LWR1 and *LWR2* Are Required for Osmoregulation and Osmotic Adjustment in Arabidopsis[1]

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With the goal of identifying molecular components of the low-water-potential response, we have carried out a two-part selection and screening strategy to identify new Arabidopsis mutants. Using a system of polyethylene glycol-infused agar plates to impose a constant low-water-potential stress, putative mutants impaired in low-water-potential induction of the tomato (*Lycopersicon esculentum*) *le25* promoter were selected. These lines were then screened for altered accumulation of free Pro. The seedlings of 22 mutant lines had either higher or lower Pro content than wild type when exposed to low water potential. Two mutants, designated *low-water-potential response1* (*lwr1*) and *lwr2*, were characterized in detail. In addition to higher Pro accumulation, *lwr1* seedlings had higher total solute content, greater osmotic adjustment at low water potential, altered abscisic acid content, and increased sensitivity to applied abscisic acid with respect to Pro content. *lwr1* also had altered growth and morphology. *lwr2*, in contrast, had lower Pro content and less osmotic adjustment leading to greater water loss at low water potential. Both *lwr1* and *lwr2* also had altered leaf solute content and water relations in unstressed soil-grown plants. In both mutants, the effects on solute content were too large to be explained by the changes in Pro content alone, indicating that *LWR1* and *LWR2* affect multiple aspects of cellular osmoregulation.

Drought exposes plants to a decrease in soil water content, quantified as a decrease in soil water potential (ψ_w), which decreases the ability of plants to absorb water from the soil (Boyer, 1982, 1985). Osmotic adjustment functions to avoid excessive dehydration through the accumulation of intercellular solutes, which lowers cellular ψ_w and maintains a favorable ψ_w gradient for water movement into the plant (Morgan, 1984; Zhang et al., 1999). Osmotic adjustment is defined as the amount of solute accumulated in response to low ψ_w excluding any increase in solute concentration that occurs solely because of cellular water loss (Morgan, 1984) and is a specific example of the more general process of osmoregulation, the mechanisms that control cellular solute and water content, and turgor. In plants at constant high ψ_w, solute content and turgor remain relatively stable (Silk et al., 1986; Kutschera, 1991). Net deposition of new solutes occurs mainly in growing tissue where it is needed to drive the uptake of water necessary for cell expansion (Silk et al., 1986). Thus, solute deposition is closely coordinated with growth and solute content subject to tight homeostatic regulation. Exposure to low ψ_w alters this regulation and leads to the accumulation of additional solutes throughout much of the plant. The increase in solute content involves many solute species including K^+, sugars, and various types of compatible solutes (Morgan, 1984; Sharp et al., 1990; Zhang et al., 1999). The upstream sensing and signaling mechanisms involved in perceiving external ψ_w and controlling cellular solute content and turgor are unknown.

Accumulation of the compatible solute Pro is a highly regulated stress response. Low-ψ_w-induced Pro accumulation involves increased Pro synthesis and decreased Pro catabolism indicated by both biochemical (Rhodes et al., 1986) and gene expression studies (Delauney and Verma, 1993; Rentsch et al., 1996; Yoshiba et al., 1997). In specific tissues, changes in import or export of Pro (Girouse et al., 1996; Verslues and Sharp, 1999) may also be important. There is strong evidence that Pro accumulation is dependent on abscisic acid (ABA; Bray, 1993; Ober and Sharp, 1994; Strizhov et al., 1997), although ABA is likely not the only regulatory factor involved (Savoure et al., 1997). Understanding the regulation of Pro accumulation is likely to be useful in understanding other aspects of low-ψ_w responses, including both osmotic adjustment and ABA-dependent responses.

Despite the importance of forward genetic analysis in Arabidopsis, we are not aware of any screen that has used low-ψ_w response as the primary criteria for mutant isolation. The most closely related work, performed by Zhu and colleagues (Ishitani et al., 1997; Xiong and Zhu, 2002), has identified numerous mutants with altered responses to salt stress, low temperature, and exogenous ABA. While salt stress has an osmotic component, after the first few hours of

[1] This work was supported by the National Science Foundation (grant no. GE–9355042 to E.A.B.), and by the Graduate Division and the Department of Botany and Plant Sciences, University of California, Riverside (P.E.V.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.045856.

exposure, ion toxicity, caused by excess Na^+, is the main factor causing plant stress (Munns, 2002). A mutant screen that identifies loci that alter the response to low-ψ_w treatment has the potential to find new loci specifically involved in the low-ψ_w response. Using a system of polyethylene glycol (PEG)-infused agar plates to impose reproducible, constant low-ψ_w treatments (van der Weele et al., 2000), we combined negative selection based on expression of a stress-induced promoter with screening for altered Pro accumulation to isolate mutants in the low-ψ_w response.

Twenty-two lines were identified in which induction of a low-ψ_w-regulated promoter was decreased and low-ψ_w-induced Pro accumulation was either increased or decreased relative to wild type. Detailed characterization of two mutants found that total solute content and osmotic adjustment were also altered. This work provides a means to understand not only Pro accumulation and other low-ψ_w responses, but also the processes underlying osmoregulation and osmosensing in plants.

RESULTS

Low-ψ_w Responses of Arabidopsis Seedlings

PEG-infused agar plates (adapted from van der Weele et al., 2000) were used to impose a precisely defined, constant, low-ψ_w treatment to Arabidopsis seedlings. This allowed the response to a particular ψ_w treatment to fully develop over time and reach a steady-state condition. Use of PEG for these experiments also has several advantages. High M_r PEG, such as the PEG-8000 used in these experiments, mimics the effects of soil drying by causing cytorrhysis (withdrawal of water from both the cell wall and cytoplasm) instead of plasmolysis (withdrawal of water only from the cytoplasm leading to separation of the cell wall and cell membrane; Carpita et al., 1979; Oertli, 1985). In addition, PEG is not taken up by the plant (Hohl and Schopfer, 1991) and has no toxic effects as long as root damage is avoided (Lawlor, 1970; Verslues et al., 1998; van der Weele et al., 2000). All the agar plate experiments reported here were completed without the addition of sugar to the media to avoid the effects of high levels of external sugars on osmoregulation and ABA response (Arenas-Huertero et al., 2000; Laby et al., 2000).

Three-day-old seedlings were transferred to PEG-infused plates for low-ψ_w treatment (−0.75 MPa) or to a new plate without PEG (−0.25 MPa) for the control treatment. Seedling relative water content (RWC) decreased in the first 24 h after transfer to low ψ_w and then partially recovered at later times (Fig. 1A). Likewise, seedling osmotic potential (ψ_s) decreased rapidly in the initial 24 h after transfer to low ψ_w, was equal to the agar ψ_w from 24 to 48 h, and then decreased further from 48 to 96 h (Fig. 1B). Pro content increased steadily until 48 h after transfer when Pro content stabilized (Fig. 1C). Even though Pro content increased approximately 10-fold, the total increase in Pro content could account for only a small portion (approximately 3%) of the total −0.33 MPa decrease in seedling ψ_s that occurred during the 96-h low-ψ_w treatment. Pro accumulation and osmotic adjustment occurred more slowly than other stress responses, such as gene expression, commonly studied in Arabidopsis. Thus, our subsequent experiments examining Pro accumulation and osmotic adjustment focused on the response 72 or 96 h after the start of low-ψ_w treatment.

