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Disruption of the cellulose synthase gene, *AtCesA8/IRX1*, enhances drought and osmotic stress tolerance in Arabidopsis

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Summary

Two allelic Arabidopsis mutants, *leaf wilting 2-1* and *leaf wilting 2-2* (*lew2-1* and *lew2-2*), were isolated in a screen for plants with altered drought stress responses. The mutants were more tolerant to drought stress as well as to NaCl, mannitol and other osmotic stresses. *lew2* mutant plants accumulated more abscisic acid (ABA), proline and soluble sugars than the wild type. The expression of a stress-inducible marker gene *RD29A*, a proline synthesis-related gene *P5CS* (pyrroline-5-carboxylate synthase) and an ABA synthesis-related gene *SDR1* (alcohol dehydrogenase/reductase) was higher in *lew2* than in the wild type. Map-based cloning revealed that the *lew2* mutants are new alleles of the *AtCesA8/IRX1* gene which encodes a subunit of a cellulose synthesis complex. Our results suggest that cellulose synthesis is important for drought and osmotic stress responses including drought induction of gene expression.

Keywords: *AtCesA8/IRX1*, drought and osmotic stresses, cellulose synthesis.

Introduction

Drought and salt stresses are environmental factors that greatly limit crop production and plant distribution in the world. The cellular and molecular responses of plants to drought and salt stresses have been analyzed intensively at biochemical and physiological levels (Shinozaki *et al.*, 2003; Xiong *et al.*, 2002; Zhu, 2002). Both drought and salt stresses can lead to water deficit in plant cells, and delay plant growth and development. Plants have evolved several ways to cope with these unfavorable conditions. The accumulation of compatible osmolytes helps protect cells from the harmful effects caused by the stresses. Among the osmolytes, proline and sugars often accumulate to a high level under water stress conditions (Zhu, 2002). The phytohormone abscisic acid (ABA) also accumulates to a high level under these stresses. Although ABA-independent pathways exist, the increase of ABA concentration in cells would induce the expression of many stress-inducible genes. Moreover, ABA plays vital roles in regulating transpirational water loss by controlling stomatal movement (Xiong *et al.*, 2002).

Plants sense water stress through several different pathways. The organ that is exposed to soil water shortage first is the root. Water stress in roots promotes the production of ABA which can be transported to leaves for regulating stomatal movement (Sauter *et al.*, 2001). Furthermore, low water potentials caused by water deficiency in soil inhibit shoot growth and promote root growth. The increased root:shoot ratio of plants is considered to be an adaptation of plants to water shortage condition as elongated roots can take up more water from the soil (Wu and Cosgrove, 2000). A putative osmosensor localized at the cell membrane was suggested for sensing the osmotic change in Arabidopsis cells (Urao *et al.*, 1999).

Cellulose is the most abundant polysaccharide produced by plants. It constitutes the major part of plant cell walls, determining cell shape and plant morphology. Cellulose is synthesized by cellulose synthase complexes localized on the plasma membrane (Doblin *et al.*, 2002). Work on cellulose synthase catalytic subunits (CesA) in Arabidopsis has identified 10 genes. *AtCesA1* (*RSW1*), *AtCesA2*, *AtCesA3*

(*IXR1/CEV1*), and *AtCesA6* (*IXR2/PRC1*) are suggested to be required for catalyzing the biosynthesis of cellulose deposited to the primary wall (see review, Doblin *et al.*, 2002). *AtCesA4* (*IRX5*), *AtCesA7* (*IRX3/FRA5*) and *AtCesA8* (*IRX1/FRA6*) are essential for cellulose synthesis in the secondary cell well (Taylor *et al.*, 1999, 2000, 2003; Turner and Somerville, 1997; Zhong *et al.*, 2003). Cellulose content is reduced dramatically and secondary cell wall becomes thinner in mutants defective in these genes. The thinner cell wall is unable to withstand the negative pressure produced during the course of water transport, and xylem in *irx1*, *irx3* and *irx5* mutants becomes collapsed (Taylor *et al.*, 1999, 2000, 2003; Turner and Somerville, 1997). Recently, Cochard *et al.* (2004) provided experimental data showing that under severe dehydration, tracheid walls of pine needles were severely collapsed. Their data suggest that wall collapse might be one mechanism for plants to respond to water stress. Nonetheless, there is little direct evidence linking cellulose synthesis and drought stress.

During a screen for mutants with a leaf-wilting phenotype, we isolated two Arabidopsis mutants, *lew2-1* and *lew2-2*. We studied the *lew2-1* allele in detail and found that *lew2-1* plants accumulate higher levels of osmolytes and ABA, and are more drought tolerant than the wild type. The transcripts of stress-inducible marker genes, *RD29A*, *P5CS* (pyrroline-5-carboxylate synthase) and ABA2/*SDR1* (alcohol dehydrogenase/reductase), are more induced in *lew2-1* than in the wild type. We cloned the *LEW2* gene by map-based cloning, and found that it is a new allele of the *AtCesA8/IRX1* gene (Holland *et al.*, 2000; Taylor *et al.*, 2000). Our results suggest that cellulose synthesis plays an important role in plant responses to drought and osmotic stresses.

Results

Isolation of lew *mutants*

We conducted a genetic screen to find mutants that lose water quickly by looking for the leaf-wilting phenotype in an ethyl methyl sulfonate (EMS)-mutagenized Arabidopsis M2 population in a growth room. Plants were grown in pots for 2 weeks at 22°C under 50 μmol m^{-2} sec^{-1} light and then 120 μmol m^{-2} sec^{-1} light at 28°C (see Experimental procedures). Plants showing a leaf-wilting phenotype were selected as putative mutants. In the M3 generation, we confirmed one recessive mutant, *lew1* in a Columbia *gl1* background (*lew1* is not studied in this report). We used the same strategy to recheck the putative mutants previously isolated using the *RD29A-LUC* system (Ishitani *et al.*, 1997). We found two mutants that showed a similar leaf-wilting phenotype, one in a C24 background and another in a Columbia *gl1* background. Both mutants were backcrossed to the wild-type plant in their original background, and the resulting F_1 seedlings exhibited the wild-type phenotypes. F_2 progeny from self-fertilized F_1 plants showed an approximately 3:1 segregation of wild type:*lew2* mutant phenotypes, which indicates that the mutations are recessive and in single nuclear genes. Complementation test by crossing the two mutants revealed that they were allelic and so were named *lew2-1* (C24 background) and *lew2-2* (Columbia background). All subsequent physiological and phenotypic analyses were carried out on *lew2-1* and *lew2-2* mutants that had been backcrossed to the wild type for four times to eliminate other mutations. Both *lew2-1* and *lew2-2* mutants were analyzed simultaneously and showed similar results. Here only results for *lew2-1* are shown.

