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# The Plant Cuticle Is Required for Osmotic Stress Regulation of Abscisic Acid Biosynthesis and Osmotic Stress Tolerance in *Arabidopsis* [W]

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**Osmotic stress activates the biosynthesis of abscisic acid (ABA). One major step in ABA biosynthesis is the carotenoid cleavage catalyzed by a 9-*cis* epoxycarotenoid dioxygenase (NCED). To understand the mechanism for osmotic stress activation of ABA biosynthesis, we screened for *Arabidopsis thaliana* mutants that failed to induce the *NCED3* gene expression in response to osmotic stress treatments. The *ced1* (for 9-*cis* epoxycarotenoid dioxygenase defective 1) mutant isolated in this study showed markedly reduced expression of *NCED3* in response to osmotic stress (polyethylene glycol) treatments compared with the wild type. Other ABA biosynthesis genes are also greatly reduced in *ced1* under osmotic stress. *ced1* mutant plants are very sensitive to even mild osmotic stress. Map-based cloning revealed unexpectedly that *CED1* encodes a putative α/β hydrolase domain-containing protein and is allelic to the *BODYGUARD* gene that was recently shown to be essential for cuticle biogenesis. Further studies discovered that other cutin biosynthesis mutants are also impaired in osmotic stress induction of ABA biosynthesis genes and are sensitive to osmotic stress. Our work demonstrates that the cuticle functions not merely as a physical barrier to minimize water loss but also mediates osmotic stress signaling and tolerance by regulating ABA biosynthesis and signaling.**

## INTRODUCTION

The phytohormone abscisic acid (ABA) regulates many aspects of plant growth and development as well as responses to the environment (Borsani et al., 2002; Finkelstein et al., 2002; Zhu, 2002; Nambara and Marion-Poll, 2005). Plants maintain a low level of ABA under normal growth conditions. This level of ABA may be required for normal plant growth and development since in strong ABA-deficient mutant backgrounds, or when ABA signaling is blocked, plants have difficulty surviving even under relatively normal growth conditions (Fujii and Zhu, 2009). In response to environmental stress, such as drought or high salinity, ABA levels increase dramatically to cope with the stress. This stress regulation of ABA biosynthesis primarily occurs at the transcriptional level, although other levels of control, such as protein stability or activity, conjugation, and catabolism, may also play important roles in regulating ABA levels (Finkelstein et al., 2002; Xiong and Zhu, 2003; Okamoto et al., 2009; Cutler et al., 2010).

De novo ABA biosynthesis in higher plants proceeds through the cleavage of a $C_{40}$ carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Taylor et al., 2000; Seo and Koshiba, 2002; Schwartz et al., 2003; Nambara and Marion-Poll, 2005; Finkelstein and Rock, 2008). All these steps in the ABA biosynthesis pathway may be regulated to various extents, as it was discovered that ABA biosynthesis genes are upregulated both by stress and by ABA (Xiong et al., 2001, 2002; Xiong and Zhu, 2003). The stress regulation and self-regulation of the pathway may thus provide plants a mechanism for rapid response to environmental challenges. Although ABA biosynthesis may be regulated at multiple steps, the cleavage step catalyzed by a 9-*cis* epoxycarotenoid dioxygenase (NCED) is generally considered to be the rate-limiting step (Tan et al., 1997; Qin and Zeevaart, 1999; Iuchi et al., 2001). Thus, determining how *NCED* genes are regulated by osmotic stress will be instrumental for understanding the mechanisms of plant acclimation to stress.

To begin to dissect the regulatory circuits for stress induction of ABA biosynthesis, we adopted a genetic approach using an *Arabidopsis NCED3* promoter driven firefly luciferase (LUC) reporter system to isolate mutants defective in stress regulation of ABA biosynthesis. Since osmotic stress is an important factor for drought stress, and osmotic stress treatment could be administered in a more quantitative and reproducible way using

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polyethylene glycol (PEG) in agar media, we chose to investigate osmotic stress induction of the *NCED3:LUC* reporter gene. In this study, we identified and characterized a regulator of *NCED3* expression, CED1 (for 9-*cis* epoxycarotenoid dioxygenase defective 1). In *ced1* mutant plants, the induction of *NCED3* by osmotic stress was greatly reduced. The mutant plants also had a lower ABA level and were extremely sensitive to osmotic stress. Unexpectedly, *CED1* was found to encode a putative $\alpha/\beta$ hydrolase domain-containing protein and is allelic to *BODYGUARD* (*BDG*), which has been shown to be essential for cutin synthesis and disease resistance. Although it has long been known that cuticle plays important roles in minimizing water loss and increasing plant resistance to both biotic and abiotic challenges, the discovery of BDG/CED1 as important for osmotic stress induction of ABA biosynthesis and osmotic stress tolerance suggests that this thin physical barrier is actively involved in signaling of not only biotic but also abiotic stresses to enhance plant survival under adverse environmental conditions.

## RESULTS

### *ced1* Mutant Plants Are Impaired in Osmotic Stress–Induced ABA Accumulation

To understand the mechanisms of stress regulation of ABA biosynthesis, we took a genetic approach to analyze stress induction of the *NCED3* gene. Transgenic *Arabidopsis thaliana* plants expressing the firefly LUC reporter gene under control of the *NCED3* promoter (*NCED3:LUC*) (referred as the wild type hereafter) were generated, and the resulting seeds were mutagenized using ethyl methanesulfonate. We initially screened and obtained a large number of mutant lines with an altered regulation of *NCED3:LUC* in response to osmotic stress. In subsequent generations, however, the expression of the LUC reporter gene in these mutants and the wild type was silenced probably due to increased DNA methylation in the *NCED3* promoter region of the transgene since treatment with the DNA methylation inhibitor 5-Aza-C could restore *NCED3:LUC* expression (see Supplemental Figure 1 online). With the expectation that changes in *NCED3* expression may affect ABA biosynthesis and stress resistance, we rescreened these putative mutant lines for altered ABA accumulation and resistance to low water potential stress. This resulted in the identification of a group of mutants that show lower levels of ABA accumulation compared with the wild type when treated with osmotic stress. One such mutant, *ced1*, was chosen for further characterization in this study.

*ced1* mutant plants were backcrossed with wild-type plants. All the F1 plants displayed a wild-type phenotype in osmotic stress response, and about three-quarters of the F2 progeny from self-pollinated F1 showed a wild-type phenotype (data not shown). The result suggests that *ced1* is a recessive mutation in a single nuclear gene.

