Dolichol Biosynthesis and Its Effects on the Unfolded Protein Response and Abiotic Stress Resistance in Arabidopsis

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Dolichols are long-chain unsaturated polyisoprenoids with multiple cellular functions, such as serving as lipid carriers of sugars used for protein glycosylation, which affects protein trafficking in the endoplasmic reticulum. The biological functions of dolichols in plants are largely unknown. We isolated an Arabidopsis thaliana mutant, lew1 (for leaf wilting1), that showed a leaf-wilting phenotype under normal growth conditions. LEW1 encoded a cis-prenyltransferase, which when expressed in Escherichia coli catalyzed the formation of dolichol with a chain length around C80 in an in vitro assay. The lew1 mutation reduced the total plant content of main dolichols by ~85% and caused protein glycosylation defects. The mutation also impaired plasma membrane integrity, causing electrolyte leakage, lower turgor, reduced stomatal conductance, and increased drought resistance. Interestingly, drought stress in the lew1 mutant induced higher expression of the unfolded protein response pathway genes BINDING PROTEIN and BASIC DOMAIN/LEUCINE ZIPPER60 as well as earlier expression of the stress-responsive genes RD29A and COR47. The lew1 mutant was more sensitive to dark treatment, but this dark sensitivity was suppressed by drought treatment. Our data suggest that LEW1 catalyzes dolichol biosynthesis and that dolichol is important for plant responses to endoplasmic reticulum stress, drought, and dark-induced senescence in Arabidopsis.

INTRODUCTION

Dolichols are long-chain unsaturated polyisoprenoids present in all eukaryotic cells; they consist of 15 to 23 isoprenic units depending on the species (Chojnacki and Dallner, 1988; Swiezewskaa and Danikiewicz, 2005). In animals, dolichols are broadly distributed in different cells. They are localized within the two halves of the phospholipid bilayer, with high contents in the plasma membranes, peroxisomes, Golgi vesicles, and lysosomes. Dolichols exist as free alcohols or products derived by phosphorylation, esterification, or glycosylation (Chojnacki and Dallner, 1988; Swiezewskaa and Danikiewicz, 2005). The biosynthesis of dolichols takes place mainly in the endoplasmic reticulum (ER) and to a lesser extent in peroxisomes (Chojnacki and Dallner, 1988). This biosynthesis is initiated by the formation of farnesyl diphosphate (FPP), primarily through the mevalonate pathway (Chojnacki and Dallner, 1988; Swiezewskaa and Danikiewicz, 2005). The formation of longer chain polyprenyl diphosphate is catalyzed by enzymes called cis-prenyltransferases, which sequentially add isopentenyl diphosphates (IPPs) onto FPP (Grabinska and Palamarczyk, 2002). The number of IPPs added is species-dependent. Polyprenyl diphosphate is further converted to dolichol and dolichyl phosphate by a series of reactions (Grabinska and Palamarczyk, 2002). Dolichyl phosphate serves as a glycosyl carrier lipid in the biosyntheses of protein C- and O-mannosylation, in glycosylphosphatidylinositol anchors, and is one of the rate-limiting factors in N-linked protein glycosylation in yeast and mammalian cells (Burd and Aebi, 1999; Jones et al., 2005). The first eukaryotic cis-prenyltransferase-encoding gene, RER2, which is responsible for dolichol synthesis, was cloned

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from the yeast mutant rer2 (Sato et al., 1999). The multicopy suppressor of rer2, STR1, encodes a protein similar to RER2, but with different physiological roles during cell growth (Sato et al., 2001). rer2 shows many phenotypes, including temperature and hygromycin sensitivity, slow growth, defects in N- and O-glycosylation, and abnormal accumulation of ER and Golgi membranes (Sato et al., 1999). By contrast, the str1 mutant does not have noticeable phenotypes (Sato et al., 2001). Through comparison of cis-prenyltransferase sequences with plant genomic sequences, a homologous gene (AT2G23410) was cloned from Arabidopsis thaliana (Cunillera et al., 2000; Oh et al., 2000). Expression of this gene in yeast rer2 complements its thermosensitive phenotype (Cunillera et al., 2000). A recombinant protein produced from AT2G23410 shows dehydrodolichyl diphosphate synthase activity, and dolichols could be synthesized in an in vitro assay (Cunillera et al., 2000; Oh et al., 2000). It should be noted that Escherichia coli cis-prenyltransferase synthesizes undecaprenyl diphosphate (C10p), which functions as a carrier lipid in cell wall polysaccharide synthesis (Kato et al., 1999). However, no dolichol-deficient mutants have been isolated from any multicellular organisms, including plants, and the physiological roles of dolichols in plants are largely unknown.

N-Glycosylation of secreted or membrane proteins occurs, shortly after synthesis, in the lumen of the ER. Inhibition of N-glycosylation often reduces the folding efficiency, enhances incorrect folding, and accelerates the degradation of the hypoglycoprotein. Chaperone proteins such as BiP (for BINDING PROTEIN) play crucial roles in assisting protein folding during ER stress, which can result from N-glycosylation defects (Wilson, 2002). Increases in secretory activity and in the accumulation of unfolded proteins induce the transcripts of chaperones through the unfolded protein response (UPR) pathway (Wilson, 2002). Recently, two BASIC DOMAIN/LEUCINE ZIPPER (bZIP) transcription factor genes, bZIP60 and bZIP28, were isolated, both of which have been shown to activate BiP expression, probably through ER stress response element-like sequences (Iwata and Koizumi, 2005; Liu et al., 2007a). bZIP60 and bZIP28 are membrane proteins that are anchored to the ER membrane under normal conditions. Upon ER stress, bZIP28, and probably also bZIP60, is cleaved and the bZIP domain translocates to the nucleus to activate the expression of BiP (Iwata and Koizumi, 2005; Liu et al., 2007a).

Drought stress is one of the most damaging environmental conditions that limit plant growth and distribution. Drought stress induces the accumulation of the phytohormone abscisic acid, which, as an endogenous signal, plays crucial roles in mediating plant responses in order to adjust the water deficit (Xiong et al., 2002; Christmann et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). During drought stress, many genes are upregulated. Expression of some of these genes is mediated by abscisic acid, while others are abscisic acid–independent (Xiong et al., 2002; Christmann et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). The expression of BiP was found to be induced by water stress in soybean (Glycine max) (Cascardo et al., 2000), and overexpression of BiP in transgenic tobacco (Nicotiana tabacum) improves plant drought tolerance (Alvim et al., 2001). However, the upregulation of BiP is not necessarily related to increased drought tolerance (Koiwa et al., 2003). Gene expression profiling of soybean leaves shows that some genes are induced by both osmotic and UPR stresses, suggesting that the two pathways may converge in plants (Irsgler et al., 2007). These results suggest complex crosstalk between drought stress responses and the UPR pathway (Alvim et al., 2001; Kiwa et al., 2003; Irsgler et al., 2007).

Through a genetic screen, we isolated the Arabidopsis mutant ler1 (for leaf wilting1). Here, we show that ler1 is altered in a gene encoding a cis-prenyltransferase for dolichol synthesis. ler1 mutant plants showed a leaf-wilting phenotype under normal growth conditions due to impaired membrane integrity. ler1 mutant plants were hypersensitive to tunicamycin and had reduced levels of protein glycosylation. Drought stress induced higher expression of the UPR genes BiP and bZIP60 as well as earlier expression of the abiotic stress–responsive genes COR47 and RD29A in ler1 than in the wild type. Furthermore, drought treatment suppressed dark-induced senescence in ler1. These data implicate LEW1 as having crucial roles in dolichol synthesis, the UPR pathway, and the abiotic stress response in Arabidopsis.