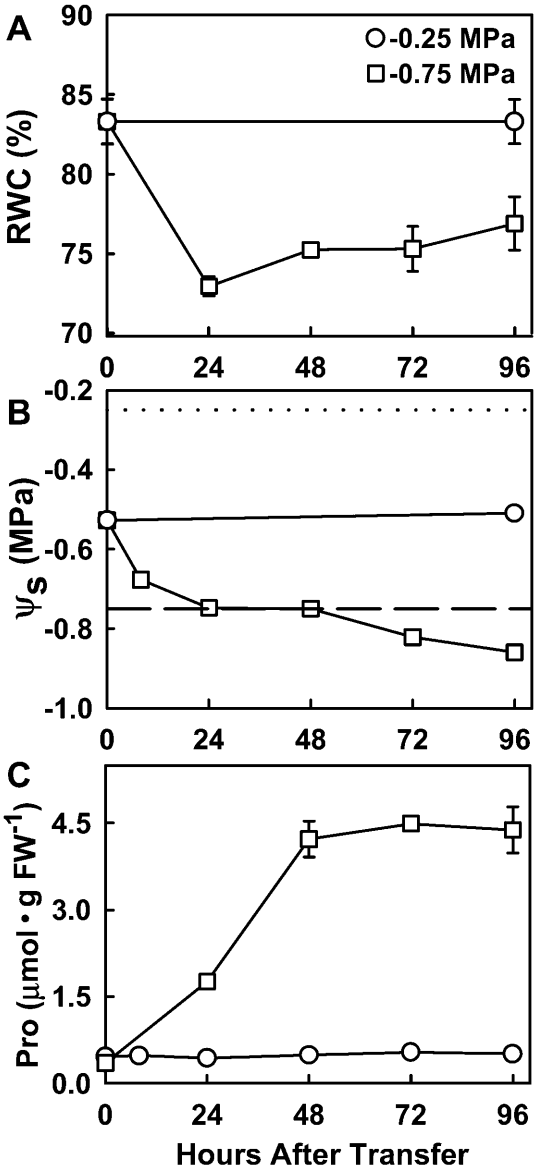

Figure 1. Wild-type response to low-ψ_w treatment. Three-day-old wild-type seedlings (*le25:ADH* Ben) were transferred to either −0.25 MPa (control) or −0.75 MPa PEG-infused agar plates, and low-ψ_w responses were measured over a 96-h period. A, Seedling RWC. Data are means ± SE (n = 6). B, Seedling ψ_s. Dotted line shows the agar ψ_w of the −0.25 MPa treatment. Dashed line shows the agar ψ_w of the −0.75 MPa treatment. Data are means ± SE (n = 3–4). C, Seedling Pro content. Data are means ± SE (n = 4–6). Error bars are not shown when smaller than symbols.

Mutant Isolation

A negative selection scheme followed by a screen for altered Pro accumulation was used to isolate mutants affected in low-ψ_w responses. An *adh*$^-$ line of Arabidopsis (ecotype Bensheim) was transformed with a construct containing the *ADH*-coding region (Chory et al., 1995) controlled by the ABA- and stress-inducible *le25* promoter of tomato (*Lycopersicon esculentum*). In *le25:ADH* transgenic Arabidopsis seedlings, ADH activity increased after seedlings were transferred to low ψ_w and reached a maximal level at approximately 48 h after transfer (data not shown), demonstrating that the *le25* promoter was active in Arabidopsis. *le25*-driven ADH activity was measured 48 h after seedlings were transferred to plates of varying severities of low ψ_w. The highest ADH activity was measured at −1.7 MPa and was 10-fold greater than the activity of seedlings exposed to −0.25 MPa (Fig. 2A). Untransformed *adh*$^-$ plants had no detectable ADH activity at any ψ_w (data not shown). *le25:ADH* seedlings exposed to −1.2 MPa were sensitive to 1.2 mM allyl alcohol (Fig. 2B) because ADH metabolizes allyl alcohol to acrolein, a phytotoxic compound (Chory et al., 1995). The untransformed *adh*$^-$ or *le25*:*ADH* seedlings exposed to control media (−0.25 MPa) were not killed by this treatment (Fig. 2B). This *le25*:*ADH* line is hereafter referred to as wild type or Ben.

Seeds of the wild-type line were ethyl methanesulfonate mutagenized and M_1 seed collected in pools of 35 to 40 plants. Approximately 600 seeds from each of more than 300 pools were germinated, the seedlings transferred to low-ψ_w agar plates (−1.2 MPa) for 48 h, treated with 1.2 mM allyl alcohol for 2 h, and transferred to high-ψ_w plates for 7 d. Of the seedlings surviving the allyl alcohol treatment, approximately 1,000 were chosen and transferred to soil. M_2 seed was harvested from approximately 600 individual plants. A total of 135 of these lines having either higher or lower seedling Pro content than wild type after a 72 h −1.2 MPa treatment were planted in soil. M_3 seed was collected from each line and rescreened for altered Pro accumulation. Thirty-five lines, which showed substantial differences in Pro accumulation relative to wild type in both the M_2 and M_3 generations, were selected for further analyses. These lines were twice backcrossed to wild type, twice selfed, and allyl alcohol resistance and Pro accumulation at −1.2 MPa was quantified in seedlings from each of the backcrossed lines.

Twenty-two lines with increased allyl alcohol resistance (24% to 78% seedling survival after allyl alcohol treatment compared to 21% for Ben wild type) were chosen for further study (Fig. 3A). Of these lines, one-half had increased Pro accumulation (125% to 205% of the Ben wild type) and one-half had decreased Pro accumulation (50% to 80% of Ben wild type) in response to media of −1.2 MPa (Fig. 3B). The remainder of this report focuses on two mutants, designated as *low-water-potential response1* (*lwr1*) and *lwr2*. *lwr1* and *lwr2* are recessive, nonallelic mutants (Table I).

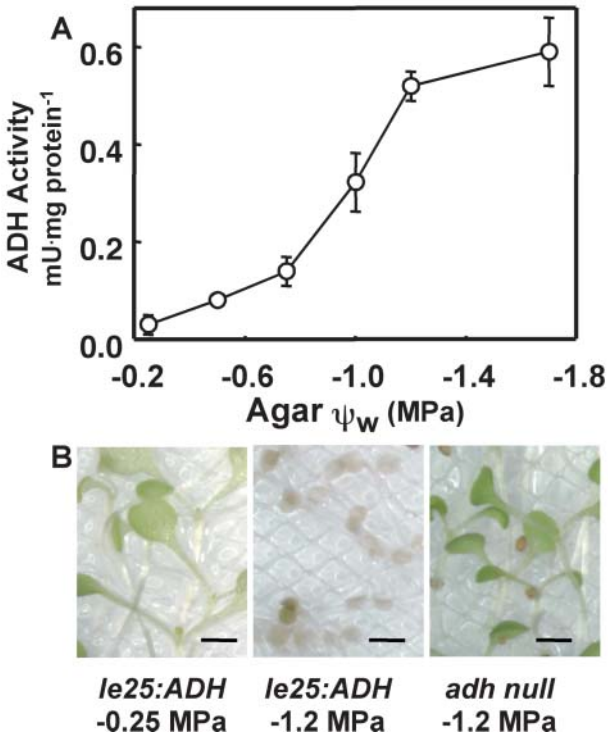

Figure 2. ADH activity and allyl alcohol responses of *le25*:*ADH* Ben seedlings. A, Induction of ADH activity by low ψ_w. ADH activity was assayed 48 h after seedlings were transferred to a range of agar ψ_w. Data are means ± SE (n = 5–7). B, Appearance of *le25:adh* and Ben *adh* null seedlings 7 d after a 2-h, 1.2 mM allyl alcohol treatment. Prior to the allyl alcohol treatment, 3-d-old seedlings were exposed to either control (−0.25 MPa) or low-water-potential stress (−1.2 MPa) for 2 d. After the allyl alcohol treatment, seedlings were transferred to control plates. Scale bars in pictures indicate 1 mm. The nylon mesh used to transfer seedlings between plates can be seen in the background.