Wild-type and *ced1* seedlings grown side-by-side on a half-strength Murashige and Skoog (MS) nutrient agar medium were measured for their ABA content under either normal or osmotic stress conditions. Under normal conditions, ABA in *ced1* seedlings was found to be at a low level similar to that in the wild type (Figure 1A). To impose osmotic stress on the seedlings, 16-d-old seedlings grown in MS control plates were transferred to plates containing 40% PEG (average molecular weight 8000) solution. When incubated for 6 h in PEG solution, ABA levels in the wild-type seedlings increased dramatically. However, ABA levels in *ced1* seedlings were considerably lower than in the wild type under the same treatment conditions (Figure 1A), indicating that the mutant was impaired in osmotic stress–induced ABA accumulation.

In *Arabidopsis*, the *NCED3* gene was induced by osmotic stress and the encoded enzyme is thought to control the level of ABA under osmotic stress (Iuchi et al., 2001). To ascertain whether the lower ABA accumulation in *ced1* mutant plants under osmotic stress is a result of reduced *NCED3* expression, we examined the steady state levels of the endogenous *NCED3* transcript.

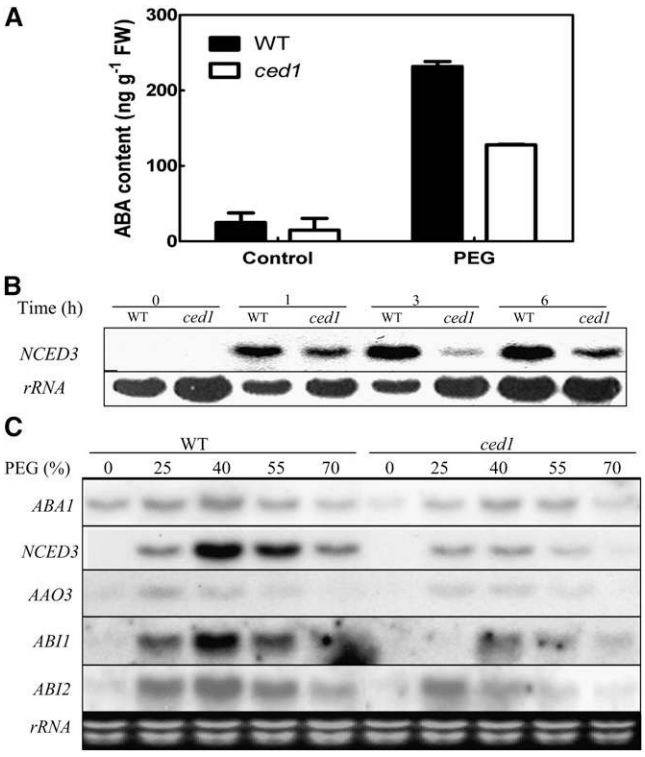


**Figure 1.** The *ced1* Mutation Decreased ABA Accumulation and the Expression of ABA Biosynthesis and Signaling Genes in Response to Osmotic Stress Treatment.

**(A)** ABA contents in control or PEG-treated seedlings. Sixteen-day-old seedlings were transferred to plates containing 40% PEG solution for 6 h. Data are means ± SE ($n$ = 4 to 8). FW, fresh weight; WT, wild type.

**(B)** RNA gel blot analysis of a time course of *NCED3* expression in *ced1* and wild-type plants after transferring to 40% PEG. Twenty micrograms of total RNA were used per lane. rRNA was used as loading control.

**(C)** Transcript levels of ABA biosynthesis and signaling genes in response to osmotic stress in *ced1* and wild-type seedlings. Sixteen-day-old plants grown on MS agar plates supplemented with 3% sucrose were transferred to plates containing PEG solutions of various concentrations and incubated for 1 h. Ethidium bromide–stained rRNAs were used as loading control.

Sixteen-day-old seedlings of wild-type and *ced1* mutant plants were treated with 40% PEG solution for various time periods, and total RNA was extracted for RNA gel blot analysis. Indeed, the *NCED3* transcript level was found to be substantially lower in the *ced1* mutant than in the wild type (Figure 1B).

### Osmotic Stress Induction of ABA Biosynthesis and Signaling Genes in *ced1*

Since *ced1* was impaired in osmotic stress induction of *NCED3* expression and ABA accumulation, we further determined the transcript levels of other ABA biosynthesis genes under osmotic stress conditions. These genes include *ABA1* (encoding zeaxanthin epoxidase) (Marin et al., 1996) and *AAO3* (encoding ABA aldehyde oxidase) (Seo et al., 2000) in addition to *NCED3*. Sixteen-day-old seedlings were treated with various concentrations of PEG for 1 h, and RNA gel blot analysis was performed. As shown in Figure 1C, the transcript levels of *ABA1*, *NCED3*, and *AAO3* in both the wild type and *ced1* were clearly upregulated by PEG-imposed osmotic stress, yet the levels decreased as the PEG concentrations further increased, perhaps as a result of severe damage to the seedlings. Nonetheless, the expression levels of these three genes were clearly lower in *ced1* than in the wild type under the same treatment conditions. These data suggest that *CED1* is required for full induction of multiple ABA biosynthesis genes by osmotic stress.

Since the *ced1* mutation compromises stress induction of ABA biosynthesis genes, we were interested in testing whether it also affects the regulation of early ABA signaling genes. The ABA signaling pathway is mediated by ABA receptors in the PYR1/RCAR1 family, which, upon binding ABA, inhibit the type 2C protein phosphatases, such as ABI1 and ABI2 (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). *ABI1* and *ABI2* are negative regulators of the ABA signaling pathway (Gosti et al., 1999; Saez et al., 2004) and are induced by osmotic stress. Consistent with earlier observations, *ABI1* and *ABI2* were induced by osmotic stress in the wild type, and they were also induced in *ced1* plants (Figure 1C). However, the expression levels of these two genes were substantially lower in *ced1* than in the wild type, indicating that osmotic stress induction of these ABA signaling genes also requires normal CED1 functions (Figure 1C).