We observed that the *lew2-1* plants grown under normal conditions (see Experimental procedures for conditions used in this study) showed weaker or no wilting phenotype. However, the leaves of *lew2-1* became wilty after the plants were transferred from normal conditions to higher transpiration conditions for 6 h, whereas wild-type plants did not show the leaf-wilting phenotype under the same conditions (Figure 1a,b). Wilting occurred primarily at the leaf tips and then spread around the leaf margins (Figure 1b,c). We further determined whether water loss was affected in *lew2* mutants by comparing the rates of change of the fresh weight of detached leaves during dehydration. In contrast to leaf wilting, water loss in *lew2-1* leaves was a little slower than in wild-type leaves (Figure 1d). Moreover, *lew2-1* plants showed turgid leaves than wild-type leaves that were seriously wilting after 2 weeks of withholding water (Figure 1e). After more than 1 week drought treatment, all wild-type plants died, whereas all mutant plants survived. These results suggest that *lew2* mutants are more resistant to drought.

The lew2-1 *mutant is more tolerant to osmotic stress in both germination and growth*

Drought-tolerant phenotypes for *lew2* mutants led us to check whether *lew2* mutants are more tolerant to high osmotic stress. Seedlings grown for 4 days on MS medium were transferred to MS medium supplemented with different concentrations of NaCl for 4 days. The *lew2-1* and wild-type seedlings showed no apparent difference with 150 mM NaCl treatment. With 200 or 250 mM NaCl, the cotyledons of *lew2-1* were still green, whereas those of wild-type seedlings became bleached and were dead (Figure 2a). Wild-type plants were killed by 300 mM NaCl, but *lew2-1* plants still survived.

We also tested the growth sensitivity of *lew2-1*seedlings to osmotic reagents such as glycerol and mannitol. Seedlings grown for 7 days on MS medium were transferred to mannitol- or glycerol-containing media. Figure 2(b) shows the effects of high concentrations of mannitol on plants after 1 week. Wild-type plants grew better than *lew2-1* plants on normal MS medium, but their cotyledons became yellow

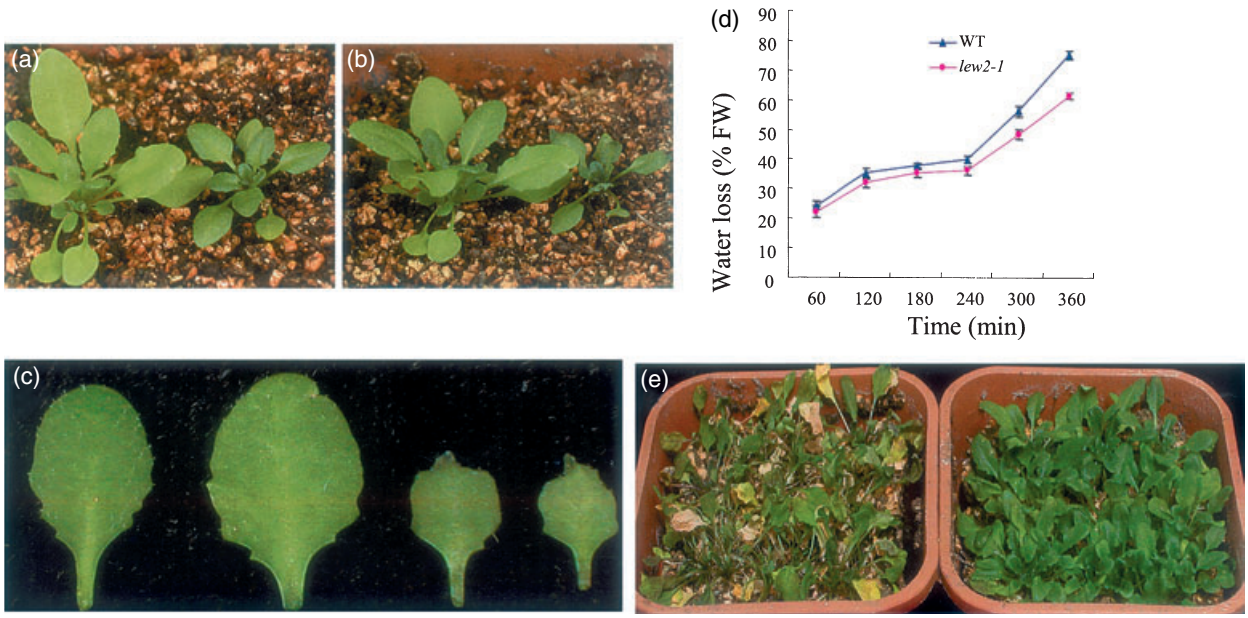

Figure 1. The leaves of *lew2-1* become wilted easily under high intensity light, but *lew2-1* plants are more tolerant to drought stress.
(a) Wild type (left) and *lew2-1* (right) plants growing in a growth room under normal conditions.
(b) Leaf-wilting phenotypes were observed only in *lew2-1* (right) but not in wild-type plants after high-transpiration condition treatment for 6 h.
(c) Closer look at leaves of (b). Leaves of wild-type plants show no wilting. However, leaf wilting was clearly observed at the tips and margins of *lew2-1* leaves but not at the vein bases.
(d) Quantitative determination of water loss of detached leaves. Values are mean ± SE, $n = 3$ independent experiments. Fifteen different leaves at similar stages were used for each experiment.
(e) *lew2-1* (right) plants are more drought tolerant than wild-type plants (left). Seedlings grown for 2 weeks were not watered, and photographs were taken after 2-week treatment.

with 400 mM mannitol, and some true leaves became yellow with 520 mM mannitol (Figure 2b). In contrast, although the growth of *lew2-1* was inhibited by high mannitol concentrations, the cotyledons and leaves of *lew2-1* plants were still green, even at 520 mM mannitol. Similar effects were observed when seedlings were grown on medium