Altered Pro Content of *lwr1* and *lwr2*

Steady-state Pro accumulation of both mutants and wild type was further characterized by quantifying Pro content 96 h after transfer of seedlings to a range of ψ_w treatments (Fig. 4). In wild type, Pro content increased in an approximately linear manner with decreasing ψ_w and was 44-fold greater at −1.7 MPa than at −0.25 MPa. The Pro content of *lwr1* seedlings was 1.4-fold greater than that of wild type when exposed to −1.2 and −1.7 MPa. The Pro content of *lwr2* seedlings was approximately 75% of the wild type at −0.75 and −1.2 MPa. Both *lwr1* and *lwr2* had significantly higher Pro content than wild type in the unstressed (−0.25 MPa) treatment.

Altered Solute Content and Osmotic Adjustment of *lwr1* and *lwr2*

To determine the extent of water loss, solute accumulation, and osmotic adjustment in the three

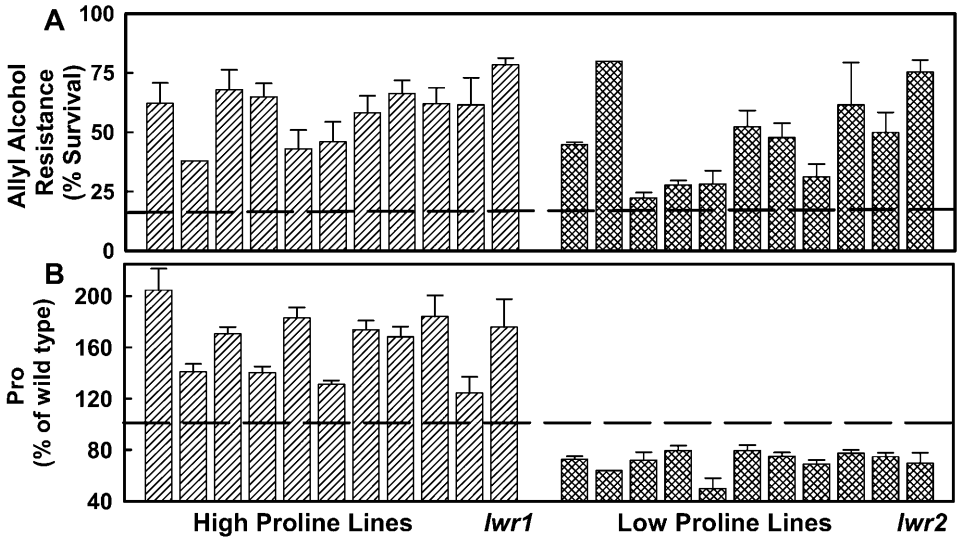

Figure 3. Allyl alcohol resistance and Pro content of 22 mutant lines. A, Percent survival of each mutant line to allyl alcohol treatment. Allyl alcohol selection was performed as in Figure 2 except that 3.0-mM allyl alcohol was used. Dashed line across the bottom of the figure indicates the wild type (unmutagenized *le25:ADH* Ben) response (21% survival). B, Pro content of each mutant line. Three-day-old seedlings were transferred to −1.2 MPa PEG-infused plates for 3 d. Pro content was determined and expressed relative to the wild-type control. The dashed line indicates the wild-type level (100%, which in these experiments was 6.42 ± 0.35 μmol g FW^{-1}). The same mutant lines are depicted in the same order in both panels. Therefore, the allyl alcohol resistance and Pro content for the same line can be compared by looking at the bars in the same horizontal position in the two sections. The *lwr1* and *lwr2* mutants are individually labeled. Data are means ± SE (n = 3–6) in both A and B.

genotypes, the RWC and ψ_s of seedlings was quantified after 96 h of exposure to a range of ψ_w treatments. RWC of wild-type seedlings steadily decreased in response to increasing severities of low ψ_w agar (Fig. 5A). As would be expected for turgid tissue, seedling ψ_s was lower than agar ψ_w from −0.2 to −0.75 MPa (Fig. 5B). At −1.2 and below, seedling ψ_s and agar ψ_w were equivalent (the dashed line in Fig. 5B indicates the points where seedling ψ_s equals agar ψ_w), indicating that seedling turgor was near zero.

To quantify seedling osmotic adjustment, we used the data in Figure 5, A and B to calculate ψ_s at 100% RWC (ψ_{s100}; Wilson et al., 1979; Babu et al., 1999) for each ψ_w treatment. Calculating ψ_{s100} removes any decrease in ψ_s caused solely by dehydration-induced increase in concentration of existing solutes. We then used regression analysis to quantify the extent that ψ_{s100} decreased in response to decreased agar ψ_w. The slope of the line when seedling ψ_{s100} is plotted against agar ψ_w is independent of the basal solute content in the unstressed (−0.25 MPa) treatment and thus provides an accurate account of low ψ_w-induced osmotic adjustment. When this analysis was performed for wild-type seedlings there was a linear relationship between ψ_{s100} and agar ψ_w ($\psi_{s100} = 0.37\psi_w - 0.37$; r^2 = 0.98). The slope of this line indicated that wild-type seedlings had just 37% (150 mM of solutes per MPa decrease in agar ψ_w; Fig. 5C inset) of the solute accumulation needed to fully osmotically adjust to decreased agar ψ_w and avoid water loss.

For *lwr1* seedlings, RWC was not significantly different from wild type from −0.25 to −0.75 MPa (Fig. 5A). In the more severe stress treatments of −1.2 and −1.7 MPa, the RWC of *lwr1* seedlings was significantly higher than wild type (Fig. 5A). The ψ_s of *lwr1* seedlings was lower than wild type above −1.2 MPa (Fig. 5B). At −0.75 MPa, for example, the solute concentration in *lwr1* seedlings was 58 mM higher than wild type. For *lwr1*, the regression line of ψ_{s100} versus agar ψ_w ($\psi_{s100} = 0.51\psi_w - 0.39$; r^2 = 0.99) was significantly different than wild type (F test, P = 0.0003). The difference in the slopes of the regression lines for wild type and *lwr1* (211 mM MPa^{-1}; Fig. 5C inset) indicated that *lwr1* had one-third greater osmotic adjustment than wild type.

In contrast, *lwr2* had reduced RWC at −0.75 and −1.2 MPa, indicating a lack of solute accumulation

Table I. *Genetic analysis of lwr1 and lwr2*

Percent survival of wild type (Ben; unmutagenized *le25:ADH*), *lwr1*, *lwr2*, and F_1 seedlings from crosses of these three genotypes after a 2-h allyl alcohol treatment. Seedlings were transferred to control media after the allyl alcohol treatment (2 mM), and survival was measured 7 d after transfer.

Genotype	Allyl Alcohol Resistance (% Seedling Survival)
Ben	35 ± 6
lwr1	66 ± 9[a]
lwr2	75 ± 5[a]
Ben × *lwr1*	6 ± 2
Ben × *lwr2*	17 ± 4
lwr1 × *lwr2*	23 ± 7

[a]Significantly greater than Ben at $P \leq 0.01$.

that would allow the seedlings to retain water. Consistent with this, the ψ_s of *lwr2* seedlings was significantly higher than wild type at −0.25, −0.5, and −0.75 MPa ($P \leq 0.001$; Fig. 5B). The regression line for ψ_{s100} plotted against agar ψ_w ($\psi_{s100} = 0.24\psi_w - 0.34$; $r^2 = 0.97$) differed from that of wild type (F test, $P = 0.003$), and the difference in slope of these lines demonstrated that osmotic adjustment of *lwr2* (97 mM MPa^{-1}; Fig. 5C inset) was one-third less than that of wild type. At −1.7 MPa, *lwr2* seedlings were visibly more dehydrated than wild-type seedlings, yet they increased in fresh weight (FW) only slightly when rehydrated indicating that the seedlings were damaged by severe water loss at this ψ_w. Thus, data for *lwr2* at −1.7 MPa were not included in the above analysis of osmotic adjustment.