## RESULTS

### ler1 Mutant Plants Exhibit a Leaf-Wilting Phenotype under Normal Growth Conditions Despite Increased Hydraulic Conductivity in Roots

The ler1 mutant was isolated during a genetic screen for leaf-wilting phenotypes (Figure 1A) in an ethyl methanesulfonate–mutagenized Arabidopsis M2 population (Chen et al., 2005). We found that ler1 plants showed a clear leaf-wilting phenotype even under well-watered normal growth conditions. The wilting in ler1 was observed mainly in the margins of old leaves but not in young leaves. This differs from leaf wilting normally caused by water deficit, in that it was not seen in all leaves and was not associated with changed water status of the seedlings. The overall size of ler1 mature plants was smaller than that of wild-type plants (Figure 1A). There was no apparent difference in flowering time between ler1 and wild-type plants (Figure 1B).

In principle, leaf wilting in plants may be caused by (1) reduced water uptake by roots, (2) low water transport through the xylem, (3) high transpiration, (4) low osmotic potential in leaf cells, or (5) impaired cell or membrane integrity. We observed that wild-type and ler1 plants showed similar root:shoot ratios (Figure 1B). This suggested that no major imbalance between water uptake and water loss was created by gross morphological differences between the two genotypes. The intrinsic capacity of roots to take up water (i.e., their hydraulic conductivity $L_p$) was determined from measurements of pressure-dependent sap flow in excised root systems (Boursiac et al., 2005). As shown in Figure 1C, ler1 plants exhibited a significantly greater (+64%) $L_p$, than wild-type plants. Previous studies have shown that aquaporin activity contributes to most of the $L_p$ in Arabidopsis (Tournier-Roux et al., 2003). In order to understand whether the enhanced $L_p$ in ler1 was due to enhanced root aquaporin activity or to enhanced flow through the cell walls, we tested the effects of two independent aquaporin inhibitors, mercury and azide. Mercury is
the most commonly described aquaporin blocker, but azide is even more potent, as it can reversibly block L_pr of wild-type plants by up to 80% (Tournaire-Roux et al., 2003). The results showed that wild-type and lew1 plants had similar levels of inhibition, particularly in the case of azide (Figures 1D and 1E), which meant that most of the increase in L_pr shown by lew1 (+64%) could be accounted for by enhanced aquaporin activity. These results disprove a possible hydraulic limitation of roots in the leaf-wilting phenotype. They further suggest that the lew1 mutation increases the efficiency of water transport across root cells.

We previously showed that leaf wilting in lew2 is caused by impeded water transport due to a collapsed xylem (Chen et al., 2005). To investigate a possible xylem defect, we examined cross sections of vascular bundles in the xylem of stems and leaves and found no difference between lew1 and wild-type plants (Figure 1F). We also performed grafting experiments to test whether leaf wilting in lew1 might have been due to an overall defect of water supply to the shoot. When lew1 shoots were grafted onto wild-type roots, leaf wilting still occurred. By contrast, when grafted onto lew1 roots, wild-type shoots did not show any wilting (Figure 1G).

In conclusion, the leaf-wilting phenotype of lew1 was not caused by defects in root water uptake or xylem transport.

**The lew1 Mutation Impairs Cell Turgor in Leaves by Causing Increased Electrolyte Leakage and Reduces Leaf Transpiration**

In contrast to the increased L_pr, increased transpiration could cause leaf wilting. Surprisingly, we found that stomatal conductance, as measured using a porometer, was reduced by ~75% in lew1 (Figure 2B). Consistent with this, spontaneous water loss from detached leaves was slower in lew1 than in the wild type (Figure 2C). Importantly, lew1 plants grown in soil were more resistant to drought stress than were wild-type plants (Figure 2A).

A closer inspection of leaf epidermis showed that, under normal growth conditions, 60% of stomata were closed in the wilted parts of lew1 leaves but only ~15% of stomata were closed in wild-type leaves (Figures 2D and 2E). Even under conditions (e.g., high light) that promote stomatal opening, when all stomata of wild-type leaves were fully open, most stomata in the wilted parts of lew1 leaves remained partly closed (Figure 2F). By contrast, no apparent difference in stomatal opening was
Figure 2. Analysis of Drought Resistance and Related Parameters in *lew1*.

(A) Comparison of wild-type and *lew1* plant growth under normal (left) and drought stress (right) conditions. For drought treatment, 3-week-old seedlings were subjected to water withholding for 2 weeks.

(B) Stomatal conductance in the wild type and *lew1*. Data are means ± SE. The numbers in parentheses indicate the numbers of analyzed plants. Two to four leaves were used per plant for the wild type, and one to two leaves were used per plant for *lew1*.

(C) Water loss from detached leaves. Three independent experiments were performed, and 15 leaves per genotype were used in each experiment. Data are means ± SE (Student’s *t*-test; ** statistically significant difference [P < 0.01]).

(D) Stomata closure rates of the wild type and *lew1*. For *lew1* plants, stomata from wilting parts were measured. About 40 stomata were measured in each of three experiments for both the wild type and *lew1*. Data are means ± SE (Student’s *t*-test; ** statistically significant difference [P < 0.05]).

(E) Stomata comparison between the wild type and *lew1*. The stomata on the wilting part of a leaf are shown. Bars = 20 μm.

(F) Comparison of stomata in the wild type and *lew1* under conditions that promote full stomatal opening. The stomata from the wilting part of a *lew1* leaf were not open under conditions when all stomata in the wild-type leaf were open. Bars = 20 μm.

(G) Comparison of stomata in the wild type and *lew1*. Stomata on nonwilting parts in the leaf base of *lew1* are shown. Bars = 20 μm.

(H) Osmotic potential in the wild type and *lew1* under normal growth conditions. Values are means ± SE, *n* = 3 (Student’s *t*-test; * statistically significant difference [P < 0.05]).

(I) Comparison of leaf electrolyte leakage between *lew1* and the wild type. Values are means ± SE, *n* = 3 (Student’s *t*-test; * statistically significant difference [P < 0.05]).

(J) Typical recording trace showing measurements of turgor in epidermal cells of a wild-type leaf. Following successful impalement of a cell with the pressure probe, the intracellular recording was continued until a stationary turgor was reached. The trace shows successive recordings in three individual cells.

(K) Mean (± SE) epidermal cell turgor in areas of leaves of wild-type and *lew1* plants showing no obvious sign of wilting (wild type, *n* = 22 cells; *lew1*, *n* = 21 cells). There is a statistically significant difference (P = 0.0005) between the two genotypes.
found in the unwilted parts of lew1 and wild-type leaves (Figure 2G). These results suggest that, in the wilted parts of lew1 leaves, guard cells and possibly surrounding cells were not able to establish a normal turgor. To assess further the basis of the apparent leaf-wilting phenotype of lew1, leaf cell turgor was investigated using the cell pressure probe technique. Because lew1 leaves could show signs of early senescence, the measurements were performed in young, growing leaves that did not show any of these symptoms. Yet, local zones of wilting, mostly in the most distal part of the blade, could be observed in lew1. Among all leaf cell types investigated, the large fusiform epidermal cells located at the edge of the blade yielded the most stable micropipette impalements and turgor recordings. In wild-type plants, cell turgor measurements could be performed all along the leaf edge, yielding a mean stationary turgor value of $P_0 = 4.70 \pm 0.21$ bars $(n = 22$ cells) (Figure 2J). By contrast, impalement of the wilted area in lew1 leaves did not allow any stable turgor recording. Measurements performed in other areas of the leaf provided a mean turgor value of $P_0 = 3.34 \pm 0.29$ bars $(n = 21$ cells), which was significantly lower ($P = 0.0005$) than the value in wild-type leaves. Thus, leaf cells of lew1 showed a significantly reduced turgor, even in areas showing no clear sign of wilting.

