following 8 d of soil drying, $K^{A_L}{}_{plant}$ for the dWT and dAS plants increased to 70% and 52% of its initial value, respectively. Following soil rewatering $E$ and $\Psi_{leaf}$ similarly recovered more completely for the dWT than for the dAS. This is in good agreement with the recovery of $HgCl_2$ sensitivity of root hydraulic conductance for *Opuntia acanthocarpa* after soil rewatering (Martre et al., 2001b). This means that the dWT plants can maintain less negative $\Psi_{leaf}$ than the dAS plants at any given $E$. The greater recovery in $K^{A_L}{}_{plant}$ at 4 d of rewetting for the dWT may be attributable in part to the growth of new roots (with full aquaporin activity), whereas 4 d may not have been sufficient time for the dAS to produce enough new roots to compensate for the lack of aquaporin activity. The incomplete recovery of $K^{A_L}{}_{plant}$ for both the dWT and dAS plants after soil rewatering may be attributable to suberization and lignification of the cell walls of the root exo- and endodermis and of the leaf bundle sheath in response to soil drying and to irreversible damage to root cortical and leaf mesophyll cells (North and Nobel, 1996). The slower and less complete recovery of water relations for the dAS plants than for the dWT plants is associated with a much lower production of new leaves at 4 d of rewatering. These results demonstrate the importance of aquaporins for quick recovery of gas exchange and growth after a drought period. This may be crucial in the field, where plants face frequent water shortages.

In conclusion, the antisense mutant Arabidopsis plants afforded us new insights into plant water relations. We corroborated the compensation of root growth that accompanies the inhibition of root hydraulic conductance to maintain the water supply to the shoot as observed by Kaldenhoff et al. (1998). We showed that aquaporins have a significant role during recovery from water shortage: dAS plants had a slower recovery and made significantly fewer leaves after rewatering.

## MATERIALS AND METHODS

### Plant Material and Culture Conditions

Seeds of the as23 PIP1 antisense line (line termed PIP1AS) of Arabidopsis ecotype C24 were kindly provided by R. Kaldenhoff (Julius-von-Sachs-Institut für Biowissenshaften, Universität Würzburg, Germany). Details of the construction of PIP2 antisense lines of Arabidopsis ecotype Columbia-0 (Col) are described below. A dAS line was obtained by crossing PIP1AS pollen with the PIP2AS-a antisense line, and a dWT line was obtained by the cross of WT C24 pollen with WT Col line. Upon request to M.J. Chrispeels, seeds of the PIP2AS-a and the dAS lines will be made available in a timely manner for noncommercial research purposes.

Seeds of the different lines were vernalized at 4°C for 4 d in Eppendorf tubes in 0.12% (w/w) agarose and were then sown in 100-$cm^3$ plastic pots filled with a 1:4 mixture of vermiculite:*Sphagnum* peat moss (Sunshine SV Mix, Sun Gro Horticulture Inc., Bellevue, WA). Pots were then placed in a walk-in growth chamber (PGV-36, Conviron, Pembina, ND). Conditions in the growth chamber were the same throughout the experiments. The photosynthetic photon flux density (PPFD) was 200 $\mu$mol $m^{-2}$ $s^{-1}$ during the 8-h photoperiod. Relative humidity was controlled at 50%/75% (light/dark), and air temperature was set at 23°C/18°C (light/dark). Plants were bottom-watered on alternate days with a 0.1-strength modified Hoagland solution no. 2 supplemented with micronutrients (Epstein, 1972).

For determination of $L_P$ for leaf and root protoplasts, plants were grown in a growth chamber under continuous light. The PPFD was 200 $\mu$mol $m^{-2}$ $s^{-1}$. Relative humidity was controlled at 45% ± 5%, and air temperature was set at 20°C. For determination of $L_P$ for leaf protoplasts, plants were grown in the 100-$cm^3$ plastic pots filled with the vermiculate-*Sphagnum* peat moss mixture and were watered with water. For determination of $L_P$ for root protoplasts, plants were grown in the 100-$cm^3$ plastic pots filled with vermiculate and were watered with 0.25-strength nutrient solution (Somerville and Ogren, 1982).