The increased solute content and osmotic adjustment of *lwr1* was unique in our mutant collection. However, we have found six other low-Pro lines that were similarly reduced in osmotic adjustment as *lwr2* (P.E. Verslues and E.A. Bray, unpublished data). Complementation testing between these other six lines and *lwr2* is currently under way. None of the 14 other mutant lines had substantial changes in osmotic adjustment despite having, in many cases, even larger changes in Pro accumulation than *lwr1* or *lwr2*.

K^+ and Other Solutes Contribute to Changes in Osmotic Content of *lwr1* and *lwr2*

The changes in solute content and osmotic adjustment of *lwr1* and *lwr2* were too large to be explained solely by the change in Pro content of the mutants. To begin to determine the types of metabolic alterations caused by the mutations in *lwr1* and *lwr2*, we determined the levels of K^+ in mutant and wild-type seedlings (Fig. 6). K^+ is present at high concentrations in plant cells and can have a large impact on overall cellular solute content and osmoregulation (Sharp

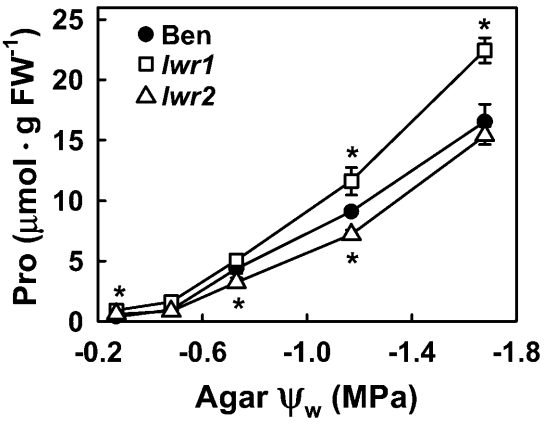

Figure 4. Pro contents of wild type (Ben), *lwr1*, and *lwr2* in response to a range of agar ψ_w. Pro content was assayed 96 h after transfer of 3-d-old seedlings to the indicated ψ_w. Data are means ± SE (n = 5–8). Significant differences between mutant and wild type ($P \leq 0.05$) are indicated by asterisks in the figure.

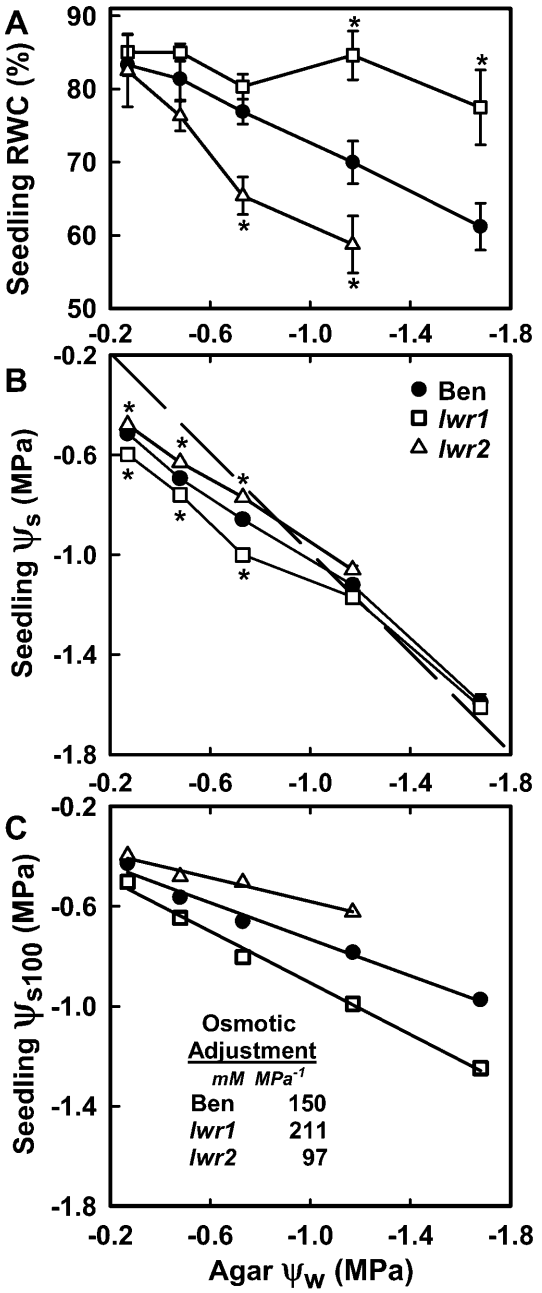

Figure 5. Analysis of osmotic adjustment of wild type (Ben), *lwr1*, and *lwr2*. A, RWC of mutants and wild type after 96 h of exposure to a range of agar ψ_w. Data are means ± SE (n = 4–10). Significant differences between mutant and wild type are indicated by an asterisk in the figure ($P < 0.05$). B, ψ_s of wild-type and mutant seedlings measured 96 h after transfer to a range of agar ψ_w. The dashed diagonal line indicates where seedling ψ_s equals ψ_w of the agar plate. Data are means ± SE (n = 4–10). Error bars are smaller than symbols for all data points. Significant differences between mutant and wild type are indicated by an asterisk in the figure ($P < 0.001$). C, ψ_s at 100% RWC (ψ_{s100}) of mutant and wild-type seedlings calculated from data in A and B. Lines are regression lines fitted to the data for each genotype ($r^2 > 0.97$ in all cases). The regression lines of both *lwr1* and *lwr2* were significantly different from that of wild type (F test; $P = 0.0003$ for *lwr1* and wild type and $P = 0.003$ for *lwr2* and wild type). Inset, Osmotic adjustment (slope of each regression line converted to millimolar solute concentration) per MPa decrease in ψ_w.

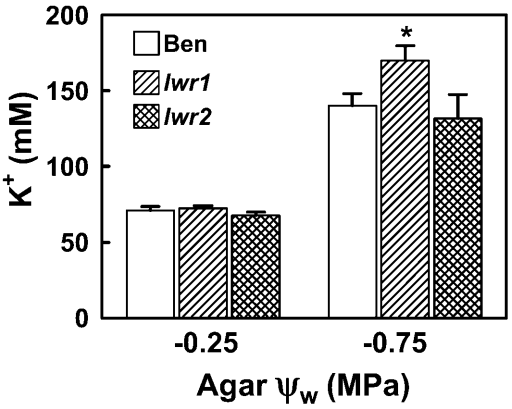

Figure 6. Concentration of K^+ in unmutagenized *le25*:ADH (Ben), *lwr1*, and *lwr2* seedlings at −0.25 and −0.75 MPa. Data are means ± SE (n = 4–8). *lwr1* was significantly different from wild type (P = 0.05) at −0.75 MPa (indicated by an asterisk).

et al., 1990). At −0.25 MPa, the K^+ concentration of both mutants and wild type were similar. When seedlings were transferred to −0.75 MPa, there was a 2-fold increase in K^+ concentration for Ben and *lwr2* but a significantly greater (2.4-fold) increase for *lwr1*.

The total difference in solute content between mutant and wild-type seedlings was calculated from the ψ_{s100} data in Figure 5C and the concentrations of K^+ and Pro at 100% RWC then used to calculate the contribution of each solute to the total change in solute content of each mutant compared to the wild type (Table II). At −0.75 MPa, one-half of the difference in solute content between wild type and *lwr1* can be explained by the higher K^+ content of the mutant (Table II). Since the anions needed to balance the charge of the K^+ are likely to be present at concentrations similar to that of K^+ itself, K^+ and its associated anions can account for nearly all of the increased solute content of *lwr1* at −0.75 MPa. The contribution of Pro to the increased solute content of *lwr1* was only about 1% of the total (Table II). In unstressed *lwr1* seedlings (−0.25 MPa), the solute content was 30 mM higher than wild type with Pro contributing about 2% of the difference. Unlike the situation with moderate stress, K^+ only accounted for approximately 7% of the increased solute content. The identity of the solutes accumulating in unstressed *lwr1* seedlings is unknown.