Under normal growth conditions, the osmotic potential of lew1 leaves was slightly higher than that of wild-type leaves (Figure 2H). This was probably due to a slight osmotic compensation in the wilted lew1 cells. We then examined cell membrane integrity by measuring electrolyte leakage. As shown in Figure 2I, lew1 leaves had a higher electrolyte ion leakage than did wild-type leaves, suggesting that the lew1 mutation impaired membrane integrity.

In conclusion, these results suggest that the leaf-wilting phenotype of lew1 is due to increased solute leakage caused by cell membrane lesions. The reduced cell turgor would lead to partial stomatal closure and less transpiration in lew1.

Mapping-Based Cloning of the LEW1 Gene

To clone the LEW1 gene, lew1 plants in the Columbia g11 accession were crossed with wild-type Landsberg plants, and F2 progeny was grown in soil under normal conditions. lew1 plants with a leaf-wilting phenotype were selected for genetic mapping. LEW1 was initially mapped to the upper arm of chromosome 1. Fine-mapping narrowed the lew1 mutation to a region covered by four BAC clones, T23J18, F25C20, F12F1, and T28K15 (Figure 3A). Further fine-mapping delimited lew1 on BAC clones F25C20 and F12F1. We sequenced all of the open reading frames from the lew1 mutant in this region and found a single G-to-A mutation in the open reading frame of AT1G11755, which contains seven exons (Figure 3A). The mutation occurred in the second exon and changed GGT (encoding Gly-21) to GAT (encoding Asp-21) in lew1 (Figure 3A).

The cDNA of AT1G11755 was amplified and overexpressed in lew1 and wild-type plants under the control of the cauliflower mosaic virus 35S promoter. We obtained 25 independent transgenic lew1 lines and found that the leaf-wilting phenotype was complemented in 21 of these lines (Figure 3B shows a representative plant of lew1+LEW1). In parallel to this, an Arabidopsis line (SALK_032276) carrying a T-DNA insertion in the second exon of AT1G11755 was obtained from the Arabidopsis stock center. Our failure to obtain homozygous T-DNA insertion plants suggested that complete disruption of LEW1 is lethal. Plants heterozygous for the T-DNA insertion were crossed with the lew1 mutant. The F2 progeny segregated into three different phenotypes, including typical wild-type and lew1 phenotypes, as expected. We also observed a third class of plants that were smaller, showed earlier senescence, and produced fewer seeds than lew1 or wild-type plants (Figure 3Ba). Genetic and DNA sequence analysis showed these plants to be heterozygous for both the T-DNA insertion and the lew1 mutation. RT-PCR analysis indicated that LEW1 transcripts were present in these heterozygous plants, perhaps at reduced levels (Figure 3Bb).

The LEW1 Gene Encodes a Novel cis-Prenyltransferase Protein

The LEW1 gene encodes a polypeptide of 254 amino acids with a predicted molecular mass of 28.52 kD. Because the genes encoding cis-prenyltransferases form a large family with an overall low level of sequence similarity, we selected for comparison only those candidates that were the most closely related to LEW1 (Figure 3C). The previously identified AT2G23410 and yeast RER2 were included in this analysis. Figure 3D shows that in Arabidopsis, LEW1 (AT1G11755) has only one copy, whereas in rice (Oryza sativa), there are two homologs (Os 03G0197000 and Os 02G0197700). The product of AT2G23410, RER2, and a RER2 homolog in E. coli (P60472) belong to another homology subgroup (Sato et al., 1999; Cuiniller et al., 2000; Oh et al., 2000). The analysis indicates that LEW1 is a new member of the cis-prenyltransferase family.

In order to confirm that LEW1 encodes a functional cis-prenyltransferase, we expressed LEW1 in E. coli (Figure 4A) and analyzed its enzyme activity in vitro. The proteins extracted from E. coli carrying LEW1 or an empty vector were incubated with $^{14}$C-labeled IPP and FPP, and the reaction products were analyzed by normal-phase thin layer chromatography (TLC) (Figure 4B). The authentic solanesols with chain lengths of C$_{45}$ and C$_{50}$ were used as standard compounds (Figure 4B). Because E. coli is able to catalyze the synthesis of dolichol with a chain length of C$_{45}$, the labeling of C$_{55}$ products was equally detected in both reactions, as expected. However, larger reaction products were detected in the extract from E. coli expressing LEW1. These products migrated slightly faster than the C$_{50}$ solanesol standards and therefore must be somewhat smaller (Figure 4B). These results indicate that LEW1 possesses cis-prenyltransferase activity for dolichol synthesis in vitro.

A previous study indicated that Arabidopsis AT2G23410 was able to complement the temperature-dependent growth phenotype of yeast mutant rer2 (Cuiniller et al., 2000). We expressed LEW1 and AT2G23410 (as a positive control) in the rer2 mutant and compared growth at 23, 30, and 37°C. As shown in Figure 4C, all of the tested yeast strains grew very well at 23°C in uracil (URA)-containing medium. By contrast, the rer2 mutant, either native or expressing the empty vector, was not able to grow at temperatures higher than 30°C. However, when transformed with a plasmid carrying the LEW1 cDNA under control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, rer2 was able to grow, albeit more slowly than the rer2 strain.
Figure 3. Position Cloning of LEW1 and Sequence Analysis of Long-Chain cis-Prenyltransferases.
expressing AT2G23410. These results indicate that LEW1 can partially complement the high temperature–sensitive phenotype of the rer2 mutant.

Dolichol Contents Are Reduced in lerw1 Mutant Plants but Are Increased in Transgenic Plants Overexpressing LEW1

Dolichols with chain lengths of C75 and C80 have been reported to be the most abundant forms in Arabidopsis (Gutkowska et al., 2004). We performed lipid analysis to compare the dolichol contents among wild-type, lerw1 mutant, and transgenic plants overexpressing LEW1 (lines OE14 and OE34). Figure 5F shows that LEW1 transcripts increased in the two overexpression lines. The dolichols in Arabidopsis exist as free lipids, lipids covalently bound to proteins, or lipid carriers linked to oligosaccharides for protein glycosylation. The total dolichols were extracted and analyzed by liquid chromatography–mass spectrometry (LC-MS) following previously established methods Figure 3. (continued).

Figure 4. Biochemical Characterization of LEW1 and Complementation of the Yeast rer2 Mutant.