### Construction of Plasmids for Transformation of Arabidopsis

The entire coding region of the PIP2;3 cDNA (Yamaguchi-Sinosaki et al., 1992) was amplified by PCR, cut with *Bgl*II and *Sal*I, and cloned in an antisense orientation between the doubly enhanced cauliflower mosaic virus 35S promoter and the nopaline synthase gene termination sequences of the binary plasmid pJIT60, which is a derivative of pBin19 and was obtained from M. Yanofsky (Division of Biology, University of California, San Diego). The specific primers used for the amplification were RD28AS 5′ (5′-agtcccgggacatccattaacac-3′) and RD28 AS 3′ (3′-gtcggttgcaaatttctagagg-5′) for the antisense construct.

### Plant Transformation and Selection

Plant transformation plasmids were directly transformed into *Agrobacterium tumefaciens* strain C58 AGL-0 (Lazo et al., 1991). The in-the-plant *A. tumefaciens*-mediated transformation was carried out as described previously (Bechtold et al., 1993) with the following modifications. WT plants of Arabidopsis (ecotype Col-0) were infiltrated for 25 min without using a vacuum, and the infiltration medium (2.3 g $L^{-1}$ Murashige and Skoog salts,

0.112 g $L^{-1}$ Gamborg's B5 vitamins, 0.5 g $L^{-1}$ MES buffer, 5% [w/w] Suc, and 0.044 $\mu$M 6-benzylaminopurine) contained 0.02% (w/w) Silwet L-77 (Union Carbide Corp., Danbury, CT).

T1 seeds were collected, dried at 29°C, and sown on sterile media containing 50 $\mu$g $mL^{-1}$ kanamycin to select the transformants. Surviving T1 plantlets were transferred to soil and allowed to set seed (T2). The segregation frequency of the T2 generation with regard to kanamycin resistance was determined on selective media. T2 plants were assumed to be homozygous for the transgene if all of 50 to 150 progeny seedlings were kanamycin resistant.

Seeds (T3) of transgenic lines segregating for kanamycin resistance in a Mendelian ratio of 3:1, typical for a single integration locus, were collected and again sown on selective media. Transgenic T3 plants were then used for all further experiments.

## Isolation of Protein Fraction and Immunodetection

The isolation of microsomes from leaves was performed according to Daniels et al. (1994). For each gel, the same amount of protein was loaded in each lane. The proteins were denatured in the presence of 1% (w/w) SDS and 100 mM ethanedithiol at 40°C. Under these conditions, PIP1 and PIP2 aquaporins ran mostly as monomers of 28 kD. After transfer to a nitrocellulose membrane, the immunodetection of PIP1 and PIP2 proteins was performed as described by Daniels et al. (1994) using, respectively, a serum against the amino acid sequence (KSLGSFRSAANV) of PIP2;3 and a serum against the N terminus of PIP1;1.

## Osmotic Hydraulic Conductivity for Leaf and Root Protoplasts

Osmotic hydraulic conductivity ($L_P$, meters per second per megapascal) for root and leaf protoplasts was determined as described by Ramahaleo et al. (1999). Root protoplasts were prepared as described by Thomine et al. (1995) and leaf protoplasts by Ramahaleo et al. (1999). Measurements were performed at 22°C, pH 5.5, and an osmotic difference of 0.2 mol $kg^{-1}$ (0.5 MPa) was used during both swelling or shrinking experiments. $L_P$ for both roots and leaves was determined on the largest protoplasts (30–60 $\mu$m in diameter); root protoplasts originated mainly from cortical cells and leaf protoplasts from mesophyll cells, as indicated by their diameter compared with the size of the corresponding cells in cross sections.

## $g_s$ and $E$

$g_s$ (millimoles per second per square meter) and $E$ (millimoles per second per square meter) were determined on the youngest fully expanded leaf with an Arabidopsis leaf chamber (6400-15 Arabidopsis Chamber, LI-COR, Lincoln, NE) mounted on an infrared $CO_2/H_20$ analyzer (LI-6400 Portable Photosynthesis System, LI-COR). The conditions in the measurement chamber were controlled as follows: flow rate, 200 $\mu$mol $s^{-1}$; PPFD during the photoperiod, 205 $\mu$mol $m^{-2}$ $s^{-1}$; $CO_2$ concentration in the sample chamber, 400 $\mu$mol $mol^{-1}$; relative humidity, 45%/65% (light/dark); and air temperature, 25.5°C/20°C (light/dark).