### Reduced Expression of Osmotic Stress–Responsive Genes in *ced1*

We performed quantitative RT-PCR (qRT-PCR) to examine the expression levels of a number of osmotic stress–responsive genes, such as *RD29A*, *KIN1*, *COR15A*, *RD22*, *RAB18*, and *P5CS1*. These genes had low basal levels of expression in the absence of stress treatment but were strongly upregulated by PEG treatment in the wild type (40% for 6 h) (Figure 2A). In *ced1* mutant plants, these genes showed no upregulation or much weaker upregulation in response to the same PEG treatment (Figure 2A). Interestingly, we also observed that expression of *RD29A*, *RD22*, *COR15A*, and *COR47* was lower in the *ced1* plants compared with the wild type in response to cold stress (see Supplemental Figure 2 online). These results suggest that CED1 may play an important role in regulating gene expression in response to osmotic stress and cold stress. To obtain a global view of the impact of *ced1* mutation on gene regulation, we performed a microarray experiment using the Affymetrix ATH1 Genechips. RNA was extracted from both *ced1* and wild-type seedlings that were treated with PEG (40%) for 6 h. Approximately 319 genes were found to have statistically significant changes in their expression levels in *ced1* mutant compared with the wild type (see Supplemental Data Set 1 online). We categorized these genes into 19 functional groups using the gene ontology search tool (http://www.Arabidopsis.org/tools/bulk/go/index.jsp) with manual adjustment when necessary (see Supplemental Table 1 online). We observed an overrepresentation of genes related to ABA response (see Supplemental Figures 3 and 4 online). A majority of the genes that showed higher expression levels in *ced1* under osmotic stress in our ATH1 array experiments (see Supplemental Data Set 1A online) are not responsive to ABA and are downregulated by osmotic stress in wild-type plants according to publicly available expression data (see Supplemental Figure 3 online). By contrast, most of the genes that showed lower expression levels in *ced1* under osmotic stress in our ATH1 array experiments (see Supplemental Table 1B online) are upregulated by ABA or osmotic stress in wild-type plants according to publicly available expression data (see Supplemental Figure 4 online). Clearly, CED1 regulates ABA-responsive gene expression under osmotic stress.

To validate the microarray results, we used qRT-PCR to analyze the expression of six genes that were found to show a significant expression difference in our microarray experiments. In agreement with the microarray data, the qRT-PCR analysis showed that At1g29395 (*COR414-TM1*), At3g05640 (*PP2Cs*), At5g15970 (*KIN2*), At5g52300 (*RD29B*), and At5g59320 (*LTP3*) were expressed at lower levels, while At5g45820 (*PKS18*) was expressed at a higher level in *ced1* than in the wild type under the osmotic stress treatment (Figure 2B). There was no significant difference in the expression of these genes under the untreated control conditions. These results indicate that *CED1* is important for osmotic stress induction of a large number of genes.

### Germination and Seedling Growth of the *ced1* Mutant Is More Sensitive to Osmotic Stress

The reduced osmotic stress induction of ABA biosynthesis genes and other osmotic stress-responsive genes in *ced1* may affect stress tolerance of the mutant. We tested the sensitivity of seed germination and early seedling growth to osmotic stress. In the absence of osmotic stress, the germination of *ced1* mutant seeds was similar to that of the wild type (Figure 3A). In the presence of osmotic stress (PEG-infused agar medium), seed germination of both *ced1* and the wild type was delayed. However, the germination rates of *ced1* seeds were substantially lower. For instance, at an osmotic potential of −0.7 MPa, only 19% of *ced1* seeds, compared with 78% of the wild type, germinated. At −1.2 MPa, none of the mutant seeds could germinate, yet 21% of the wild-type seeds were still able to germinate (Figure 3A).

In addition to inhibiting seed germination, osmotic stress also affects seedling growth. There is no obvious morphological difference between the wild type and *ced1* when growing under

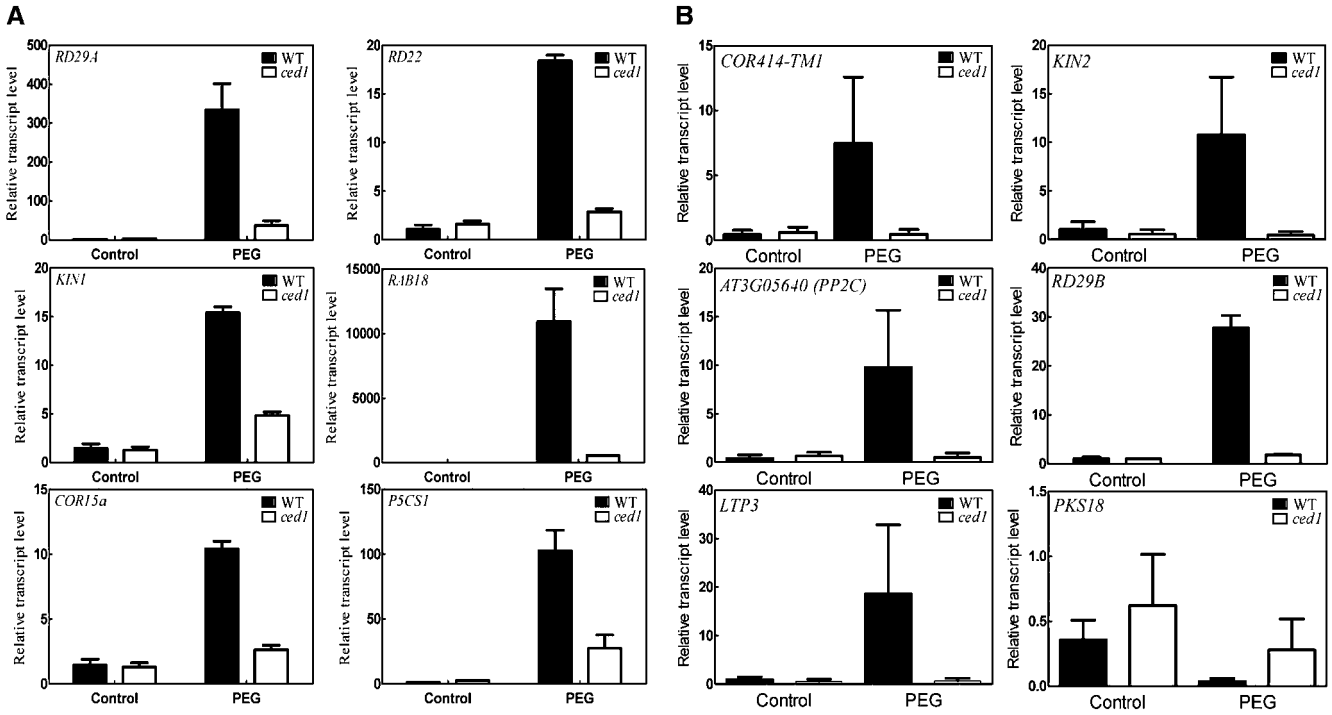


**Figure 2.** Expression of Osmotic Stress–Responsive Genes in *ced1* and Wild-Type Plants.

The qRT-PCR analysis was performed with total RNA from *ced1* and wild-type (WT) plants under untreated control or osmotic stress treatment (40% PEG, 6 h) conditions. Real-time RT-PCR quantifications were normalized to the expression of *TUB8*. Error bars represent SE from three independent experiments.