(A) Ectopic expression of LEW1 in E. coli. Proteins extracted from E. coli cells were analyzed on 12% SDS-PAGE gels and stained with Coomassie blue. The arrow points to the expressed LEW1 protein in E. coli. M, marker; Con, empty vector control; LEW1, isopropylthio-β-galactoside–induced LEW1. (B) TLC analysis of LEW1 reaction products. [1-14C]IPP and [1-14C]FPP were incubated with total proteins isolated from E. coli strains transformed with either the empty vector (Con) or LEW1. At the completion of the reaction, lipid products were extracted and subjected to TLC analysis. Solanesols C45 and C55 were used as standards. A C55 band (representing a product that can be synthesized by the native cis-prenyltransferase in E. coli) was equally detected in E. coli transformed with either empty vector or LEW1, indicating that the reactions were successful. Ori, original spot. (C) LEW1 partially complemented the temperature-sensitive phenotype of the yeast rer2 mutant. rer2 cells transformed with an empty vector, LEW1, or DPS (AT2G23410) were grown overnight. Serial decimal dilutions were spotted onto plates of synthetic dropout medium with all amino acids (URA+) or excluding URA (URA–). URA is a selection marker of the transformed clone. Plates were incubated at the indicated temperatures and photographed after 4 d.
The lew1 Mutation Impairs Protein N-Glycosylation

Dolichol acts as a lipid carrier during protein glycosylation, and mutations in the yeast RER2 lead to defects in protein glycosylation (Sato et al., 1999). First, we used periodic acid Schiff (PAS) staining to examine the glycoprotein levels (Nanjo et al., 2006) in wild-type and lew1 plants. In total protein extracts separated by SDS-PAGE and stained by PAS (Figure 6A), several relatively small bands were found to be much less abundant in lew1 than in the wild type, indicating that protein glycosylation levels were reduced by the mutation.

We then used concanavalin A-Sepharose, which binds to high-mannose-type N-glycans of glycoproteins, to enrich glycoproteins from the total protein extracts (Koiwa et al., 2003). The enriched glycoproteins were separated by SDS-PAGE. As shown in Figure 6B, more bands were detected in the wild type than in lew1. It is also noticeable that the peptide profile from lew1 was smeared, indicating that proteins in lew1 may be abnormally glycosylated. We further treated the enriched proteins with endoglycosidase H to cleave N-linked glycans, and the products were compared by SDS-PAGE. As shown in Figure 6B, similar bands were detected in both the wild type and lew1, suggesting that the differences were due to changes in protein glycosylation.

Third, we recovered from SDS-PAGE two bands that were present in the total protein extracts from the wild type but not from lew1 (Figure 6B, arrows). Tryptic peptides were resolved by matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) MS (Autoflex II TOF/TOF; Bruker) and analyzed by Mascot at www.Matrixscience.com. The smaller band corresponded to the glycoprotein β-thioglucoside glycohydrolase encoded by TGG1 in Arabidopsis (Xue et al., 1995; Husebye et al., 2002). TGG1 is localized in the vacuole, and underglycosylated TGG1 was also found in the stt3a mutant of Arabidopsis in a previous study (Koiwa et al., 2003). The partial sequence of the larger product was identical to that of a glycoprotein named SKU5 (Sedbrook et al., 2002). SKU5 is localized to both the plasma membrane and the cell wall and is involved in directional root growth in Arabidopsis (Sedbrook et al., 2002). These results further demonstrate that LEW1 plays critical roles in protein glycosylation.

lew1 Mutants Are Hypersensitive to the Glycosylation Inhibitor Tunicamycin

Tunicamycin is a specific inhibitor of UDP-N-acetylgalactosamine-dolichyl-phosphate N-acetylgalactosaminetransferase (EC 2.7.8.15), the first enzyme in the biosynthesis of dolichol-linked oligosaccharides for protein glycosylation modification (Lehrman, 1991; Koizumi et al., 1999). Blocking glycosylation by tunicamycin induces UPR, causing enhanced expression of some UPR pathway genes (Koizumi et al., 1999; Lukowitz et al., 2001; Martinez and Chrispeels, 2003). Because the lew1 mutation impairs protein glycosylation, we tested the sensitivity of lew1 to tunicamycin. As shown in Figure 6C, lew1 plants were more sensitive than wild-type plants to exposure to 0.1 μg/mL tunicamycin. Overexpression of LEW1 in the wild type did not obviously increase the resistance of transgenic plants to tunicamycin, suggesting that LEW1 is not a limiting factor for protein glycosylation.

The lew1 Mutant Is Hypersensitive to Dark Stress

Defects in protein glycosylation greatly affect the biogenesis of membrane and secreted proteins by interfering with their folding, trafficking, degradation, and targeting to their final sites in the cell; therefore, defects in protein glycosylation generally lead to ER stress. We observed that mutant plants with one copy of the T-DNA insertion allele of lew1 and the other copy as the lew1 point mutation allele showed an early-senescence phenotype (Figure 3B). We further tested whether lew1 exhibits a more severe membrane-damage phenotype in the dark, because prolonged dark treatment induces membrane damage and senescence (Fujiki et al., 2000). lew1 and wild-type plants grown under well-watered conditions for 2 weeks were transferred to darkness at 22°C for 10 d. All of the lew1 plants became yellow, but wild-type plants did not. lew1 plants transformed with the LEW1 overexpression construct (3SS-LEV1) exhibited similar phenotypes as wild-type plants (Figure 7A). Electrolyte leakage from leaves of lew1 plants was greatly increased and reached 35% after 10 d in the dark, whereas wild-type leaves showed only ~10% leakage under the same conditions (Figure 7B). These results show that more severe membrane damage occurred in lew1 than in the wild type under prolonged dark treatment.

Because tunicamycin inhibits protein glycosylation, we also investigated the effects of this compound on plant sensitivity to prolonged darkness. Spraying tunicamycin on wild-type leaves accelerated the dark-induced senescence and therefore phenocopied to some extent the dark-sensitive phenotype of lew1 (Figure 7C). In addition, tunicamycin treatment of lew1 leaves rendered their senescence in the dark more pronounced (Figure 7C). The data overall suggest that the lew1 phenotype in the dark is related to a glycosylation defect.

Darkness Stress–Responsive Genes Are Hyperinduced in lew1 by Dark Treatment

We selected two late dark-inducible marker genes, DIN2 (for dark-inducible2) and DIN9, to compare their expression patterns in lew1 and wild-type plants. DIN2 encodes a β-glucosidase, and DIN9 encodes a mannose-6-phosphate isomerase (Fujiki et al., 2000). We also examined a leaf senescence marker gene, YLS4 (for
Figure 5. Analysis of the Relative Dolichol Contents in Wild-Type, lew1, and LEW1 Overexpression Plants by LC-MS.

The peak area in the wild type was taken as 100%, and the relative contents in other plants were compared with that in the wild type.

(A) The dolichol standards (left, Dol-C_{75}; right, Dol-C_{80}). Arrows indicate the peaks.

(B) The wild type.

(C) lew1 mutant.

(D) Overexpression line 14 (OE14).

(E) Overexpression line 34 (OE34).

(F) RNA gel blot analysis of LEW1 expression in OE14 and OE34. Due to its low expression, LEW1 transcript could not be detected in the wild type and the lew1 mutant by RNA gel blot under the conditions used. OE15 and OE21, two other transgenic lines not used in this study, are also included. The bottom panel shows an RNA gel as a loading control.
YELLOW-LEAF-SPECIFIC GENE4, which is induced in old leaves by darkness (Fujiki et al., 2005). Two-week-old seedlings were kept under darkness for 24 and 48 h. RNA gel blot analysis revealed Din2, Din9, and YLS4 transcripts to be substantially induced in lew1 at 24 and 48 h in the dark, whereas the transcripts were not yet or less detected in the wild type and two transgenic lines overexpressing LEW1 at these time points in the dark (Figure 7D).