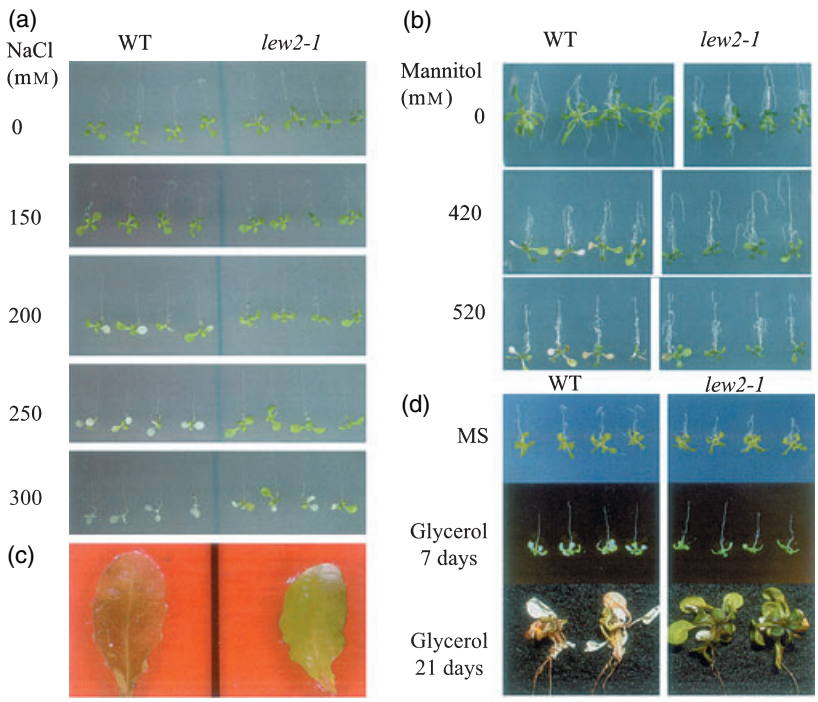

Figure 2. *lew2-1* seedlings are more tolerant to osmotic stress.
(a) Seedlings were grown first on MS agar plates for 4 days and then transferred to agar plates with or without different concentrations of NaCl. Photographs were taken after 4-day treatment.
(b) Seedlings grown for 7 days on MS medium were transferred to MS agar plates without mannitol or with mannitol at different concentrations. Photographs were taken after 1-week treatment.
(c) Phenotype of a wild type (WT) and *lew2-1* leaf soaked in 230 mM NaCl solution for 10 h.
(d) Seven-day-old seedlings were transferred to agar plates supplemented with or without 200 mM glycerol and treated for 1 or 3 weeks.

supplemented with 200 mM glycerol for 1 week (Figure 2d). After 21 days of treatment with 200 mM glycerol, most of the wild-type seedlings were completely killed, whereas virtually all *lew2-1* seedlings survived (Figure 2d).

To further examine the higher capacity of *lew2-1* in coping with osmotic stress, we carried out a plasmolysis experiment using different concentrations of NaCl. When leaves are immersed in a solution of NaCl, plasmolysis occurs, and the leaves become translucent when the concentration of NaCl exceeds a threshold value. The leaves of *lew2-1* grown under normal conditions for 3 weeks withstood a concentration as high as 230 mM NaCl and did not show plasmolysis, but wild-type leaves became completely translucent with NaCl concentrations >150 mM (Figure 2c). Consistent with this result, the osmotic potential in leaves of *lew2-1* grown in normal conditions is -1.09 ± 0.04 MPa compared with -0.77 ± 0.03 MPa in wild type ($n = 4$, $P < 0.01$).

We also wanted to know whether the *lew2* mutation affects seed germination under osmotic stress. We planted the seeds of *lew2-1* and the wild type directly on MS medium supplemented with various concentrations of NaCl, mannitol, sorbitol, or glycerol. As shown in Figure 3, seed germination did not differ between *lew2-1*and the wild type with the MS control medium. With increasing concentrations of each of these reagents, seed germination was inhibited in both *lew2-1* and the wild type. But the inhibition of germination was less in *lew2-1* than in the wild type with all osmotic stress treatments.

The lew2 *mutant contains higher levels of ABA, proline and sugars, and expresses higher levels of stress-related genes*

Because *lew2* mutant plants showed more drought and osmotic stress tolerance, we measured both proline and soluble sugar contents in the mutant. To see whether transpiration is a factor in the accumulation of these compounds, we also measured proline and sugar contents of plants growing under high humidity or in liquid culture medium. Water content in leaves of *lew2-1* is $92.16 \pm 1.13\%$ (w/w, $n = 4$) which is comparable to $92.27 \pm 2.16\%$ (w/w, $n = 4$) in leaves of wild type under normal conditions. So we measured the compound contents by using fresh leaves. As shown in Figure 4(a), under normal conditions which do not produce drought stress in wild-type plants, the proline content in *lew2-1* was more than two times as that in the wild type. But when *lew2-1* plants were grown under high humidity or in liquid culture medium, the proline content decreased to a level that was only slightly higher than wild-type plants. Drought treatment for 2 h slightly increased the proline content in both mutant and wild type. We also determined the content of soluble sugars, another class of compatible osmolytes. As shown in Figure 4(c), the contents of sucrose, fructose, or soluble sugars were all higher in *lew2-1* than in wild-type plants grown in soil, high humidity, or in liquid culture.

Abscisic acid is an important hormone for drought stress in plants. We measured the ABA content under normal