**(A)** qRT-PCR analysis of representative stress-responsive genes.

**(B)** Confirmation by qRT-PCR of the expression of genes showing significant changes between the wild type and the *ced1* mutant in microarray analysis.

control conditions in the agar plate (Figure 3B). At −0.5 MPa of osmotic potential in the medium, both shoot and root growth of *ced1* seedlings was greatly inhibited, in contrast with the much milder impacts on wild-type seedlings (Figure 3B). These results indicate that *ced1* mutant plants are more sensitive to osmotic stress during seed germination and early seedling development.

### *ced1* Plants Show Higher Transpiration Rates and Reduced Drought Resistance

When the aboveground parts were detached from roots at the rosette stage, the younger leaves of *ced1* plants withered within 20 min under our room conditions (22 ± 2°C, ~30% RH). By contrast, wild-type leaves largely remained turgid under the same conditions (Figure 3C). This observation suggests that *ced1* mutant plants may have a higher transpiration rate. Measurement of leaf water loss showed that *ced1* mutant plants lost water much faster than wild-type plants (Figure 3D). Previous studies have shown that the *snrk2.6*/*ost1* mutant was impaired in stomatal closure and had faster water loss rate than wild-type plants (Mustilli et al., 2002; Yoshida et al., 2002; Fujii et al., 2007). We found that the *ced1* mutant had even higher water loss rate than *snrk2.6* (Figure 3D). The greatly increased transpiration rate of *ced1* leaves may affect drought resistance of the mutant.

To determine their drought resistance, wild-type and *ced1* mutant plants were grown for 3 weeks in soil and then subjected to water withholding for an additional 10 d. At this time, *ced1* mutant plants displayed obvious drought-stressed phenotypes, such as leaf wilting and senescence, whereas the wild-type plants were still turgid and their leaves remained green (Figure 3E). When the plants were rewatered and allowed to recover for 2 d, almost all *ced1* mutant plants were dead, but the wild-type plants survived (Figure 3E). Quantitative analysis found that ~98 and 10% of wild-type and *ced1* mutant plants survived the treatment, respectively (Figure 3F).

### Map-Based Cloning of the *CED1* Locus

The *ced1* mutant phenotypes suggest a crucial role for *CED1* in osmotic stress regulation of ABA biosynthesis and drought stress resistance. To determine the molecular nature of *CED1*, we took a map-based cloning approach to isolate the *CED1* gene. A segregating F2 population was generated from a cross between *ced1* (in the Columbia background) and the wild type Landsberg *erecta*. A total of 734 *ced1* mutant plants were selected from the F2 population, and DNA was extracted from each plant for genetic mapping. *CED1* was mapped to the lower arm of chromosome 1 between the simple sequence length

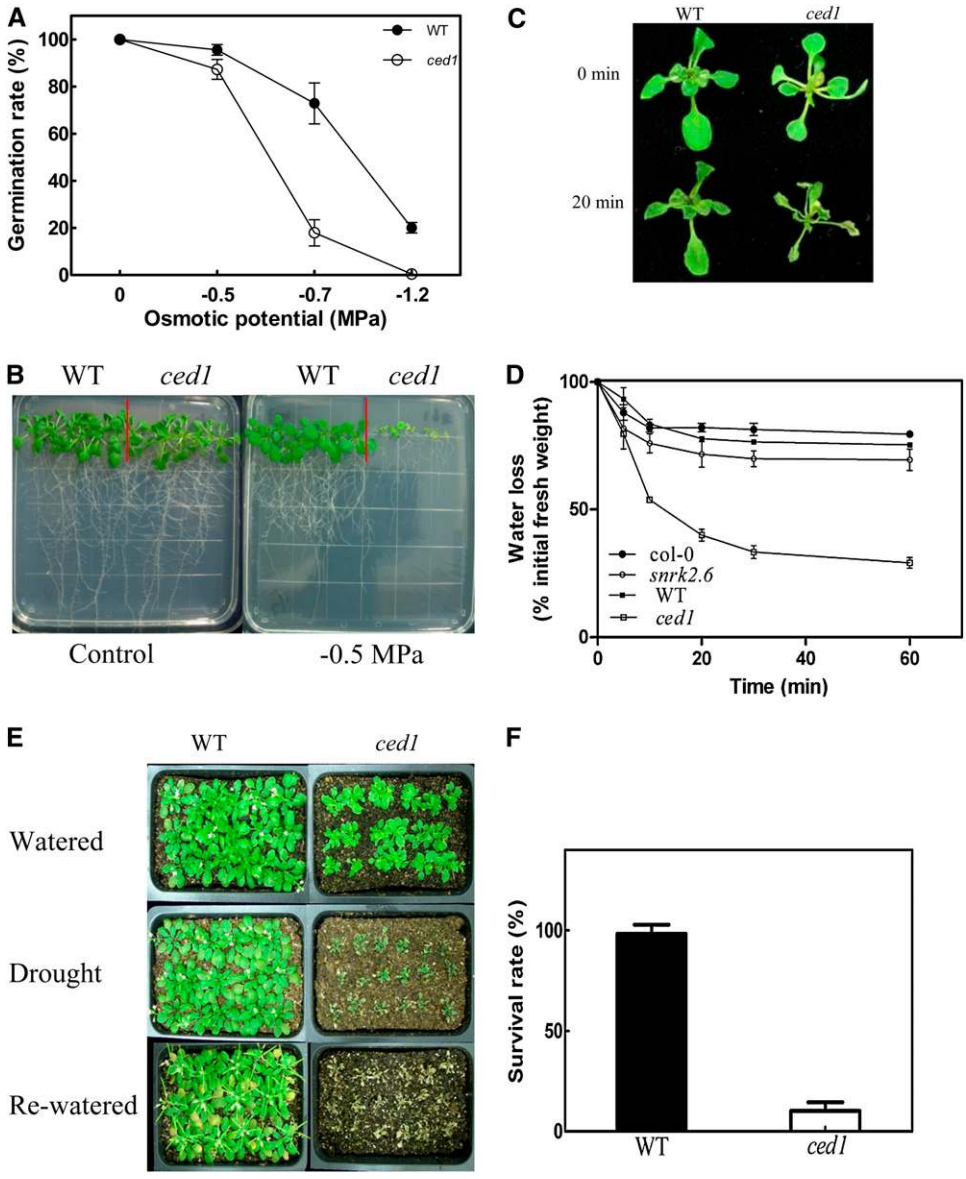


**Figure 3.** Osmotic Stress Phenotypes of the *ced1* Mutant.

**(A)** Germination of wild-type and *ced1* seeds in control or PEG-infused agar plates. Results are the average of four replicates ± SE.