For *lwr2* at −0.75 MPa, total solute content at 100% RWC was 64 mM less than wild type (Table II). K^+ content at 100% RWC was 22 mM less in *lwr2* than wild-type seedlings, indicating that accumulation of K^+ was reduced in *lwr2* compared to wild type. In terms of total solute content, reduced K^+ content accounted for one-third of the difference between mutant and wild type at both −0.25 and −0.75 MPa. Pro made a small (approximately 2% of the total) contribution to the altered solute content of *lwr2* mutant.

These calculations showed that the *lwr1* and *lwr2* mutations affected the accumulation of more than one solute species. Although differences in subcellular compartmentation and tissue distribution could not be quantified in the above calculations, the relative contributions of Pro and K^+ to the overall difference in solute content was consistent with their expected compartmentation within the cell. Pro is accumulated primarily in the relatively small volume of the cytoplasm and is an osmotically significant solute in this compartment (Leigh et al., 1981; Voetberg and Sharp, 1991; Hare et al., 1998). K^+ accumulates in the much larger vacuolar volume and hence makes a larger contribution to the bulk tissue solute content.

Carbohydrates can also be osmotically important solutes (Sharp et al., 1990). We assayed Glc content of wild type and both mutants and found that Glc content was low (2 to 4 mM) and did not change in response to low ψ_w. This provides an indication that altered sugar content is not a factor in the altered solute content of *lwr1* and *lwr2*.

lwr1 and *lwr2* Have Altered ABA Accumulation or Response

In our PEG-infused plate system, transfer of seedlings to low ψ_w elicited a rapid increase in ABA content, which peaked approximately 8 h after transfer to low ψ_w and then declined to a steady-state value that was up to 18-fold higher than the unstressed level (P.E. Verslues and A.E. Bray, unpublished data). The steady-state ABA content of *lwr1*, *lwr2*, and wild-type seedlings, assayed 96 h after transfer to a range of ψ_w treatments, linearly increased in response to decreasing external ψ_w (Fig. 7A). However, the increase was less for *lwr1* than wild type. In contrast, the ABA content of *lwr2* did not differ from wild type when expressed as a function of ψ_w (Fig. 7A). The peak ABA content at 8 h after transfer to −1.2 MPa was also reduced in *lwr1* but unaffected in *lwr2* (data not shown).

It has been suggested that the factor that induces ABA accumulation is loss of water and accompanying changes in turgor (Pierce and Rashke, 1980; Creelman and Zeevaart, 1985). Because *lwr1* and *lwr2* differ from wild type in RWC at low ψ_w, it is also of interest to examine the relationship between RWC and ABA content (Fig. 7B). Steady-state seedling ABA content was linearly related to decreasing RWC in wild type (r^2 = 0.99). In *lwr1* seedlings, RWC and ABA content were not well correlated, primarily because there was little change in the RWC of *lwr1* with decreasing external ψ_w. The decreased ABA content of *lwr1* compared to wild type at the same ψ_w may be an indirect effect of the increased RWC of *lwr1* seedlings. The ABA content of *lwr2* seedlings was also linearly related to RWC (r^2 = 0.97), but the slope of the line was less than wild type. Thus, *lwr2* seedlings were less sensitive to water loss than wild type with respect to ABA accumulation.

Table II. *Contribution of Pro and K^+ to the altered solute content of lwr1 and lwr2*

ψ_{s100} (Fig. 5) was converted to solute concentration (mM) and used to calculate the difference in solute concentration between mutants and wild type at −0.25 and −0.75 MPa. Pro (Fig. 4) and K^+ (Fig. 6) contents were adjusted to 100% RWC and used to calculate the difference in content of Pro and K^+ relative to wild type. These values were then compared to the total difference in solute content between mutant and wild type to estimate the contributions of Pro and K^+ to the altered solute content of the mutants.

	Agar					
	Ben		*lwr1* ψ_w		*lwr2*	
	−0.25	−0.75	−0.25	−0.75	−0.25	−0.75
	MPa					
ψ_{s100} (MPa)	−0.428	−0.661	−0.502	−0.803	−0.396	−0.503
Solute content (mM)[a]	175	270	205	328	162	206
Solute difference (mM)[b]			30	58	−13	−64
Pro_{100} (mM)[c]	0.31	3.20	0.76	3.86	0.49	2.03
Pro difference (mM)			0.45	0.66	0.18	−1.17
% Solute difference[d]			2	1	0	2
K^+_{100}, (mM)[e]	59	108	61	136	55	86
K^+ difference (mM)			2	29	−4	−22
% Solute difference[f]			7	50	26	34

[a]Total solute content at 100% RWC calculated using the equation: $\psi_s = -RTC$. [b]Difference in total solute content at 100% RWC between the mutant and wild type (Ben); negative values for *lwr2* indicate that wild-type solute content was greater than that of *lwr2*. [c]Pro content at 100% RWC calculated from data in Figures 4 and 5; a correction for percent dry weight (≤4%) was applied to the Pro data. [d]Percent of the total difference in solute content at 100% RWC that can be accounted for by altered Pro content. [e]K^+ content at 100% RWC calculated from data in Figures 5 and 6. [f]Percent of the total difference in solute content at 100% RWC that can be accounted for by altered K^+ content.

A change in responsiveness to ABA could also contribute to the phenotypes of *lwr1* and *lwr2*. To test this possibility, we quantified the Pro content of mutant and wild-type seedlings after 96 h of exposure to a range of ABA concentrations applied at −0.25 MPa. In the wild type, Pro accumulation saturated at approximately 10 μM exogenous ABA in which a more than 4-fold increase in Pro content was observed (Fig. 7C). In *lwr1*, the maximum increase in Pro content was similar to wild type but saturated between 0.5 and 2 μM exogenous ABA. Internal ABA content was the same for all three genotypes and increased dramatically in response to exogenous ABA (18 ng g FW^{-1} at 0.5 μM exogenous ABA, 75 ng g FW^{-1} at 2.0 μM ABA, 245 ng g FW^{-1} at 10 μM ABA, and 3,058 ng g FW^{-1} at 100 μM ABA). The increased Pro levels in response to ABA application of *lwr1* indicated an increased responsiveness to ABA in this mutant. In contrast, the Pro accumulation response of *lwr2* to exogenous ABA did not differ from wild type (Fig. 7C), raising the possibility that *lwr2* may affect low-ψ_w responses independently of ABA or may act upstream of ABA.

lwr1 and *lwr2* Affect Leaf Water Relations of Well-Watered Mature Plants

To determine whether the differences in solute content observed in seedlings were also present in adult plants, we measured water relations parameters of fully expanded leaves from mutant and wild-type plants grown under well-watered conditions (Fig. 8A). The soil ψ_w was −0.24 MPa and did not vary significantly between the three genotypes. The ψ_s of *lwr1* leaves was −0.96 MPa, while the ψ_s of wild-type leaves was −0.74 MPa. The leaf ψ_w of *lwr1* was less than wild type while the calculated ψ_p was similar to wild type. The decreased leaf ψ_w of *lwr1* plants could indicate either an increased resistance to water flow through the plant or an increased rate of leaf water loss (Boyer, 1985). Consistent with the later possibility, detached leaves of *lwr1* lost water more quickly than wild type (Fig. 8B). The difference in water loss was not caused by a difference in initial leaf hydration as leaves of all three genotypes were fully hydrated at the time of detachment (RWC > 97%). Although increased leaf water loss can explain the decreased leaf ψ_w of *lwr1*, it cannot explain the increased solute content and osmotic adjustment in seedlings grown under conditions of minimal transpiration (Fig. 5).