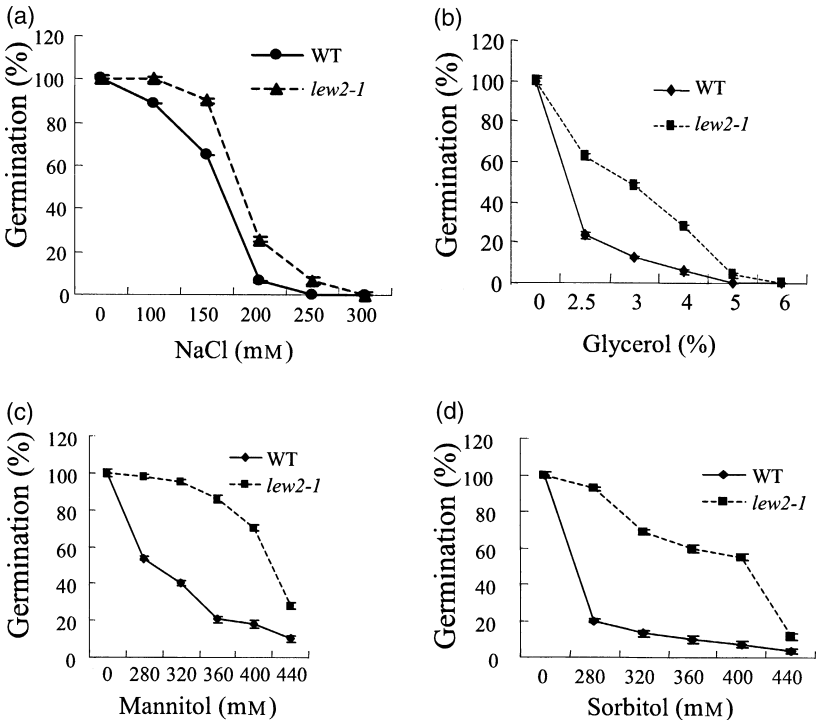

Figure 3. Germination of *lew2-1* seeds is tolerant to osmotic stress. Wild type (WT) and *lew2-1* seeds were planted on MS agar medium containing different concentrations of NaCl (a), glycerol (b), mannitol (c), and sorbitol (d). Plates were transferred to a growth chamber after 3-day imbibition. Germination percentages were determined after 4-day treatment. Values are mean $\pm$ SE, $n = 3$ independent experiments, one experiment used 100 seeds.

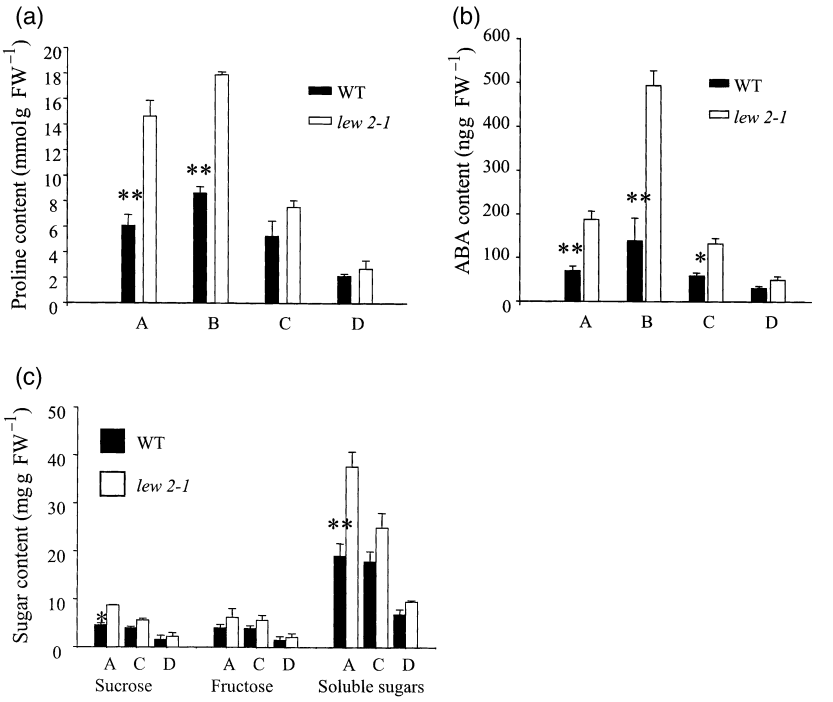

Figure 4. *lew2-1* plants contain a higher level of proline, ABA and soluble sugars than wild-type (WT) plants do. A: normal conditions; B: drought treatment; C: high humidity; D: liquid culture.
(a) Proline contents of WT and *lew2-1* seedlings.
(b) ABA contents of WT and *lew2-1* seedlings.
(c) Sucrose, fructose, and total soluble sugar contents of WT and *lew2-1* seedlings.
Values are mean ± SE, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

growth, drought treatment, high humidity, and liquid culture conditions. The ABA contents in *lew2-1* plants were significantly higher in normal and drought conditions, slightly higher in high humidity and liquid culture than that in wild-type plants (Figure 4b).

In plants, the key step in proline synthesis is catalyzed by the enzyme pyrroline-5-carboxylate synthase (P5CS). We performed Northern blot analysis to examine the transcript level of the *P5CS* gene. Consistent with a high accumulation of proline in the *lew2-1* mutant, the *P5CS* transcript levels were higher in *lew2-1* than in wild-type plants under all tested conditions including normal, high light, drought, and liquid culture conditions (Figure 5).

The alcohol dehydrogenase/reductase (*SDR1*)/*AtABA2* gene catalyzes xanthoxin to generate ABA-aldehyde in the ABA biosynthesis pathway (Xiong and Zhu, 2003). Consistent with higher ABA contents in *lew2-1* mutant, the *SDR1* transcripts were abundant in *lew2-1* than in wild-type plants in normal, liquid culture conditions (Figure 5). We also analyzed the *SDR1* expression level under high transpiration and dehydration treatment conditions. *SDR1* transcripts were induced to a high level in the *lew2-1* mutant, but not in wild-type plants in both treatments (Figure 5).

Under drought stress, some genes are induced to a high level. We selected the *RD29A* gene, which can be induced by stresses such as drought, low temperature, and salt (Yamaguchi-Shinozaki and Shinozaki, 1994). The transcripts of *RD29A* in *lew2-1* were induced to a high level under normal condition, high light condition, drought stress condition, and liquid culture condition, but almost no *RD29A* transcripts were detected in wild-type plants under these conditions (Figure 5). These results indicate that *lew2-1* plants suffer a constant stress even in favorable conditions that have no stress effect on the growth of wild-type plants.

lew2 *mutants are new alleles of the* AtCesA8/IRX1 *gene*

A segregating F_2 population was obtained from a cross between *lew2-1* in the C24 background and the wild type in the Columbia *gl1* background. The *lew2* mutants that

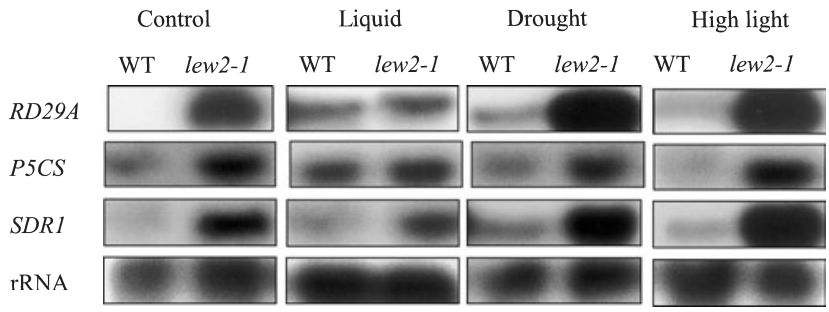

Figure 5. *lew2-1* mutant expresses more *P5CS*, *RD29A*, and *SDR1* transcripts than wild-type (WT) plants. Transcripts of *RD29A*, *P5CS*, and *SDR1* genes were increased to a higher level in *lew2-1* than in WT plants grown under normal and liquid culture conditions or after high light and drought treatments (see Experimental procedures for different treatments).