**(B)** Growth of wild-type (WT) and *ced1* seedlings on control (left) or −0.5 MPa PEG-infused agar plates (right). Seeds were planted on shown plates, and the pictures were taken 4 weeks after seed imbibition.

**(C)** Wild-type and *ced1* rosette plants were turgid immediately after detachment from roots, but *ced1* plants became wilty 20 min later.

**(D)** Water loss rates of detached *ced1* and wild-type shoots. The *snrk2.6* mutant and its wild-type background ecotype Columbia (Col-0) are shown as controls. Data are means ± SE ($n$ = 4).

**(E)** *ced1* plants were more sensitive to drought stress. Wild-type and *ced1* plants were grown in soil with sufficient water for 3 weeks (Watered), and then water was withheld for 10 d (Drought) before rewatering. Plants were then allowed to recover for 2 d (Re-watered) before taking pictures. A representative picture for each treatment is shown.

**(F)** Quantification of the survival rate of the wild type and *ced1* plants in **(E)**. Survival rates and standard deviations were calculated from the results of four independent experiments.

polymorphism markers F23H11 and F5I14. Fine mapping within this chromosomal interval delimited the *CED1* locus to an ~88-kb region within the BAC clone F1N19 (see Supplemental Figure 5A online). Within this region, 33 putative genes were predicted in The Arabidopsis Information Resource database (http://www.Arabidopsis.org/index.jsp). To find the mutation in *ced1*, 10 potential candidate open reading frames were amplified and sequenced. A single nucleotide mutation from G to A at position 1316 from the translation start site was found in *ced1* mutant in At1g64670. This mutation would create a premature stop codon in the open reading frame that results in the truncation of the encoded protein (see Supplemental Figure 5B online).

The At1g64670 gene was annotated by the Arabidopsis Genome Initiative (http://www.Arabidopsis.org/index.jsp) as encoding an epidermis-expressed extracellular protein that likely functions as an α-β hydrolase. Interestingly, this locus was defined as *BDG* that controls epidermal cuticle development (Kurdyukov et al., 2006).

To confirm that the mutation in At1g64670 is responsible for the *ced1* mutant phenotype, we transformed *ced1* plants with an ~4.8-kb genomic fragment that includes the entire At1g64670 gene. Multiple transgenic lines were obtained, and two randomly chosen independent transformants were tested for their response to osmotic stress. Both transgenic lines were found to have osmotic stress tolerance similar to the wild type, whereas the original *ced1* mutant seedlings were hypersensitive to the mild osmotic stress (−0.5 MPa) (Figure 4A). RNA gel blot analysis of these two lines confirmed the expression of the introduced gene (Figure 4B).

We also examined the osmotic stress sensitivity of the *bdg* mutant. The *bdg-2* allele contains a 7-bp deletion in the first exon (Kurdyukov et al., 2006) and is expected to be a null allele. When grown on agar plates with −0.5 MPa of water potential generated by PEG infusion, *bdg-2* phenocopied the osmotic stress–sensitive phenotype of *ced1* (Figure 4C). To provide further genetic proof that *ced1* is allelic to *bdg-2*, we crossed *bdg-2* with *ced1*. The resulting F1 seedlings were grown on −0.5 MPa PEG-infused agar plates. These seedlings exhibited increased osmotic stress sensitivity similar to the *ced1* or *bdg*-2 single mutant, indicating a lack of complementation (Figure 4D; see Supplemental Table 2 online). Therefore, we renamed *ced1* as *bdg-5* (the 5th mutant allele of *BDG*). Collectively, these data indicate that At1g64670/*BDG* is *CED1* and that *CED1*/*BDG* is required for osmotic stress tolerance.

### Sensitivity of Other Cutin Biosynthesis Mutants to Osmotic Stress

The *bdg* mutants are characterized by irregular or interrupted cuticles, increased accumulation of cutin monomers and wax, as well as increased cuticular permeability (Kurdyukov et al., 2006). Although the enzymatic activity of BDG is unknown, the phenotypes of the *bdg* mutants suggest that BDG may be essential for the polymerization of cutin monomers at a late stage of cutin biosynthesis. While cuticles are well known to be able to minimize water loss and increase drought stress resistance, their role in osmotic stress tolerance is unclear. To ascertain whether the hypersensitivity to osmotic stress in *bdg-5* is specific to the *bdg-5* mutant or is a general consequence associated with the disruption of the cuticle, we sought to examine osmotic stress sensitivity in other cuticle mutants.

The cutin biosynthesis mutant *aberrant induction of type three genes1* (*att1-2*) has a mutation in a cytochrome P450 monooxygenase that catalyzes fatty acid oxidation and has 30% of the wild-type cutin content (Xiao et al., 2004). The *long-chain acyl-CoA synthetase* (*lacs2-1*) mutant is impaired in cutin but not wax synthesis and is altered in the cuticle structure (Schnurr et al., 2004). The mutant may have a fivefold reduction in dicarboxylic acids, the typical monomers of *Arabidopsis* cutin (Bessire et al., 2007). LACERATA is also a cytochrome P450 monoxygenase (*CYP86A8*) important for the production of ω-hydroxy fatty acid components of the cutin polymer (Wellesen et al., 2001). The glycerol-3-phosphate acyltransferase double knockout mutant *gpat4gpat8* has a 60 to 70% decrease in cutin monomer content, and the cuticle membrane in leaves is completely absent (Li et al., 2007). The relative positions of function of these enzymes in the cutin biosynthesis pathway are diagrammed in Figure 5A. Recently, overexpression of the putative transcription factor At MYB41 in *Arabidopsis* was found to abolish the expression of *LACS2* and significantly reduced the expression of *ATT1*. Leaf permeability of the transgenic plants also significantly increased (Cominelli et al., 2008). A similar transgenic study also suggested that At MYB41 might negatively regulate short-term transcriptional responses to osmotic stress (Lippold et al., 2009). All of the above mutants have defects in cutin structures, with alterations either in the amount or composition of cutin. These mutants may or may not have changed wax contents. As controls, we also included two wax mutants, *mah1-1* and *cer7*. The *mah1-1* is mutated in the cytochrome P450 midchain alkane hydroxylase (*CYP96A15*) required for producing the wax components secondary alcohols and ketones (Greer et al., 2007). The *cer7* mutation is in a putative 3′-5′ exoribonuclease of core exosome that degrades the transcript of a negative regulator of the wax gene *CER3*/*WAX2*/*YRE* (Hooker et al., 2007).