There were also alterations in leaf water relation parameters of *lwr2* plants grown at high ψ_w. Interestingly, the ψ_s of *lwr2* leaves was −0.1 MPa (40 mM) greater than wild type (Fig. 8A) and calculated leaf turgor was 0.17 MPa higher than wild type.

lwr1 Plants Have Altered Growth and Morphology

lwr1 plants exhibited several conspicuous alterations in growth and morphology. Unstressed *lwr1* seedlings had reduced elongation of the hypocotyl and root (Fig. 9A). Well-watered soil-grown plants had

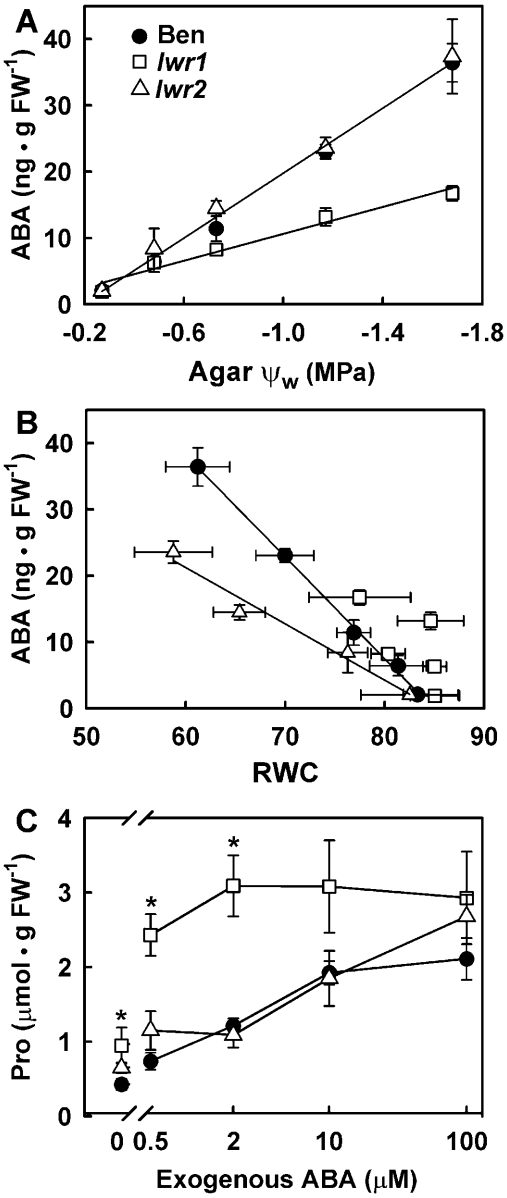

Figure 7. ABA content and response of wild type (Ben), *lwr1*, and *lwr2* seedlings to applied S(+)-ABA. A, ABA content of 3-d-old seedlings after 96 h at the indicated ψ_w. Data are means ± SE (n = 4–9). Regression lines were fitted to the combined data of wild type and *lwr2* (r^2 = 0.99) and *lwr1* (r^2 = 0.97). The two regression lines were significantly different (F test, $P = 5 \times 10^{-12}$). B, ABA content expressed as a function of RWC. Regression lines were fitted for each genotype (Ben, r^2 = 0.99; *lwr1*, r^2 = 0.44 [this line is not shown in the figure]; *lwr2*, r^2 = 0.97). The wild-type regression line was significantly different from that of *lwr2* (F test, P = 0.0003) C, Pro content 96 h after transfer to plates containing the indicated concentrations of S(+)-ABA. Data are means ± SE (n = 7). Significant differences between mutant and wild type are indicated by an asterisk in the figure ($P < 0.002$).

altered leaf shape and appearance including a crinkled, uneven leaf surface and shorter petiole (Fig. 9B), and the production of additional rosette leaves compared to wild type (Fig. 9C). *lwr1* plants also bolted later than wild type and were reduced in stature after bolting (Fig. 9D). Analysis of F_3 seedlings collected from more than 100 plants in a segregating F_2 population indicated that these changes in leaf morphology cosegregated with the increased seedling solute content described above (data not shown). We have not observed any changes in morphology or development in the *lwr2* mutant.

DISCUSSION

The isolation of mutants with altered low-ψ_w responses is a key step in understanding perception of water status and the mechanisms of adaptation to suboptimal water availability. In this study, several critical factors allowed us to isolate a group of mutant lines with altered low-ψ_w responses. Our experimental system permitted a steady low-ψ_w treatment to be applied over a sufficient period of time for Pro accumulation to reach steady-state levels, allowing this trait to be used for isolating and characterizing new mutant lines. Also, combining allyl alcohol selection with a screen for altered Pro accumulation allowed us to rapidly generate a population enriched for

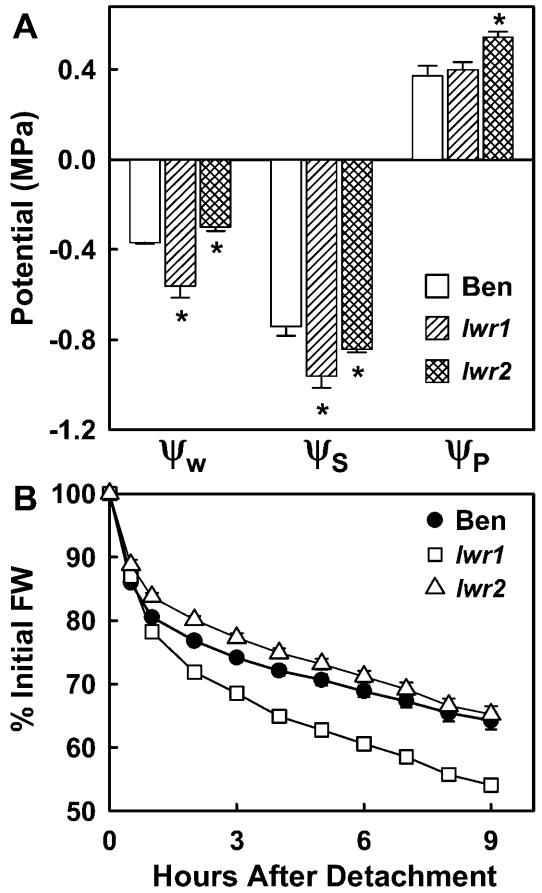

Figure 8. Leaf water relations of wild type (Ben), *lwr1*, and *lwr2* under well-watered conditions (soil ψ_w = −0.24 MPa). A, Leaf ψ_w, ψ_s, and calculated ψ_p. Data are means ± SE (n = 4−5). Significant differences ($P < 0.05$) of mutants versus wild type are marked with an asterisk. B, Water loss of detached leaves. Data are means ± SE (n = 14). Error bars are not shown when smaller than symbols.