Drought or Osmotic Stress Suppresses the Darkness Sensitivity of lew1 Plants, and lew1 Seedlings Are More Tolerant to Osmotic Stress

Because lew1 plants are more tolerant to drought stress, we next analyzed the response of lew1 to drought stress in the dark. Ten-day-old seedlings grown in the light were pretreated by withholding water for 7 d prior to the dark treatment, which produced only a modest drought stress. Control plants were watered every third day, and both control and drought-treated plants were kept in the dark or light for various times. After an additional 15 d of drought treatment, wild-type plants grown in the light were seriously wilted (Figure 8A, 15 d in light) and did not recover growth after rewetting. By contrast, lew1 plants, which developed clear drought-tolerant phenotypes in light, were able to regrow after watering (see Supplemental Figure 1 online). These results are consistent with previous data (Figure 2A) and indicate that lew1 is tolerant to drought stress at different developmental stages.

lew1 plants growing under well-watered conditions in the dark for 15 d became yellowish and were seriously damaged (Figure

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**Figure 6.** lew1 Impairs Protein N-Glycosylation, and lew1 Plants Are Hypersensitive to Tunicamycin.

(A) Comparison of protein glycosylation patterns between the wild type and lew1 by PAS staining. Thirty micrograms of total protein were resolved by SDS-PAGE and detected by PAS staining. The arrow points to the bands missing from lew1.

(B) Concanavalin A–Sepharose binding assay for N-glycosylated proteins. Total proteins extracted from the wild type and lew1 were incubated with concanavalin A-Sepharose, and the bound proteins were eluted, treated with (+) or without (−) endoglycosidase H (Endo H), resolved by SDS-PAGE, and stained with Coomassie blue. The arrows at left point to the two protein bands found in the wild type but not in lew1. These two bands were recovered and analyzed by MALDI-TOF MS. The top one corresponds to SKU5, and the bottom one corresponds to TTG1. Endoglycosidase H treatment resolved the proteins found in both the wild type and lew1. M, marker.

(C) lew1 seedlings are sensitive to tunicamycin (TM). Seedlings were germinated on MS medium or MS medium containing 0.1 μg/mL tunicamycin and grown for 10 d in a growth chamber. WT, wild type (gl1); lew1, lew1 mutant; 35S-LEW1, a wild-type line overexpressing LEW1 under the control of the 35S promoter (line 14); lew-35S-LEW1, a lew1 mutant line overexpressing LEW1 under the control of the 35S promoter (line 5).
8A, 15 d in dark). However, lew1 plants that had been pretreated by drought were able to survive the dark treatment and were green and healthy after 15 or even 20 d in the dark (Figure 8A). These plants grew very well after being transferred to normal conditions and irrigated in light (Figure 8B, 20 d in drought + dark). These results indicate that drought stress relieved dark-induced senescence in lew1.

We further tested the growth of lew1 mutant plants on medium supplemented with various osmotic stress agents. As controls, both lew1 and wild-type seedlings grew well on Murashige and Skoog (MS) medium containing 3% sucrose (Figure 8C). When no sugar was added to the MS medium, lew1 seedlings grew poorly and died after 10 d in culture. The growth of wild-type seedlings was also inhibited in the absence of sugar in the medium, but in contrast with lew1, all wild-type seedlings survived and still grew (Figure 8C). When grown on MS medium (without sugar) supplemented with 100 mM mannitol, 180 mM NaCl, or 1.5% glycerol for 10 d, all lew1 seedlings survived, as did the wild type (Figure 8C). These results indicate that hyper-osmotic stress is able to suppress the sensitivity of lew1 seedlings to conditions of no sugar in culture medium.

We also compared the growth of lew1 and the wild type on MS medium with 3% sucrose and different concentrations of NaCl. As shown in Figure 8D, lew1 seedlings showed similar sensitivity to NaCl concentrations of <120 mM as the wild type. However, lew1 seedlings grew better than wild-type seedlings on 180 mM NaCl. This result suggests that unlike stt3a and other N-glycan defect mutants, which are sensitive to salt stress (Koiwa et al., 2003; Kang et al., 2008), lew1 mutants were more resistant to high NaCl than the wild type.

Drought Stress Induces Higher Expression of BiP and bZIP60 in lew1 Than in the Wild Type

Cells have evolved elaborate mechanisms to ensure the accuracy of protein folding and assembly. When unfolded proteins are accumulated to a certain level, UPR signaling is activated to induce the expression of specific proteins to protect cells from the ER stress or to induce cell death if homeostasis cannot be reestablished (Lin et al., 2007). A previous study found that overexpression of BiP improved the drought tolerance of transgenic plants, suggesting that UPR signaling might be involved in
drought stress tolerance (Alvim et al., 2001). In order to determine whether drought stress could provoke UPR signaling or not, we compared the transcripts of genes in the UPR pathway in the wild type and *lew1* under drought stress conditions. We used a BiP probe that detects the transcripts of both *BiP1* and *BiP2* because of their high sequence identity. *BiP* transcripts were hardly detected in the wild type after a 5-h drought treatment under our conditions, whereas the *BiP* transcripts were clearly detected at 2 h and highly expressed at 5 h in *lew1* (Figure 9A). *BiP* transcripts were also induced by a mannitol treatment at higher levels in *lew1* than in the wild type (Figure 9B). bZIP60 is a key bZIP transcription factor responsible for the ER stress response, and its expression is induced by tunicamycin (Iwata and Koizumi, 2005). The expression of *bZIP60* was induced to a higher level in *lew1* than in the wild type under drought treatment (Figure 9A). The expression of *PROTEIN DISULFIDE ISOMERASE (PDI)*, encoding a disulfide isomerase, is also induced by tunicamycin in Arabidopsis (Martinez and Chrispeels, 2003). However, the level of *PDI* did not change under our drought stress conditions (Figure 9A). These results indicate that osmotic stress produced
from either drought or mannitol treatment can activate the expression of some genes in the UPR pathway, and this response is enhanced by the lew1 mutation.

Because of the sensitivity of lew1 to dark stress, we further tested whether the expression of the UPR marker genes BiP and bZIP60 is induced by dark treatment. RNA gel blot analysis indicated that the expression of neither BiP nor bZIP60 was detected in the wild type or lew1 under dark treatment (data not shown). These results suggest that the dark treatment is not able to provoke the expression of UPR pathway genes.

Stress-Responsive Genes Are Induced to Higher Levels and at Earlier Time Points by Drought Stress in lew1 Than in the Wild Type

We further investigated whether the lew1 mutation also alters the expression of drought-responsive genes. In these experiments, 4-week-old seedlings were removed from soil and subjected to different water stress treatments, and the expression of the drought-responsive genes RD29A and COR47 was analyzed by RNA gel blots. Under drought conditions, the transcripts of RD29A and COR47 were induced to higher levels in lew1 than in wild-type plants (Figure 9C, Dro1 and Dro2). In addition, NaCl and mannitol treatments resulted in earlier induction of RD29A and COR47 in lew1 than in the wild type (Figure 9D).

Because tunicamycin treatment causes ER stress, we also tested the expression of RD29A and COR47 under tunicamycin treatment. We detected higher expression levels of RD29A and COR47 in lew1 than in the wild type (Figure 9D). Because tunicamycin treatment causes ER stress, we also tested the expression of RD29A and COR47 under tunicamycin treatment. We detected higher expression levels of RD29A and COR47 in lew1 than in the wild type (Figure 9D).