These mutants were grown under osmotic stress conditions, and their stress sensitivity was examined. In the absence of osmotic stress treatment, the postgermination growth of all mutants was similar to that of the wild type (Figure 5B). Under the PEG-imposed osmotic stress (−0.5 MPa), the growth of cutin mutants and the wild type were inhibited. However, the inhibition on all cutin mutants was much more severe than on the wild type (Figures 5B). The plants overexpressing At MYB41 (at-MYB41-OX) were also more sensitive to osmotic stress (see Supplemental Figure 6 online). These results indicated that these cutin-defective plants are much more sensitive to osmotic stress, similar to the *bdg-5* mutant. By contrast, the sensitivity to osmotic stress of the wax mutants *mah1-1* and *cer7* was similar to that of the wild type (see Supplemental Figure 6 online).

### Cutin but Not Wax Biosynthesis Mutants Are Impaired in Osmotic Stress Induction of ABA Biosynthesis and ABA Signaling Genes

The above data indicated that all of the cutin mutants examined are, like *bdg-5*, more sensitive to osmotic stress than the wild type. Since *bdg-5* is defective in osmotic stress regulation of ABA biosynthesis genes as well as ABA signaling genes (Figures 1B

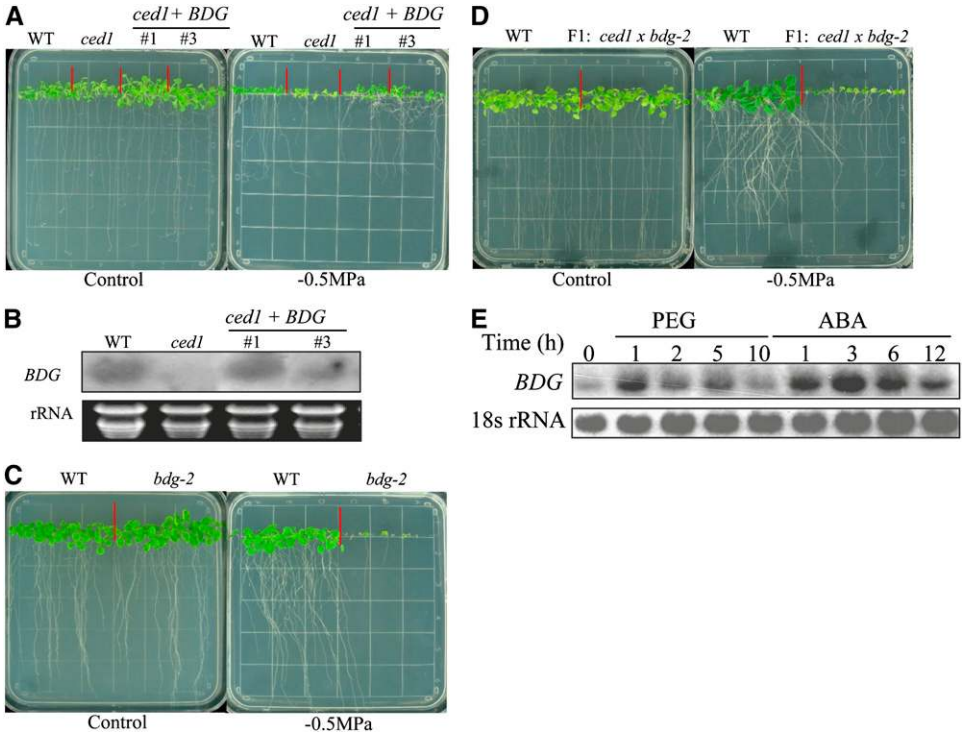


**Figure 4.** Functional Complementation of the *ced1* Mutant and Expression of *CED1*/*BDG* in Response to Osmotic Stress or ABA.

**(A)** Complementation of *ced1* by the wild-type *CED1*/*BDG* gene. The wild type (WT), *ced1*, and two homozygous *ced1* transgenic lines (#1 and #3) expressing the wild type *CED1*. Plants were grown on MS (Control) or −0.5 MPa PEG-infused agar plates. The photograph was taken 4 weeks after seed imbibition.

**(B)** RNA gel blot analysis of the *CED1*/*BDG* gene expression level in the wild type, *ced1*, and the two homozygous transgenic lines (#1 and #3). rRNA was used as a loading control.

**(C)** The *bdg-2* mutant was more sensitive to osmotic stress. Seeds were germinated and seedlings were grown on MS (Control) or −0.5 MPa PEG-infused agar plates for 4 weeks before taking the pictures.

**(D)** The *ced1* mutant is allelic with *bdg-2*. Wild-type and *F1* plants *(ced1* × *bdg-2*) were grown on MS (Control) or −0.5 MPa PEG-infused agar plates for 4 weeks before taking the pictures.

**(E)** Regulation of *CED1*/*BDG* expression by osmotic stress and ABA. Sixteen-day-old plants were treated with 40% PEG or 100 μM ABA and incubated for the indicated time. 18S rRNA was used as a loading control.

and 1C), we asked whether the other cutin mutants are similarly impaired in osmotic stress regulation of these genes.

As shown in Figure 6, the transcript levels of *ABA1*, *NCED3*, *ABI1*, and *ABI2* were clearly upregulated by osmotic stress in both the wild type and cutin mutants. Remarkably, the expression levels of these genes were all lower in the cutin mutants than in the wild type. Consistent with the negative roles of At MYB41 in regulating cutin biosynthesis, transgenic plants overexpressing At *MYB41* showed a lower induction of ABA biosynthesis and signaling genes, similar to what was seen with other cutin biosynthesis mutants.

The wax mutants *mah1-1* and *cer7* did not show any significant reduction in the expression of these ABA biosynthetic and signaling genes in response to osmotic stress (Figure 6). This is consistent with the unaltered sensitivity of the mutants to osmotic stress compared with the wild type (see Supplemental Figure 6 online). These data suggest that cutin but not wax biosynthesis mutants are unable to appropriately respond to osmotic stress in activating ABA biosynthesis and signaling genes, which may result in their increased sensitivity to even mild osmotic stress.