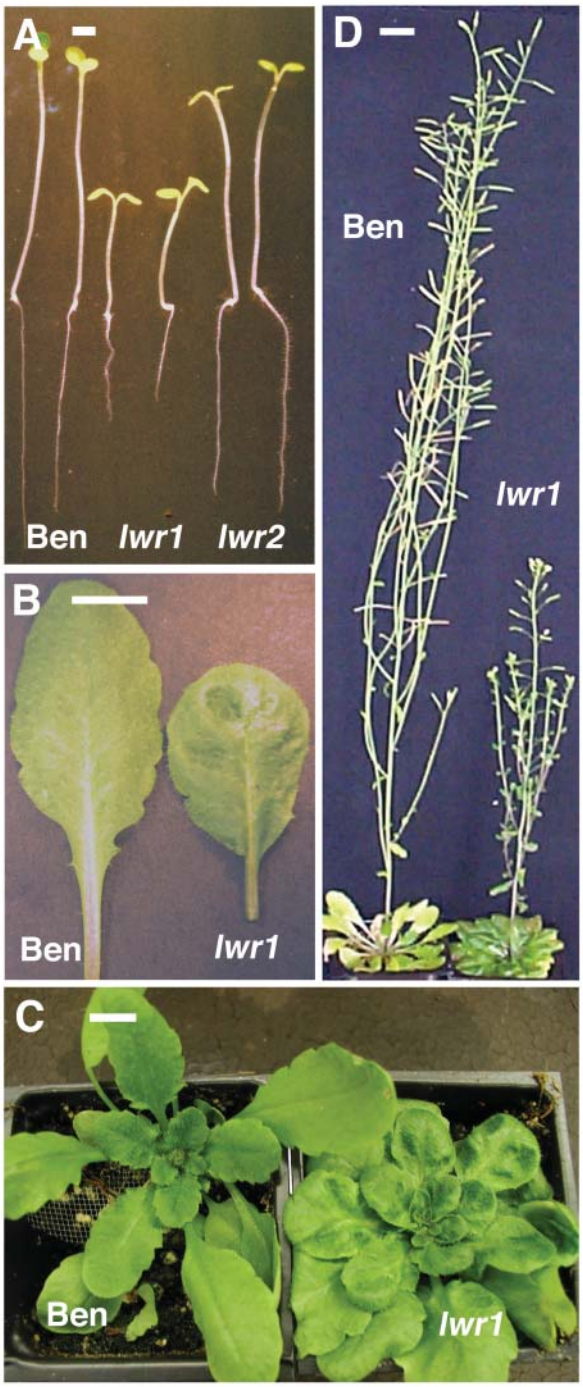

Figure 9. Morphological differences of *lwr1* compared to wild type (Ben). A, Five-day-old seedlings of Ben, *lwr1*, and *lwr2* at −0.25 MPa. Scale bar indicates 1 mm. B, Fully expanded rosette leaves of Ben and *lwr1*. Scale bar indicates 1 cm. C, Rosette stage plants of Ben and *lwr1*. Scale bar indicates 1 cm. D, Flowering plants of Ben and *lwr1*. Scale bar indicates 2 cm.

mutations affecting low-ψ_w responses, making the relatively laborious Pro screen feasible. Using two low-ψ_w-regulated traits together favored the isolation of mutants, such as *lwr1* and *lwr2*, which are altered in multiple aspects of the low-ψ_w response.

Physiology and Genetics of Osmoregulation

The observations that content of solutes other than Pro is altered in *lwr1* and *lwr2* and that many of our other mutants did not have changes in osmotic adjustment despite having similar changes in Pro accumulation support the conclusion that the osmotic adjustment phenotypes observed in *lwr1* and *lwr2* are specific effects of these mutations and not simply a consequence of the altered Pro accumulation. Our calculated values of osmotic adjustment are consistent with values reported for several plant species using several methods of calculation (Zhang et al., 1999). In particular, our data of seedling ψ_s versus agar ψ_w follow the same pattern as previous data of ψ_s versus tissue ψ_w (Morgan, 1991).

Solute deposition is normally well coordinated with growth (Kutschera, 1991) and in both growing and nongrowing tissues the constancy of solute content over time indicates the existence of solute homeostatic regulation. *lwr1* exhibits increased solute content in both nongrowing, unstressed leaves and seedlings at high and low ψ_w. The altered solute content in nongrowing leaves strongly suggests that the altered solute content of *lwr1* is not solely an effect of altered growth. In unstressed seedlings where substantial growth is occurring, the increased solute content of *lwr1* suggests that the normally rigid homeostatic mechanisms that coordinate growth and solute deposition have been altered. Like *lwr1*, the freezing-tolerant mutant *esk1* has decreased leaf ψ_s, elevated Pro at high ψ_w, and reduced growth compared to wild type (Xin and Browse, 1998), although it does not appear to have the other morphological changes present in *lwr1*. When the water-stress- and ABA-inducible homeobox gene *ATHB7* is constitutively overexpressed the plants are morphologically similar to *lwr1*, although it is not known if these plants have increased solute content (Hjellstrom et al., 2003). Future experiments will need to address the question of whether both the altered growth and increased solute content are direct effects of the *lwr1* mutation. In either case, identification and further characterization of *lwr1* will yield information on how solute deposition is regulated and coordinated with growth.

Because of the possibility of manipulating osmotic adjustment to increase productivity during drought stress, osmotic adjustment has been mainly studied in crop species where a number of studies have identified loci controlling osmotic adjustment (Morgan, 1991; Lilley et al., 1996; Teulat et al., 1998). While these studies have not conclusively shown that enhanced osmotic adjustment is useful in conferring stress resistance (Munns, 1988; Serraj and Sinclair, 2002), they have established that a relatively small number of loci explain most of the variation in osmotic adjustment. Grumet and Hanson (1986) selected barley (*Hordeum vulgare*) isolines based on differences in Gly betaine content. These barley isolines were found to also have alterations in ψ_{s100}, which could not be explained by

the difference in Gly betaine content alone, similar to the altered ψ_{s100} of *lwr1* and *lwr2*. Grumet and Hanson (1986) concluded that Gly betaine content is controlled at least in part by osmoregulatory genes, which determine the overall cellular solute content. Our experiments suggest that Pro accumulation in Arabidopsis may also be controlled in part by osmoregulatory genes such as those proposed by Grumet and Hanson (1986). We now have the advantage that the well-developed molecular genetic resources available in Arabidopsis will allow the LWR1 and LWR2 gene products to be identified. Toward this goal, we have generated mapping populations by crossing *lwr1* and *lwr2* in the Ben ecotype to the Columbia ecotype, and scoring of these populations with appropriate molecular markers is proceeding.

Mechanisms of Osmosensing

In plant low ψ_w responses, the type of stimulus that is sensed and the type of sensors and signal transduction components involved remain to be identified (Wood, 1999). Evidence that loss of turgor is well correlated with ABA accumulation (Pierce and Rashke, 1980; Creelman and Zeevaart, 1985) and the fact that other possible stimuli such as water activity and structure change little over the range of ψ_w sensed by plants (Hsiao, 1973) have led to the idea that a change in turgor is the primary stimuli that elicits ABA accumulation and other low ψ_w responses. The hypothesis that *lwr2* was either less able to sense water loss or turgor or less able to transmit the stimulus and activate downstream responses offers a compelling explanation for the observed phenotypes. A reduced ability to respond to low ψ_w-induced changes in water content or turgor could explain why *lwr2* seedlings accumulated less ABA than wild type at a given RWC as well as the reduced solute content and osmotic adjustment of *lwr2* across a range of ψ_w. In *lwr2* source leaves at high ψ_w, disrupted sensing of turgor or water content may lead to the build up of solutes, either produced by photosynthesis or arriving in the transpiration stream, which otherwise would be exported or metabolized. This in turn would lead to higher leaf turgor in the mutant compared to wild type. The fact that *lwr2* did not show altered response to exogenous ABA suggests the intriguing possibility that *lwr2* acts upstream of ABA action or acts independently of ABA. Also, the observation that *lwr2* did not have increased leaf water loss shows that the reduced solute content and osmotic adjustment of *lwr2* are regulated independently of leaf water loss. This is consistent with previous observations that regulation of stomatal conductance is not dependent on a change in leaf water status (Wilkinson and Davies, 2002) and that ABA-induced stomatal closure is independent of ABA-induced Pro accumulation (Maggio et al., 2002).

In the case of *lwr1*, increased Pro and solute content could be caused by an overactivation of the osmosensing or osmoregulatory mechanism. The reduced expression from the *le25* promoter can be explained by the reduced ABA content of the mutant at −1.2 MPa. The decreased ABA content may have been caused by the higher RWC of *lwr1*. Thus, the reduced ABA accumulation in *lwr1* could be an indirect effect of the increased solute content resulting in reduced loss of water in the mutant compared to the wild type instead of a direct regulatory effect of *lwr1* on ABA content.