DISCUSSION

Here, we report on the genetic and functional analyses of LEW1, a gene encoding a long-chain cis-prenyltransferase in dolichol biosynthesis in Arabidopsis. There are 10 cis-prenyltransferases...
related to the yeast RER2, and LEW1 belongs to a subgroup that is different from the other 9 cis-prenyltransferases in Arabidopsis. LEW1 is an essential gene in Arabidopsis, since plants with a homozygous T-DNA insertion in LEW1 were nonviable. In yeast, RER2 and STR1 encode two similar cis-prenyltransferases catalyzing the biosynthesis of dolichols with different chain lengths: RER2 for C70 to C85 and STR1 for C95 to C100 (Sato et al., 1999, 2001). The rer2 mutant showed various phenotypes that were not observed in the str1 mutant. Although STR1 overexpression could suppress the growth and glycosylation defects of rer2, the biological functions of RER2 and STR1 are considered to be different (Sato et al., 1999, 2001). These results suggest that different cis-prenyltransferases in Arabidopsis might play distinct roles in plant growth and development.

Our in vitro assay indicated that LEW1 is able to catalyze the biosynthesis of dolichol with a chain length of less than C90. Comparison of total dolichol contents between lew1 and the wild type showed that the dolichols with chain lengths of C75 to C80 were reduced to about one-quarter of the wild-type level, suggesting that LEW1 plays a predominant role in catalyzing the biosynthesis of dolichol C75 to C80 in Arabidopsis. In mammals, the chain lengths of dominating dolichyl phosphates used as glycosyl carrier lipids are around C85 to C105 (Schenk et al., 2001). Our results indicate that the chain lengths of dolichols in Arabidopsis are similar to those in yeast. Although LEW1 catalyzes the biosynthesis of dolichols with chain lengths similar to those produced by RER2, overexpression of LEW1 only partially complemented the temperature-sensitive phenotype of rer2. By contrast, AT2G23410, which catalyzes the formation of dolichol with a chain length of C105, was able to complement the rer2 mutant phenotype (Cunillera et al., 2000; Oh et al., 2000). Similarly, a cDNA that encodes a cis-prenyltransferase for catalyzing the production of dolichols with a chain length of C95 to C105 from human brain complemented the growth and other phenotypes of the rer2 mutant (Shridas et al., 2003). Studies on the rer2 mutant overexpressing SR71 indicated that yeast cells are able to utilize the longer dolichols in protein glycosylation when shorter ones (C70 to C80) are lacking (Sato et al., 2001). Sequence analysis suggested that LEW1 was more diverged from RER2 than AT2G23410. We speculate that LEW1 expressed in yeast might have low catalytic efficiency for dolichol synthesis, possibly because the LEW1 protein may not be folded correctly in yeast or LEW1 may need some specific cofactors or modifications for full activity.

One of the important functions of dolichols is to serve as the lipid carrier of carbohydrates in the assembly of Dol-P-linked oligosaccharides for protein N-glycosylation (Burda and Aebi, 1999). In plants and animals, some genes involved in the dolichol-related N-linked glycosylation pathway have been identified, and defects in the human genes lead to serious diseases called congenital disorders of glycosylation (Koiva et al., 2003; Kranz et al., 2004, 2007; Leroy, 2006). In this study, we found that the lew1 mutation reduced protein glycosylation levels and identified two specific proteins with altered glycosylation. There are 10 RER2-related cis-prenyltransferase homologs in Arabidopsis, and at least one of them, AT2G23410, was shown to also be able to catalyze the biosynthesis of dolichols in an in vitro assay (Cunillera et al., 2000; Oh et al., 2000). It is not known yet whether all of these genes play some roles in N-linked protein glycosylation as LEW1 does.

More than 70% of all proteins in eukaryotic cells are predicted to be N-glycosylated (Apweiler et al., 1999). Genes encoding some of the enzymes functioning at various steps of the protein glycosylation pathway have been characterized. These include, for example, GT1 (for N-ACETYL GLUCOSAMINYLTRANSFERASE I, encoded by COMPLEX GLYCAN1 [CGL1]) (von Schaewen et al., 1993; Wenderoth and von Schaewen, 2000; Strasser et al., 2005), GMII (an α-mannosidase II, encoded by HYBRID GLYOSYLCATION1 [HGL1]) (Strasser et al., 2006), STT3a (Koiva et al., 2003), DGL1 (for DEFECTIVE GLYCOSYLATION1), an Arabidopsis homolog of an oligosaccharyltransferase complex subunit (Lerouxel et al., 2005), CYT1 (for CYTOKINESIS-DEFECTIVE1, mannose-1-phosphate guanylyltransferase) (Lukowitz et al., 2001), GCS1/KNF (glucosidase I), and RSW3 (glucosidase II; glucosidases I and II trim the terminal glucose of N-glycans). Mutations in these genes have some overlapping, yet differential, effects on plant growth, development, and stress responses. For instance, a lesion in GT1 caused by the cgl1 mutation impairs the synthesis of Golgi-modified complex N-linked glycans, and cgl1 mutant plants do not show phenotypes under normal conditions but are more sensitive to heat stress or dark treatment (von Schaewen et al., 1993). Similarly, hgl1 mutants do not show any obvious phenotypes under normal conditions (Strasser et al., 2006), whereas dg1 and cyt1 mutants do (Zablackis et al., 1996; Bonin et al., 1997; Lukowitz et al., 2001; Lerouxel et al., 2005). Interestingly, some mutants, such as dg1, gcs1/knf, rsw3, and cyt1, show a clear cell wall–defective phenotype, suggesting that N-glycan processing plays crucial roles in plant cell wall formation. Mutations in STT3a, a subunit of the oligosaccharyltransferase complex responsible for protein N-glycosylation, may also affect the cell wall, particularly under salt stress (Koiva et al., 2003). stt3a mutants are hypersensitive to salt and osmotic stress due to cell swelling and cell cycle arrest at the root tip (Koiva et al., 2003). The stt3a mutations were also reported to cause increased expression of BinP in the root tip (Koiva et al., 2003). lew1 mutant plants were not hypersensitive to salt or osmotic stress. Rather, the mutant showed increased drought resistance and enhanced survival under high-salt conditions. These results suggest that defects in different steps of the protein N-glycosylation pathway have different consequences on plant stress responses.

Although we compared the amounts of cellulose and lignin from lew1 and wild-type cell walls and did not find a clear difference (see Supplemental Figure 2 online), we cannot exclude the possibility of a subtle change in cellulose or other cell wall components in lew1 that could not be detected in our experiments. Recent studies suggest that the plant cell wall could be important for stress signaling (Ellis et al., 2002; Zhong et al., 2002; Chen et al., 2005; Hernandez-Blanco et al., 2007; Li et al., 2007). Mutations in genes for cell wall biosynthesis or modifications can activate jasmonate, ethylene, and/or abscisic acid signaling, all of which could be connected to sugar responses (Ellis et al., 2002; Zhong et al., 2002; Chen et al., 2005; Hernandez-Blanco et al., 2007; Li et al., 2007). Our results on lew1 suggest possible complex crosstalk among the different signaling pathways, which may regulate the various stress responses observed in this study.
Dolichols are involved in many different cellular processes. In addition to their roles in protein glycosylation and other modifications, free dolichols also have important biological functions as lipids (Chojnacki and Dallner, 1988; Bizzarri et al., 2003). Because it is an unsaturated lipid, dolichol was postulated to function as a free radical scavenger against reactive oxygen species either produced by chemicals or UV light or accumulated upon aging. However, there is no direct evidence for such a function in vivo (Bergamini et al., 1998; Bizzarri et al., 2003; Sgarbossa et al., 2003). Although we did not observe any phenotypes, such as sensitivity to H$_2$O$_2$ treatment (see Supplemental Figure 3 online), we still cannot exclude that the pleiotropic phenotypes observed in lew1 might be a comprehensive outcome of changing lipid peroxidation, plasma membrane characteristics, protein dolichylation, and/or protein glycosylation.