### Increased Osmotic Sensitivity in *bdg* Mutant Plants Is Not Caused by Increased Permeability

Increased permeability was observed in aerial tissues of *bdg-2* mutant plants (Kurdyukov et al., 2006; MacGregor et al., 2008). Similar to *bdg-2*, we found that aerial tissues of *bdg-5* mutant plants show increased permeability (see Supplemental Figure 7 online). To examine whether the increased osmotic sensitivity in *bdg-5* plants might be due to the increased permeability, we sowed the seeds in osmotic stress media immediately adjacent to strips of Parafilm as described (MacGregor et al., 2008). Under this condition, the aerial tissues will grow only onto the Parafilm and will not be in contact with the medium. Consistent with the previous observation in *bdg-2* (MacGregor et al., 2008), lateral root formation in *bdg-5* is reduced with the use of Parafilm in control media (see Supplemental Figure 8A online). However, the *bdg-5* seedlings remain hypersensitive to mild osmotic stress imposed by PEG-infused agar media (see Supplemental Figure 8A online). These results suggest that increased osmotic stress

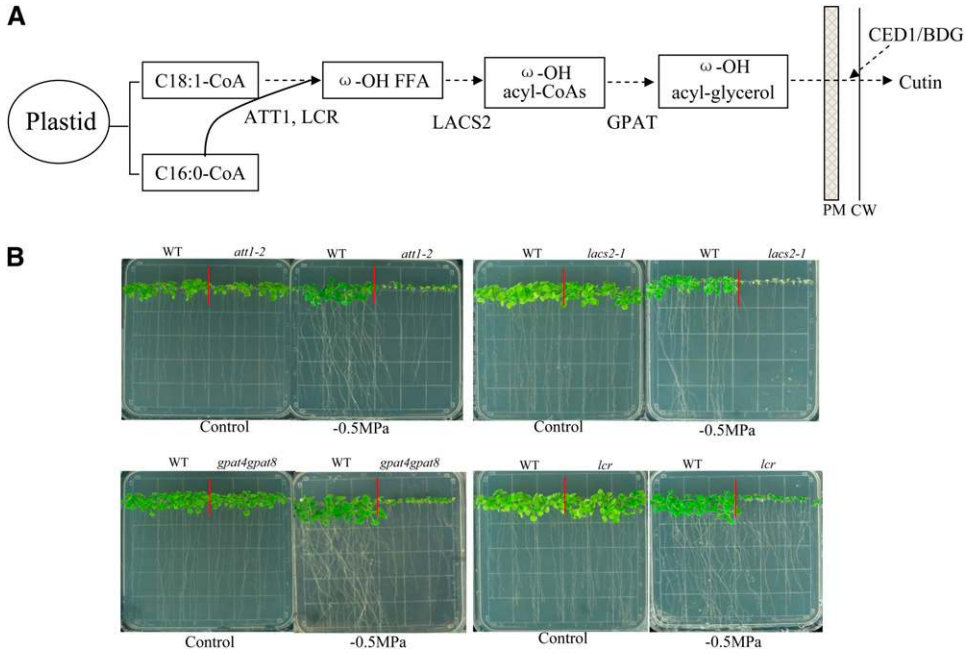


**Figure 5.** Osmotic Stress Sensitivity of Cutin Mutants.

**(A)** Schematic diagram of a possible cutin biosynthesis pathway. Shown are the synthesis of ω-hydroxy fatty acid–based cutin monomers and the enzymes that catalyze the reactions. The relative positions of these biosynthesis steps have not been completely determined (Pollard et al., 2008). CED1/BDG is proposed to function in the polymerization of the cutin monomers and is localized extracellularly. FFA, free fatty acid.

**(B)** Growth of wild-type (WT) and cutin mutant seedlings on MS (Control) or −0.5 MPa PEG-infused agar plates. Seeds were planted on the plates, and the pictures were taken 4 weeks after seed imbibition.

sensitivity in *bdg-5* plants is not caused by increased aerial tissue permeability. We observed similar results in *bdg-2* mutant when seeds of the *bdg-2* were germinated next to strips of Parafilm in the presence of osmotic stress (see Supplemental Figure 8B online). We also tested another mutant, *gpat5*, which is defective in glycerol-3-phosphate acyltransferase. The *gpat5* mutant displayed severalfold reduction in suberin in its seed coats and but no change in surface wax (Beisson et al., 2007). Seed coats of *gpat5* mutant plants had a great increase in permeability (Beisson et al., 2007). We found that seed germination and postgermination seedling development of *gpat5* are not affected by osmotic stress conferred by PEG (see Supplemental Figure 8C online). The result suggests that increased seed coat permeability does not necessarily lead to increased osmotic stress sensitivity in germination. Together, the above data support that increased permeability is not the cause of hypersensitivity of *bdg* mutants to osmotic stress conferred by PEG.

### *BDG* Expression Is Induced by Osmotic Stress and ABA

The critical role of *BDG* in osmotic stress tolerance and ABA biosynthesis prompted us to investigate how the *BDG* gene is regulated in response to osmotic stress and ABA. RNA gel blot analysis was performed with total RNA obtained from the wild-type seedlings grown on the MS agar media for 16 d. As shown in Figure 4E, the transcript level of *BDG* was substantially upregulated after 1 h of osmotic stress but returned to the untreated control level after prolonged stress. ABA induced a higher expression level of *BDG*, which peaked at 3 h after the treatment and gradually decreased thereafter.

## DISCUSSION

Plant aerial parts are covered with a layer of hydrophobic cuticle that prevents the epidermal cells from direct contact with the dry environment of the air. This physical barrier minimizes water loss, discourages pathogens and herbivores, and protects the cells below from damage by UV and other environmental stresses (Kolattukudy, 2001; Kosma and Jenks, 2007; Pollard et al., 2008). The cuticle also prevents organ fusion (Sieber et al., 2000) and is thus required for normal morphogenesis of the aerial organs. Although the cuticle has been known to play important roles as a physical barrier to enhance plant resistance to abiotic stress and cutin was recently shown to be important for plant resistance to pathogens, a role for cuticle in regulating ABA biosynthesis and osmotic stress resistance was unexpected.