showed the leaf-wilting phenotype under strong light were selected, and DNA extracted from each mutant was used for genetic mapping with simple sequence length polymorphism markers (data not shown). These studies resulted in mapping *lew2* to the same region as the *AtCesA8/IRX1* gene which encodes a subunit of cellulose synthesis complex on chromosome 4 (Holland *et al.*, 2000; Taylor *et al.*, 2000). Genomic DNA sequencing of the *AtCesA8* gene identified a single mutation of G to A in the *lew2-1* allele, which changes W217 to a stop codon (from TGG to TGA). Therefore, mutation in *lew2-1* may produce either a truncated protein without 297 amino acids (M298 is the first Met after W217) at the N-terminal end, with consequently severely reduced or no activity, or disrupt the AtCeSA8 protein completely. We also sequenced the *AtCesA8* gene amplified from the *lew2-2* mutant and found a C to T mutation, which makes a missense amino acid change from L792 to F792 (CTC to TTC). Compared with the phenotypes of *irx1-1* and *irx1-2* as described by Turner and Somerville (1997), both *lew2-1* and *lew2-2* mutants show a more severe phenotype, with less rigid stems, which are not able to stand upright and easily fall over when grown to a certain height (data not shown). Cellulose content in stems of *lew2-1* is 9.87 ± 0.69 (percentage of stem dried weight, $n = 4$) compared with 33.35 ± 2.67 (percentage of stem dried weight, $n = 4$) in stems of wild-type plants. Therefore, mutations in both *lew2-1* and *lew2-2* may impair protein function more severely than those in *irx1-1* and *irx1-2*.

Cross sections of roots and mature stems showed xylem walls that appear collapsed in *lew2-1* growing in soil under normal conditions (Figure 6). Because the collapsed xylem phenotype displayed in *lew2* mutants may be related to transpiration, we further checked the xylem structure in plants grown in liquid culture medium, when little or no transpiration would occur. The seeds of *lew2-1* and wild-type plants were sterilized and cultured directly in Murashige and Skoog (MS) liquid medium. To keep whole seedlings under the water surface, only a few plants were cultured in one 250-ml flask with 100 ml of medium. Cross sections were cut from the roots of 2-week-old seedlings of *lew2-1* and wild-type plants and viewed under a microscope. The xylem in liquid-cultured root was collapsed in the *lew2-1* mutant but not in the wild type (Figure 6). A similar collapsed xylem was also observed in the veins of leaves (data not shown). These results indicate that the *lew2-1* mutant shows collapsed xylem phenotype in both transpiration and no transpiration conditions.

Due to the collapsed xylem of *lew2* mutants, we measured the sap exudate rate from cut stems. Because both root size and xylem structure may affect liquid flow through the vascular structure, we measured the root weight, number and length of both mutant and wild-type plants grown in

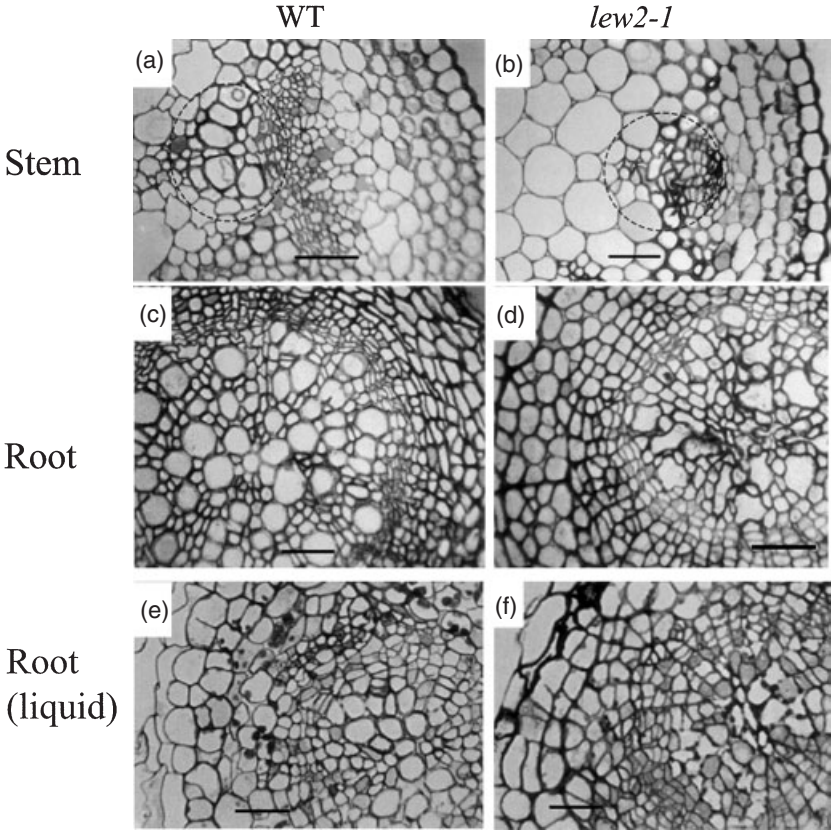

Figure 6. Cross sections of vascular bundles of parts of wild type (WT) and *lew2-1* plants. Stems of WT (a) and *lew2-1* (b) grown under normal conditions. Xylem circled with broken line. Roots of WT (c) and *lew2-1* (d) grown under normal conditions. Roots of WT (e) and *lew2-1* (f) grown in liquid culture medium. Bars = 50 μm.

soil but did not find any apparent difference in roots (Figures 7a,b and 8d), even though the *lew2-1* plants are smaller than wild-type plants (Figure 1a). We selected blooming *lew2-1* and wild-type plants with similar stem sizes and measured the sap exudate rate by covering the plants in a plastic box in order to keep 100% humidity, so that the exudates might be xylem sap due to root pressure (Shi *et al.*, 2002). The rate of sap flowing out from the inflorescence stems of wild-type plants was more than two times that of the *lew2-1* plants (Figure 7c).