A striking phenotype, which served in the initial isolation of lew1, is leaf wilting even under normal growth conditions. We found that the leaf wilting was not due to a defect in water uptake by roots or in water transport from root to shoot through the xylem. The phenotype was also not caused by excessive transpiration or a low leaf cell osmotic potential. On the contrary, lew1 plants displayed enhanced water permeability in roots and had a reduced stomatal conductance. Our results suggest that the main cause of the leaf-wilting phenotype is probably cell membrane lesions that result in electrolyte leakage and thus impair the ability to maintain cell turgor. The turgor defect may explain, at least partly, the reduced stomatal conductance and enhanced drought resistance of the lew1 mutant. The cell membrane lesions reflect a critical role of dolichol in membrane function and/or its function in protein glycosylation. Because virtually all membrane proteins and secreted proteins are folded and assembled in the ER before being exported and transported to final destinations in the cell, defects in protein glycosylation would greatly affect protein folding, induce ER stress, and eventually reduce the levels and/or activities of certain membrane proteins. Membrane trafficking is known to play crucial roles in abiotic stress responses (Carter et al., 2004). One of the two identified proteins that are affected by lew1, SKU5, is localized in the plasma membrane (Sedbrook et al., 2002). Other plasma membrane proteins, such as H$^+$-ATPases and aquaporins, which are critical in turgor maintenance, may also be altered by the lew1 mutation.

The increased dark sensitivity of lew1 and the early-senescent phenotype exhibited by heterozygous plants carrying lew1 and a T-DNA insertion also support membrane defects in the mutant. The dark-inducible genes DIN2 and DIN9 and the late senescence marker YLS4 were induced to higher levels in lew1 than in the wild type by the dark treatment. Leaf senescence is considered to be a type of programmed cell death, with plasma membrane breakage as an earlier symptom (Lim et al., 2007). In yeast, defects in protein N-glycosylation induce apoptosis (Hauptmann et al., 2006). Apoptosis, or programmed cell death, is strongly induced by reduced activities of ER and the Golgi apparatus in plant cells (Crosti et al., 2001; Malerba et al., 2004). The fact that tunicamycin-treated wild-type plants mimicked the leaf-senescence phenotype of lew1 in the dark suggests that protein N-glycosylation defects in lew1 contribute to the increased dark-sensitivity and early-senescent phenotypes of the mutant. Nevertheless, dark treatment did not upregulate UPR pathway genes. Interestingly, drought but not tunicamycin treatment greatly improved the survival of lew1 plants during prolonged dark treatment. This suggests that drought treatment suppresses the dark sensitivity of the mutant by a mechanism independent of the UPR pathway.

The transcriptional upregulation of a large number of genes involved in the UPR pathway is the most prominent strategy for the cell to cope with ER stress (Urade, 2007). BIP is a chaperone protein that is induced by chemicals such as the specific N-glycosylation inhibitor tunicamycin (Koizumi et al., 1999). Previous studies have indicated that UPR pathway genes are important for both biotic and abiotic stress responses (Alvim et al., 2001; Wang et al., 2005; Fujita et al., 2007; Liu et al., 2007b). In this study, we observed that drought stress induced the expression of the UPR pathway genes BIP and bZIP60, and the induction was much stronger in lew1 than in the wild type. On the other hand, tunicamycin treatment was able to induce the expression of the drought-responsive genes RD29A and COR47, with stronger induction in the lew1 mutant. These stress marker genes were also more responsive to drought in the mutant. Taken together, these results suggest that drought stress may cause ER stress and activate UPR responses, and part of the drought responses (e.g., induction of RD29A and COR47) may be mediated by the UPR pathway. The enhanced inducibility of UPR genes and drought-responsive genes in lew1 may contribute to the drought resistance and salt/osmotic tolerance phenotypes of the mutant.

**METHODS**

**Plant Growth Conditions**

*Arabidopsis thaliana* ecotype Columbia (gl1) was grown in long-day conditions (16-h-light/8-h-dark cycle) with forest soil and vermiculite (1:1) or on MS medium (M5519; Sigma-Aldrich) containing 3% (w/v) sucrose and 0.8% (w/v) agar at 22°C. All seeds were kept at 4°C for 3 d before sowing. For drought treatment in soil, we grew Arabidopsis seedlings in short-day conditions (12-h-light/12-h-dark cycle) at 22°C. For drought treatment, 10-d-old seedlings in soil were not watered for 7 d to give a modest drought treatment in the light. Then, the plants were moved to darkness for 15 or 20 d, or continually grown in the light for 15 d, without watering. At the end of treatment, plants were watered and moved to light for an additional 3 d. For osmotic stress, 4-d-old seedlings grown on MS medium with 3% sucrose were moved to MS medium without sucrose, with sucrose (3%), or without sucrose but with 100 mM mannitol, 180 mM NaCl, or 1.5% glycerol and cultured for 7 d. For salt treatment, 4-d-old seedlings were moved to the MS medium containing 3% sucrose with different concentrations of NaCl (0, 75, 120, or 180 mM) and cultured for 10 d.

**Isolation of the lew1 Mutant and Genetic Analysis**

Ethyl methanesulfonate–mutagenized M2 seeds were sterilized with 0.5% NaClO for 15 min, washed five times with distilled water, sown on MS medium, kept at 4°C for 3 d, and then moved to a plant growth chamber to grow for 5 d. The seedlings were finally transferred to soil in a greenhouse. After growing for 2 weeks, water was withheld from the seedlings. lew1 was chosen because it showed the leaf-wilting phenotype. lew1 was backcrossed with the wild type, and F2 plants showed a...
segregation of ~1:3 lew1:wild type (a total 159 seedlings counted, 38 lew1 and 121 wild-type seedlings), indicating that lew1 is recessive and caused by a single nuclear gene. The lew1 mutant was backcrossed with the wild type four times before being used for detailed analysis. We ordered the SALK_065628 heterozygous seeds from the ABRC. We isolated genomic DNA from the progeny of SALK lines and performed PCR analysis using primer pair 5'-CCGAGACGTCGCTACACTCTC-3' and 5'-GCTTCCGTCGAGCGCAAC-3', which covered the T-DNA insertion region, but did not find the homozygous lines, suggesting that the null mutation of LEW1 is lethal. Crossing the heterozygous T-DNA line with lew1, we recovered the heterozygous plants in which the LEW1 gene was impaired by both lew1 mutation and T-DNA insertion. RT-PCR was used to compare the LEW1 transcripts in the wild type, lew1, and a heterozygous plant of lew1 crossed with the T-DNA line. LEW1 transcripts were amplified for 30 cycles with the primers 5'-GGTCTAGCGAGAAAGGCGCATG-3' and 5'-GGGAAACCGAGTGGTCCCTC-3', using the first-strand cDNA reverse transcribed from mRNAs (M-MLV RTase cDNA synthesis kit, D6130; Takara). Products were visualized on ethidium bromide-stained gels.

**Genetic Mapping and Complementation**

lew1 mutant plants were crossed with the Landsberg accession, and 1638 mutant plants were chosen from the F2 generation according to the leaf-wilting phenotype. Simple sequence length polymorphism markers were designed according to the information in the Cereon Arabidopsis Polymorphism Collection and used to analyze recombination events (Bell and Ecker, 1994; Jander et al., 2002). The lew1 mutation was first mapped to chromosome 1 between T7123 and F2J6. To narrow the lew1 mutation, markers in BAC clones F21M12, T23J18, T28K15, F21M12, T20H2, T19E23, and F14M2 were designed. Finally, the lew1 mutation was delimited between BAC clones F25C20 and F12F1. We sequenced all candidate genes in this region from the lew1 mutant and compared the sequences with those in GenBank in order to find the lew1 mutation.