## Plant and Soil Water Potential

$\Psi_{leaf}$ (megapascals) was determined using a Scholander-type pressure chamber. After the balance pressure was determined, the leaf was rapidly placed in a 0.1-mL insulin syringe (whose bottom was covered with a layer of glass fiber to retain the cell wall fragments) and then frozen in liquid nitrogen. After thawing, the tissue was squeezed through the glass fiber filter, and the osmolality of the expressed liquid was measured with a vapor pressure osmometer (5500 Vapro, Wescor, Logan, UT) and used in the Van't Hoff relation to calculate the osmotic pressure (megapascals). The leaf turgor pressure (megapascals) was calculated as the sum of $\Psi_{leaf}$ and the osmotic pressure. $\Psi_{soil}$ (megapascals) was determined on 7-$cm^3$ soil samples collected in the center of the pot using a dewpoint hygrometer (WP4 Dewpoint PotentiaMeter, Decagon Devices, Pullman, WA). Two soil samples were collected for each pot.

## Hydraulic Conductances

The hydraulic conductance from soil to leaf ($K^{AL}_{plant}$, based on the leaf area per plant, millimoles per second per square meter per megapascal), that from stem to leaf ($K^{AL}_{leaf}$, based on the leaf area per plant, millimoles per second per square meter per megapascal), and that from soil to stem ($K^{AL}_{root}$, based on the leaf area per plant, millimoles per second per square meter per megapascal, or $K^{MR}_{root}$, based on the root dry mass per plant, millimoles per second per gram per megapascal) were calculated with the evaporative flux method under steady-state conditions (Tsuda and Tyree, 2000). One leaf per plant was covered before the beginning of the photoperiod with self-adhesive tape and aluminum foil to prevent transpiration (the bagged leaf). $E$ was determined with the LI-6400 infrared $CO_2/H_2O$ analyzer on two leaves per plant 5 h after the beginning of the photoperiod. One hour before $E$ was determined, the plant was immersed to nearly the height of the pot in the nutrient solution. One-half hour after $E$ was determined, the bagged leaf and the two leaves whose transpiration were measured were excised at the base of the stem. The balance pressure of the bagged leaf and the transpiring leaves were determined in a pressure chamber. The pressure potential of the bagged leaf and the transpiring leaves were assumed to provide estimates of $\Psi_{stem}$ (megapascals) and average $\Psi_{leaf}$, respectively. $\Psi_{soil}$ was assumed to be equal to the osmotic pressure of the nutrient solution, which was measured with the vapor pressure osmometer. $K^{AL}_{plant}$, $K^{AL}_{leaf}$, $K^{AL}_{root}$, and $K^{MR}_{root}$ were calculated as $E/(\Psi_{soil} - \Psi_{leaf})$, $E/(\Psi_{stem} - \Psi_{leaf})$, $E/(\Psi_{soil} - \Psi_{stem})$, and $E \cdot A_L/M_R \cdot (\Psi_{soil} - \Psi_{stem})$, respectively. Under soil drying and rewatering conditions, the pots were not immersed in the nutrient solution, and $\Psi_{soil}$ was determined using the dewpoint hygrometer.

## Leaf Area and Leaf and Root Dry Mass

The leaf blades of 5-week-old plants of all genotypes were detached and digitized with a digital camera, and their projected area ($A_L$) was calculated with an image analysis software (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD). The soil mixture around the root systems was gently removed by soaking the root system in successive batches of water. Leaf ($M_L$) and root ($M_R$) dry mass were determined after oven drying at 70°C to a constant mass.

## Statistics

All statistical analyses were done using SigmaStat 2.03 (SPSS, Chicago). Data with non-normal or inhomogeneous variance were log or square root transformed, as needed. Differences in $L_P$ were analyzed using one-way ANOVA ($\alpha = 0.05$) followed by a Dunn's test. Differences in leaf number, median leaf size, $A_L$, $M_L$, $M_R$, and $M_R$:$M_L$, and hydraulic conductances under wet conditions were analyzed using one-way ANOVA ($\alpha = 0.05$) followed by a Tukey's test. Differences between the dWT and dAS plants in water potentials, $g_s$, $E$, and $K_{plant}$ were analyzed using unpaired $t$ tests. Differences in water potentials $g_s$, $E$, and $K_{plant}$ for the dWT or dAS plants during soil drying and after soil rewatering were analyzed using one-way repeated measures ANOVA ($\alpha = 0.05$) followed by a Tukey's test.

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