Growth and development of the lew2-1 *mutant*

When grown on MS agar plates, in MS liquid culture or in soil for 1 week or on MS medium in the dark for 1 week, *lew2-1* plants grew in a manner similar to wild-type plants (Figure 8a,b). When grown in soil or liquid medium for more than 2 weeks, *lew2-1* plants showed a retarded growth phenotype (Figures 1a and 8c). *lew2-1* plants showed dwarf phenotypes, with dark green and smaller leaves and shorter siliques compared with wild-type plants.

The development of *lew2-1* mutants was greatly delayed; *lew2-1* plants flowered about 2 weeks later and produced fewer seeds than the wild type under normal growth conditions (data not shown). The length of the first internode is not much different between *lew2-1* and wild-type plants (Figure 8e,h), but the length from the second internode is much shorter in *lew2-1* than in the wild type (Figure 8f,h). Consistent with inflorescence stem lengths in different parts, the length of epidermal cells at the base of the stem is similar between *lew2-1* and wild-type plants (Figure 8g,i). However, in the middle and top parts of the stem, the length of epidermal cells is shorter in *lew2-1* than in wild-type plants (Figure 8g,i). Even so, root growth in soil was not affected in *lew2* mutants (Figures 7a,b and 8d), but the roots of *lew2-1* plants grown in liquid were somewhat shorter than that of wild-type plants (Figure 8c). We also grew the *lew2-1* plants under high humidity conditions by covering the plants with a plastic bag so that *lew2-1* plants could grow with less water transpiration. Under these conditions, we found that the dwarf phenotypes were reduced slightly, and the color of *lew2-1* leaves was no longer dark green but almost the same as that of wild-type leaves (data not shown).

Discussion

The molecular and genetic basis of plant tolerance or sensitivity to drought and osmotic stress is poorly understood. The identification and characterization of Arabidopsis mutants have contributed greatly to the understanding of plant growth and development, and responses to some environmental stresses such as salinity (Meyerowitz and Somerville, 1994). However, it has been difficult to identify mutants with strong phenotypes in drought and osmotic stress tolerance or sensitivity. Our results show that *lew2-1* plants are more tolerant to drought and osmotic stresses. Under normal growth conditions in soil, wild-type plants grow very well and do not suffer from water stress, but *lew2-1* plants are affected at both the physiological and molecular levels. Two water stress-related marker genes, *RD29A* and *P5CS*, are constitutively expressed in *lew2-1* but not in wild-type plants grown under normal conditions. *lew2-1* accumulates more ABA, proline and total soluble sugars than the wild type under these conditions.

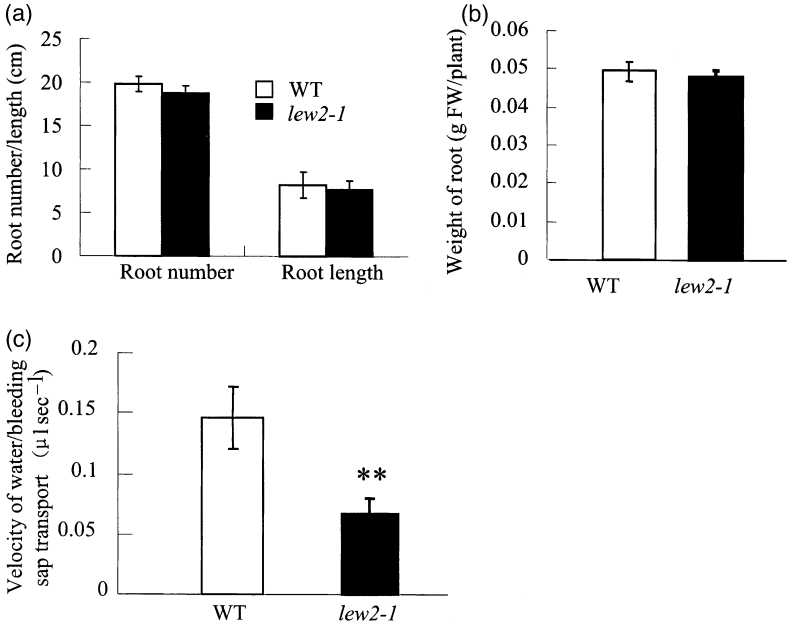

Figure 7. Comparison of velocity of bleeding sap transport in xylem between *lew2-1* and wild-type (WT) plants.
(a) Root number and root length of WT and *lew2-1*. Mature plants grown under normal conditions were carefully removed from soil, and soil was washed out. Roots longer than 5 cm were counted or the length measured. At least 10 plants were used.
(b) Root weight. The total roots from (a) were weighed freshly.
(c) Velocity of bleeding sap transport. About 20 plants, each with about 15-cm length of inflorescence stem, were selected for *lew2-1* and WT. Cross sections were made in the middle of the first internode; the sap was collected for each plant at the first minute. Sap amount was measured by use of a microinjector. Values are mean ± SE, $n = 20$, $^{**}P < 0.01$.