For complementation of the lew1 mutant, LEW1 cDNA was amplified from reverse-transcribed cDNA with the primer pair 5'-CACCTATG-GATTTGAAATACGATGCGGTCCTC-3' (added CACC at the 5' end) and 5'-GATCTCTGAAATCTGGTCTAGCACC-3'. The amplified fragment was cloned into Gateway vector pMDC32 (Curts and Grossniklaus, 2003) with the Gateway Technology system (Invitrogen). Then, the cloned construct was transformed to *Agrobacterium tumefaciens* and transferred into plants using the floral dip method (Clough and Bent, 1998).

**RNA Gel Blot Analysis**

Shoots of 4-week-old seedlings from soil were dipped into solution containing 300 mM mannitol or 150 mM NaCl for different times. For drought treatment, the removed shoots were put on a laboratory bench for 1 h, which resulted in the loss of ~20% of their water (relative air humidity was ~70%) (drought 1 h), then the seedlings were covered with a transparent film in order to prevent seedlings from quickly losing water for 1 or 4 h (drought 2 h and 5 h). Shoots lost ~30% of their water at the end of drought treatment for 5 h. For dark treatment, seedlings in soil were moved to a plant growth chamber with no light. After brief sonication, 1.5 units of potato (*Solanum tuberosum*) 8RI at 16°C for 5 min. When the mixture was cooled to room temperature, it was ligated to pQE-2T vector that had been excised with BanHI and EcoRI at 16°C overnight. The modified pQE-2T vector has the multiple clone sites BanHI, KpnI, SacI, NotI, SphI, Ncol, SalI, XbaI, HindIII, and EcoRI. LEW1 cDNA was amplified with forward primer 5'-GGGAGATGCGGCGGTTAGA-3' and reverse primer 5'-GCTCTAGAGTGACATTGAG-3' and then cloned to a modified vector pQE-2T in BanHI and XbaI sites. The plasmids were transformed into BL21 *Escherichia coli* cells, and the bacteria were induced at 37°C with 1 mM isopropyl-thio-β-galactoside for 4 h, centrifuged at 3200g at 4°C for 15 min, and suspended in the buffer (pH 8.0) containing 50 mM Tris-HCl, 0.25 M NaCl, 1 mM EDTA, and 1 mM DTT. Phenylmethylsulfonyl fluoride to a final concentration of 0.5 mM was added, and the suspended bacteria were broken with an ultrasonic crusher (Sonics and Materials; model vcv500). NaCl to a final concentration of 0.5 mM was added to the broken bacteria, which were then centrifuged at 3200g at 4°C for 60 min. The upper phase was used for LEW1 activity assays as described previously (Sato et al., 1999) with some modifications. The proteins were analyzed on 12% SDS-PAGE gels. One hundred microliter reaction mixtures including 100 μg of crude proteins, 25 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 1.25 mM DTT, 2.5 mM sodium orthovanadate, 1.4 nmol of farnesyl pyrophosphate (F6892; Sigma-Aldrich), and 5 nmol of [1-14C] isopentenyl pyrophosphate (I4143; Sigma-Aldrich) were incubated at 30°C for 1 h, and then 2 μl of chloroform:methanol (2:1) was added to stop the reaction. Next, 0.8 μL of 0.9% NaCl was added to the reaction mixtures to extract the organic (lower) phase, which was washed three times with chloroform:methanol:water (3:4:8:47) and dried under nitrogen.

The dried samples were dissolved with 5 μL of buffer containing 100 mM sodium acetate (pH 4.8), 0.1% (v/v) Triton X-100, and 60% (v/v) methanol. After brief sonication, 1.5 units of potato (*Solanium tuberosum*) acid phosphatase was added to treat the products at room temperature overnight. Lipid products were extracted three times with 2 mL of n-hexane. All extracts were washed twice with water and dried under nitrogen. The final products were dissolved in 100 μL of n-hexane and analyzed by reverse-phase thin layer chromatography on a Silica Gel
G-60 plate (Merck) developed with benzene/ethyl acetate (90:10). The standard solaneols (C<sub>46</sub> and C<sub>49</sub>; Sigma-Aldrich) were marked under 254-nm UV light, and the radiolabeled products were detected with x-ray film for 15 d.

**Analysis of Dolichols in Arabidopsis**

Dolichol analyses were performed using LC-MS as reported previously (Skorupinska-Tudek et al., 2003; Gutkowska et al., 2004). Briefly, 150 mg of freeze-dried seedlings was ground and extracted three times with 5 mL of CHCl<sub>3</sub>:methanol (1:1). After centrifugation, 1.5 mL of methanol and then 1.5 mL of 20% (w/v) KOH were added to the organic layer and residues. After incubating at 80°C for 1 h, the lipids were extracted three times with 4 mL of hexane. The extracts were analyzed by HPLC/electrospray ionization-MS. A mixture of dolichols 13 to 21 was purchased from Avanti Polar Lipids and used as a standard compound. The experiments were repeated three times, and similar results were obtained. Here, we show one representative result.

**Complementation Study with Yeast and Plants**

LEW1 cDNA was excised from pGEX-2T with BamHI and HindIII and cloned into yeast vector p426-GPD. DPS (AT2G23410) cDNA was amplified from RNA-transcribed cDNA product with primer pair 5'-CGG-GATCCATGTTGTCTCTTCTTTCTTCTGATTCTTCTCTATTATCAC-3' and 5'-GCCGTGACTCAACCCGACAACAAATCGTCTTTCC-3'. The amplified cDNA was cut by BamHI and SacI and then cloned into p426-GPD. The cloned constructs and empty vector were transformed into yeast mutant strain SNH23-7D (MATalpha rer2-2 mfalpha1::ADE2 mfalpha2::TRP1 bar1::HI3 HIS3 ade2 trp1 his3 leu2 ura3 lys2; American Type Culture Collection catalog number MYA-2727) by the lithium acetate procedure (Gietz and Woods, 2002). Selected URA<sup>+</sup> transformants were grown at 320 kPa.

**Water Transport Measurements**

Lp<sub>0</sub> was calculated from 1000 replicates are shown. Bootstrap values calculated on the basis of the initial weight. Leaf osmotic potential of 4-week-old seedlings was measured as described previously (Guo et al., 2002). Three independent experiments were done.

**Phylogenetic Analysis**

We used the LEW1 amino acid sequence to query the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). The amino acid sequences were aligned using MEGA (version 4.0) (Tamura et al., 2007 with the default settings. The alignment is shown in Supplemental Data Set 1 online. A phylogenetic tree was constructed with the neighbor-joining method using the default settings of MEGA (version 4.0; http://www.kumarlab.net/publications) (Tamura et al., 2007). Bootstrap values calculated from 1000 replicates are shown.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers NC_003070 (LEW1/AT1G11755) and NM_127905 (AT2G23410).

**Supplemental Data**

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

**Supplemental Figure 1.** Phenotypes of <i>lew1</i> and Wild-Type Plants under Drought Treatment after Rewatering.

**Supplemental Figure 2.** Acid Detergent Fiber (ADF) Contents of <i>lew1</i> and Wild-Type Plants.

**Supplemental Figure 3.** Comparison of <i>lew1</i> and Wild-Type Plants on H<sub>2</sub>O<sub>2</sub> Medium.

**Supplemental Data Set 1.** Multiple Alignments of LEW1 Homologs.
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