MATERIALS AND METHODS

Plant Growth Conditions and Low ψ_w, Allyl Alcohol, and ABA Treatment

The PEG-infused plate system was a modification of that described by van der Weele et al. (2000). All experiments were done without the addition of any carbon source to the media. PEG-infused plates were made by dissolving solid PEG-8000 (Sigma, St. Louis) in a sterilized solution of basal media (half-strength Murashige and Skoog salts with 2 mM MES buffer) and adjusting the pH to 5.7. This PEG solution was then overlaid on agar-solidified (15 g L^{-1} Bacto agar) basal media (3:2, v/v). The agar media and PEG solution were then allowed to equilibrate for at least 12 h before the excess PEG solution was removed. ψ_w of the agar was verified using a vapor pressure osmometer as described below.

Seeds were surface sterilized and spread on basal agar media that had previously been overlaid with a nylon mesh to facilitate transfer of seedlings between plates. After stratification for 3 d at 4°C in low light, plates were moved to a growth room at 23°C having a 16-h light period (light intensity of 150–180 μE cm^{-2} min^{-1}) and kept vertically so that seedlings grew along the surface of the agar. Plates were kept within a Plexiglas enclosure lined with wet paper towels. After 3 d of growth, seedlings were transferred on the nylon mesh to either fresh agar-solidified basal media (unstressed control) or PEG-infused agar.

Allyl alcohol treatment was performed 48 h after transfer of seedlings to a −1.2 MPa PEG-infused agar plate. At this time, plates were removed from the growth room and a solution of allyl alcohol dissolved in basal media was pipetted onto the top of the agar, covering the seedlings. After a 2-h incubation, seedlings were rinsed three times with sterile water, transferred to a fresh plate of agar-solidified basal media, and returned to the growth room. The number of surviving seedlings was scored 7 to 9 d later.

ABA treatments were performed by adding the indicated concentrations of S (+)-ABA (Lomon Bio Technology, Sichuan, China) dissolved in a small volume of ethanol to the basal growth media. Seedlings were transferred to the ABA-containing plates after 3 d of growth on basal media. Controls with ethanol only added to the basal media showed no effect on seedling Pro or ABA content.

Soil-grown plants were kept under long-day conditions (23°C, 16-h photoperiod).

Construction and Mutagenesis of *le25:ADH* Transgenic Arabidopsis

le25, which encodes a late-embryogenesis abundant protein, is induced by low-ψ_w treatment only under conditions of ABA accumulation (Cohen et al., 1991, 1999; Imai et al., 1995) and is partially induced by ABA application at high ψ_w (Imai et al., 1995). Deletion analysis and promoter mutagenesis showed that the *le25* promoter contains a functional ABA-response element as well as at least one other cis-acting element required for full stress induction. The 392-bp fragment from the 3′ end of the *le25* promoter, which was shown to confer full transcriptional activity in transgenic tomato plants (*Lycopersicon esculentum*; Shih, 1998), was fused to the ADH coding region (from plasmid pMY425 provided by the laboratory of M. Yanofsky). The fusion construct was ligated into pBI-101 and was used to transform *Agrobacterium tumefaciens* strain EHA105. The T-DNA fragment also contained a fusion of the *Leu amino peptidase A* promoter from tomato to the green fluorescent protein-coding region, which was not used in this study. An *adh*$^-$ line of Arabidopsis (Bensheim R002 provided by J. Chory) was transformed by root cocultivation. One transgenic line exhibiting stress induction of the *ADH* transgene was

chosen and confirmed to have a single T-DNA insertion by DNA blotting of restriction enzyme digested genomic DNA using a probe to the *ADH*-coding region and by single copy reconstruction (data not shown).

Seeds of this *le25:ADH* transgenic line were ethane methylsulfonic acid mutagenized and M_0 seed planted to soil. M_1 seed was collected as pools each containing seed from 20 to 35 M_0 plants. An aliquot (approximately 600 seeds) from each pool was plated and used for the initial allyl alcohol resistance selection.

Analysis of Plant Water Relations

Measurements of ψ_w and ψ_s were carried out using a vapor pressure osmometer (Model 5100C; Wescor, Logan, UT). ψ_s of plant tissue (approximately 20–50 seedlings or 3 10-mm-diameter leaf discs) was determined by twice freezing and thawing the tissue, grinding and briefly centrifuging to remove insoluble material, and measuring the osmolarity of the cell sap. For ψ_w measurements of soil or leaf tissue, a larger size sample chamber was used. Leaf ψ_w was measured on fully expanded leaves collected during the middle of the dark period. Leaf discs (4 discs of 10-mm diameter) were collected, immediately transferred to the sample chamber, and allowed to equilibrate in the instrument for 3 h. A stable instrument reading was obtained at that time. Solute concentration was converted to ψ_s using the van't Hoff equation $\psi_s = -RTC$, where R is the gas constant, T is absolute temperature, and C is the molar solute concentration. Turgor (ψ_P) was calculated using the equation $\psi_w = \psi_s + \psi_P$.

To quantify RWC, seedlings were removed from the agar plate using the nylon mesh, gently blotted, weighed, and placed in ice-cold water to rehydrate for 2 to 3 h. Seedlings, still on the nylon mesh, were then blotted and reweighed and dried at 65°C for 12 h. After obtaining the total dry weight, the weight of the nylon mesh without seedlings was measured and subtracted from the other weights before calculating RWC. RWC was multiplied by ψ_s or Pro or K^+ concentration to determine the ψ_{s100} or Pro or K^+ concentration at 100% RWC. Because Pro was routinely expressed on a fresh-weight basis, a correction was applied for the percent dry weight (as determined during the RWC measurements) of the seedlings. The correction was small (less than 4%) in all cases and did not affect the interpretation of the data.

To determine leaf water loss, individual rosette leaves were weighed immediately after detachment and then at 30 min or 1 h intervals thereafter. In between measurements, leaves were kept on the laboratory bench. Leaves were collected near the end of the light period and were fully hydrated (RWC $> 97\%$) at the beginning of each experiment.

Metabolite, ADH, and ABA Analysis

Pro was assayed on water-extracted seedling samples (Bates et al., 1973). K^+ was assayed by atomic adsorption spectrometry. ADH activity was assayed by the ethanol-dependent reduction of NAD^+ (Bailey-Serres and Dawe, 1996). ABA was assayed by radioimmunoassay (Bray and Beachy, 1985). For ABA quantification, seedlings were removed from the agar plate using the nylon mesh and twice rinsed (each rinse < 10 s) with a NaCl solution of the same ψ_w as the agar to remove any PEG or exogenous ABA, which may interfere with the assay of seedling internal ABA. Seedlings were then blotted dry and weighed.

Data reported for these assays represent the combined mean of at least 3 independent experiments with two or three samples collected in each experiment. Seedling samples typically consisted of 20 to 100 seedlings (15–200 mg of tissue) depending on assay and experimental treatment. Significant statistical differences were determined by standard two-tailed T-test with *P* values as noted in text or figures. Significant difference of regression lines was determined by F test (www.graphpad.com/curvefit).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We thank Dr. Kelly Hershey for generating the *le25:adh* transgenic line used in this study and completing the mutagenesis; Dr. David Parker for use of equipment and assistance with the K^+ analysis; Drs. Linda Walling, Patricia Springer, and Christina Walters for useful advice and discussion; and Rui Yu, Mayuki Tanaka, Suzie Kim, and Ramon Barajas for assistance in the laboratory.

Received May 6, 2004; returned for revision June 24, 2004; accepted June 24, 2004.

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