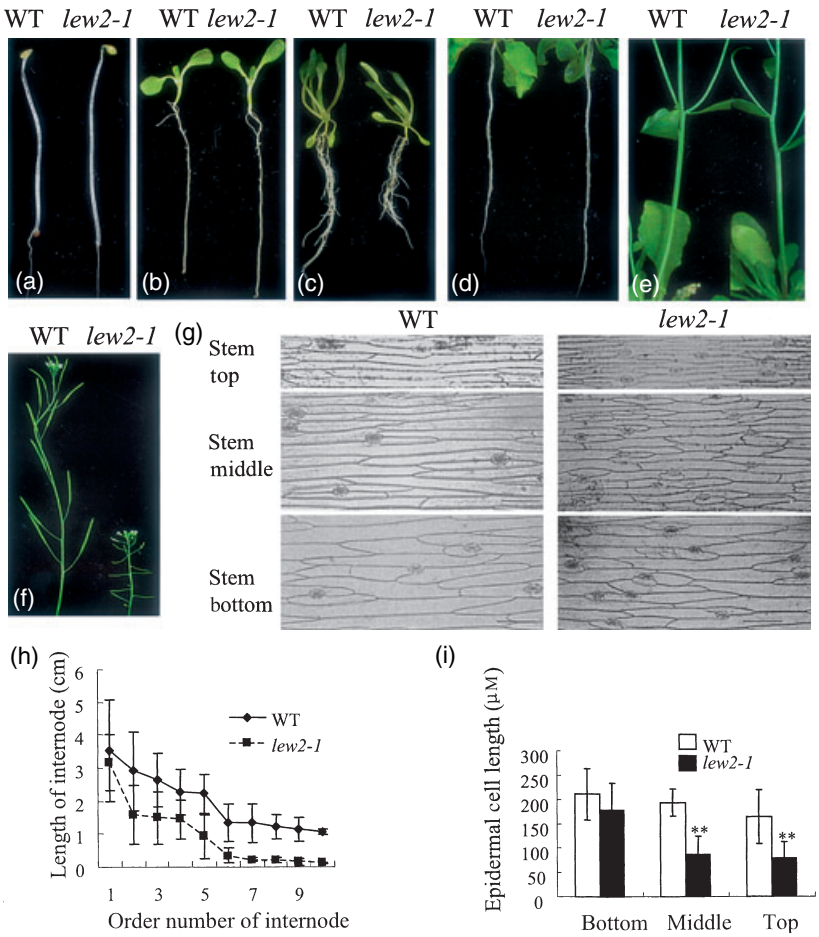

Figure 8. Phenotypic characterization of *lew2-1* plants.
(a) Seedlings of wild type (WT) and *lew2-1* grown in dark for 1 week.
(b) Seedlings of WT and *lew2-1* grown in MS liquid medium for 1 week.
(c) Seedlings of WT and *lew2-1* grown in MS liquid medium for 2 weeks.
(d) Root length of WT and *lew2-1* mature plants grown in soil.
(e) Comparison of the first internode between WT and *lew2-1* plants. Picture of plants of the same developmental stage. *lew2-1* plants flower about 2 weeks later than WT.
(f) Comparison of the florescence stem and silique sizes between WT and *lew2-1* plants.
(g) Comparison of epidermal cells from WT and *lew2-1* mature florescence stems. Bottom, middle of the first internode; middle, middle of the fourth internode from stem base; top, cells between the fifth and sixth silique from the top.
(h) The internode length of WT and *lew2-1* plants. Ten different mature plants were used for determining internode length.
(i) Comparison of epidermal cell length of WT and *lew2-1* mutant in different parts of internodes as in (g). Values are mean ± SE, $n = 30$, $^{**}P < 0.01$.

Map-based cloning identified *LEW2* as a new allele of one subunit of the cellulose synthesis complex. The cell wall cellulose is the main sink of soluble sugars produced by photosynthesis in plants (Babb and Haigler, 2001). Previous studies indicate that drought stress can increase the content of some sugars, probably through inhibiting some enzyme activities involved in cellulose synthesis (Foyer *et al.*, 1998). Mutation in the *AtCesA8/IRX1/LEW2* gene led to the accumulation of soluble sugars to a higher level in cells, and subsequently increased drought and osmotic tolerance. The increase of sugars in cells could stimulate the expression of the *P5CS* gene and ABA synthesis-related gene *SDR1*, and cause the accumulation of proline and ABA which enhance drought and osmotic tolerance (Cheng *et al.*, 2002; Hellmann *et al.*, 2000). Our results suggest that modulating cell wall cellulose synthesis may be one of the main adaptations of plants to drought and osmotic stresses.

One of the main functions of xylem is to transport water from roots to different parts of the plant. It is reasonable to think that the collapsed xylem in mutant plants would impede water transport, but previous studies did not show any direct evidence for this (Turner and Somerville, 1997). Our results clearly indicate that the collapsed xylem in *lew2-1* mutants impedes proper water transport. Impeded water transport in shoots could cause water supply shortage which, in turn, may affect subsequently physiological and molecular changes, including increasing ABA, proline and soluble sugar accumulation in plant cells, because the expression of stress-related genes such as *P5CS*, *RD29A* and some ABA synthesis genes is induced by water stress (Ishitani *et al.*, 1997; Shinozaki *et al.*, 2003). It is generally accepted that at an early stage of drought stress in soil, ABA is synthesized in the roots and transported along the xylem to the shoots as a stress signal to regulate stomatal movement and stress-related gene expression (Sauter *et al.*, 2001). However, when proper water transport is impeded, which can lead to water shortage in shoots or plants are suffering a constant stress as in *lew2* mutants, it is possible that ABA can be synthesized in the shoots and transported down to the roots. After receiving these stress signals, root growth in soil would be promoted (Sauter *et al.*, 2001). This might partly explain why the roots of *lew2-1* grew in a manner similar to wild-type roots in normal conditions. However, *lew2-1* roots in liquid culture were shorter than

wild-type roots, probably because the *lew2* plants in liquid culture medium might produce ABA only through blocking the cellulose synthesis pathway, whereas in normal conditions, *lew2* plants might produce more ABA because of water deficit caused by collapsed xylem in addition to blocking the cellulose synthesis pathway.

It is also possible that the molecular phenotypes seen in *lew2* mutant plants (enhanced ABA content, constitutive expression of *P5CS*, *SDR1*, and *RD29A*) are partly the result of changes in the generation and/or perception of osmotic stress signals, as a consequence of cell wall defect. A previous study in a cellulose synthase-defective mutant *cev1* has suggested that cell wall may signal stress responses in Arabidopsis (Ellis *et al.*, 2002).

The *lew2* mutants are smaller with dark green leaves when grown in normal conditions at later developmental stages. The accumulation of sugars, ABA and proline in *lew2* mutants may be one of the reasons affecting plant growth and development. Transgenic plants with elevated levels of proline and total soluble sugars have increased stress tolerance but often retarded growth (Gilmour *et al.*, 2000). The impeded proper water transport in *lew2-1* mutants would influence cell turgor and in turn diminish cell elongation. A water supply defect in *lew2-1* could explain many of the plant growth and development effects observed under transpiration conditions. The length of the first internode in *lew2-1* is only slightly shorter than that of the wild type, but the length of the second internode is affected much more in *lew2-1* than in the wild type. This observation can be explained in part by the water supply shortage, because more distant parts of the plant would not have as much water transported through the xylem in the mutants. In addition, leaf wilting began at the tip and spread to the margin but did not reach the base of the leaf during short-term treatment under high transpiration conditions. Interestingly, under normal growth conditions, the *lew2* mutants were more drought tolerant than the wild type. The higher accumulation of sugars, ABA and proline in *lew2* mutants may be the main reason for its stress phenotype. However, we did not observe any developmental difference between *lew2-1* or *lew2-2* (data not shown) mutants and wild-type plants at earlier stages (1 week after germination), suggesting that the *AtCesA8/IRX1/LEW2* gene may function differently at different developmental stages.

Compared with *irx1-1* and *irx1-2* (Turner and Somerville, 1997), *lew2-1* might be a more severe mutant. Under normal growth conditions, the collapsed xylem was observed in roots, stems, and leaves (data not shown) of *lew2-1*. Surprisingly, when *lew2-1* plants were grown in liquid culture, where the water transport rate in xylem should be very low because plants are surrounded by water and no transpiration would occur, we still observed the collapsed xylem phenotype in *lew2-1*. Two possibilities could explain this result: *lew2-1* mutation severely affects the deposit of cellulose on the secondary cell walls, which become much thinner, and even a little negative pressure produced by water transport in xylem would alter the shape of the xylem elements. Another possibility is that the negative pressure may be produced by the force difference between the xylem conduit and surrounding cells but not by water transport in this liquid culture condition.

Experimental procedures

Plant growth conditions

lew2-1 and *lew2-2* mutants were identified by examining putative mutants isolated during a screening for deregulation of *RD29A-LUC* gene expression from EMS mutagenesis of the *Arabidopsis thaliana* ecotype C24 (for *lew2-1*) or Columbia *gl1* (for *lew2-2*). Seedlings growing on MS medium for 7 days were transferred to soil and grown in a greenhouse under 50 μmol m^{-2} sec^{-1} light at 22°C under long-day conditions (16-h-light/8-h-dark cycle). For the high transpiration conditions, potted seedlings were placed close to (10 cm below) the white light. In that position the light intensity was approximately 120 μmol m^{-2} sec^{-1}, and temperature was 28°C. In this study, we refer normal growth conditions as 22°C, 16-h-light/8-h-dark cycle, relative humidity 70%, and light intensity 50 μmol m^{-2} sec^{-1}, and high transpiration conditions as 28°C, relative humidity 50%, light intensity 120 μmol m^{-2} sec^{-1} with constant light. The high humidity condition was achieved by covering plants with a plastic bag of 30 × 40 × 35 cm. Inside the bag the light intensity was approximately 24 μmol m^{-2} sec^{-1} and relative humidity nearly 100%. For liquid culture, seeds were sterilized and cultured in 250-ml flasks with 100 ml MS medium supplemented with 30 g sucrose per liter, at a shaking speed of 170 rpm in the same growth room as above. For drought treatments, potted seedlings were grown for 2 weeks with constant watering, then the water was withheld, and pictures were taken after 2-week treatment. For NaCl treatments, 5-day-old seedlings grown on MS medium were transferred to MS medium supplemented with different concentrations of NaCl. For glycerol or mannitol treatment, 10-day-old seedlings were transferred to MS medium supplemented with 10 g l^{-1} agar and 30 g l^{-1} sucrose and different concentrations of glycerol or mannitol.

For seed germination assays, at least 100 seeds from *lew2-1* and wild-type plants were sterilized and planted on MS triplicate plates supplemented with 30 g l^{-1} sucrose, 6 g l^{-1} agar, and different concentrations of NaCl, sorbitol, mannitol, or glycerol. After 2-day stratification at 4°C, the plates were cultured in a growth chamber at 22°C under long-day conditions (16 h light/8 h dark) at 80 μmol m^{-2} sec^{-1} light. The germinated seeds (emergence of radicals) were counted after 4 days.

RNA gel blot analysis

Wild type and *lew2-1* plants were grown in soil under normal conditions for 3 weeks and drawn out of the soil and exposed to dehydration condition for 0 h (control) and 2 h (in a room at 23°C with relative humidity 70%), or plants in soil were treated with high light for 6 h (120 μmol m^{-2} sec^{-1}, 28°C, relative humidity 50%). For liquid culture, wild type and *lew2-1* mutant seedlings were grown in liquid medium at 21°C for 3 weeks. Total RNA was isolated and analyzed as previously described (Gong *et al.*, 1997). The partial *RD29A* fragment (967 bp) was amplified by PCR with the forward

5′-GACGAGTCAGG AGCTGAGCTG-3′ and reverse 5′-CGATGCTG-CCTTCTCGGTAGAG-3′ primers used as probes. The partial fragment (115 bp) of the *P5CS* gene was amplified with the forward 5′-AGCCTTGGCACAGGAGCAACG-3′ and reverse 5′-TGAGACCAG-TGACAGCATCAAACA-3′ primers used as probes. The partial fragment (771 bp) of the *SDR1* gene was amplified with the forward 5′-TGATCACTGGAGGAGCCACAGG-3′ and reverse 5′-CTCCGCTT-ATGTACCGCGAGTC-3′. rRNA on the membrane was used as an equal loading control.

Microscopy

Light and electron microcopy were as described (Wang and Kollmann, 1996) with some modifications. Briefly, leaves, roots, and inflorescence stems of *lew2-1* and C24 were cut into small pieces and fixed at room temperature for 2 h in 5% glutaraldehyde plus 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After being washed in the same buffer, samples were post-fixed overnight at 4°C in buffered 1% osmium tetroxide. After being dehydrated in a graded ethanol series, the samples were embedded in Spurr's resin. For light microscopy, 1-μm-thick sections were cut with glass knives, stained with 0.5% crystal violet and photographed under a light microscope (B5-223.IEP; Motic China Group Co. Ltd., Xiamen, China).

ABA measurement

Rosette leaves (1 g) were excised from 3-week-old mutant and wild-type plants grown under normal conditions, drought treatment (see above for RNA gel blot analysis), high humidity or liquid culture. ABA was extracted immediately (Yang *et al.*, 2001). The isolated ABA was measured by an enzyme-linked immunosorbent assay (ELISA) as previously described (Yang *et al.*, 2001).

Water loss and osmotic potential measurements

Rosette leaves of *lew2-1* and wild-type plants grown under normal conditions for 3 weeks were detached and weighed immediately on a piece of weighing paper and then placed on a laboratory bench (RH 40%) and weighed at designated times. Three replicates were made for each line. The percentage loss of fresh weight was calculated on the basis of the initial weight of the leaves. Ten young rosette leaves (0.25 cm^2) were excised from seedlings of wild type and *lew2-1* plants grown for 3 weeks, respectively. Leaf osmotic potential was measured as previously described (Ruggiero *et al.*, 2004).

Carbohydrate and proline analyses

Leaves of *lew2-1* and wild-type plants grown for 3 weeks under different conditions (normal, high humidity, and liquid culture) were harvested at 10 AM. Sucrose, fructose, and soluble sugars were extracted and determined as previously described (Strand *et al.*, 1999). Otherwise, harvested leaves were treated under dehydration condition for 2 h (see above for RNA gel blot analysis). Proline contents were determined as previously described (Bates *et al.*, 1973).

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