### Cutin and Cuticle Are Responsible for the Defects in Osmotic Responses in *bdg-5* and Other Cuticle Mutants

With the critical role of *CED1* in regulating *NCED3* gene expression, one may expect *CED1* to encode a typical regulatory component such as a transcriptional factor. Unexpectedly,

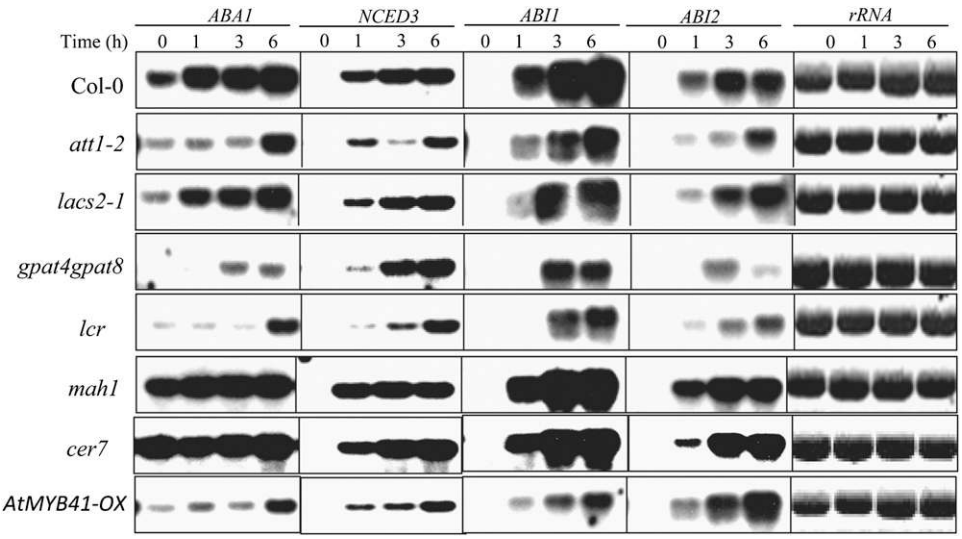


**Figure 6.** Steady State Transcript Levels of ABA Biosynthesis and Signaling Genes in Cutin or Wax Mutants.

Seedlings were treated with 40% PEG for the indicated time. Ten micrograms of total RNA were used per lane for the RNA gel blot analysis. rRNA was used as a loading control. Identities of probe sequences are indicated above the images, and the genotypes of different plants are indicated at left. Col-0, ecotype Columbia (wild-type plants).

*CED1* was found to be allelic to *BGD*, which encodes a putative $\alpha/\beta$ hydrolase fold protein recently shown to be essential for cutin synthesis. Although the catalytic activity of this putative enzyme is unknown, its tissue-specific expression and localization (epidermis and extracellular) and the mutant phenotypes (accumulation of cutin monomers and waxes) support that *CED1*/*BDG* may be an enzyme that catalyzes either the polymerization of cutin monomers or some yet unknown process in the cutin biosynthesis pathway (Kurdyukov et al., 2006), although the possibility that *BDG* could directly be a signaling component cannot be completely ruled out.

Unlike *BDG*, whose catalytic activity is still unknown, most of the other cutin biosynthesis loci encode enzymes belonging to well-defined catalytic groups, although their detailed reactions and their relative positions in the overall cutin biosynthesis pathway may not be clear. For instance, *ATT1* is a cytochrome P450 monooxygenase (CYP86A2) catalyzing $\omega$-oxidation of fatty acids and the *att1* mutant has 30% of the wild-type cutin level (Xiao et al., 2004). *LACS2* is a long-chain acyl-CoA synthase that produces $\omega$-hydroxy fatty acyl-CoA for cutin synthesis, and its mutation reduces the thickness of the cutin membrane (Schnurr et al., 2004). Our finding that all these cutin biosynthesis mutants are similarly impaired in osmotic stress induction of ABA biosynthethic genes and osmotic stress tolerance indicates that most likely it is the cutin integrity that is responsible for the defects in osmotic stress responses. Since these mutants are affected in different steps of cutin biosynthesis, it is less likely that a common substrate or product in cutin biosynthesis acts as a signal for the activation of ABA biosynthesis.

While it has long been accepted that plant cuticles function in increasing drought resistance by minimizing nonstomatal transpiration water loss, because the cuticle constitutes a physical water-repelling barrier to prevent water escape, it is unexpected that the cuticle may play an active role in regulating ABA biosynthesis and osmotic stress resistance. The exact mechanism for cuticle regulation of osmotic stress activation of ABA biosynthesis and other stress responses is unknown at this time. However, our study clearly indicates that an intact cuticle is essential for osmotic stress to activate ABA biosynthesis as well as for plants to develop osmotic stress resistance, providing an unexpected link between cuticle integrity and cell signaling. This discovery may have some resemblance to the cell wall integrity response in yeast, where cell wall damages elicit a signaling cascade that activates cell wall remodeling-related genes (Levin, 2005). Recently, it was also found that the high osmolarity glycerol pathway is also involved in cell wall damage response (Bermejo et al., 2008; Garcia et al., 2009), which implicated a connection between cell wall integrity and osmotic stress response in yeast cells.

### Possible Connections between Cuticle and Cell Wall in Osmotic Stress Regulation of ABA Biosynthesis

It is well recognized that the growth of a plant cell is largely determined by the balance between turgor pressure from the protoplasts and the constriction imposed by the cell wall. The direction for signaling cell growth is from within the cell to the cell wall so that the wall can be loosened and expanded. Whether and how the cell wall may send a retrograde signal to the cell to make appropriate adjustment to cope with external osmotic stress is currently unknown. Although the conventional idea is that cellular responses to osmotic stress start with the perception of the stress at the plasma membrane, recently, however, several genetic studies revealed connections between the cell wall and osmotic stress responses. Mutations in a cellulose synthase-like gene *SOS6*/*CSLD5* caused the mutants to be

hypersensitive to osmotic stress, although the cell wall composition of this mutant was only subtly affected (Zhu et al., 2010). A mutation in the cellulose synthase gene *CesA8*/*IRX1*, on the other hand, led to increased osmotic stress tolerance in the mutant (Chen et al., 2005). These studies suggest a critical role for cell wall in osmotic stress tolerance, although the mechanisms involved are unknown.

A role for the cell wall in osmotic stress induction of ABA biosynthesis has also been implicated in several studies. It seems that a net change (reduction) in turgor pressure is required for osmotic stress induction of ABA biosynthesis in detached leaves (Pierce and Raschke, 1980). Penetrating solutes equally lower the osmotic potential of the cell but failed to elicit ABA biosynthesis (Creelman and Zeevaart, 1985). Similarly, osmotic stress treatments failed to increase ABA biosynthesis in *Vicia faba* (Lahr and Raschke, 1988) or barley (*Hordeum vulgare*) protoplasts (Loveys and Robinson, 1987), but the same treatments could activate ABA biosynthesis in leaf discs in these studies. Nonetheless, one other study reported that ABA biosynthesis could occur in protoplasts of rose petals or *Amaranthus* leaves (Bianco-Trinchant and Le Page-Degivry, 1998). Aside from the difference in plant materials and experimental conditions, it is not clear why these research results differ.

Unlike a complete lack or a general compromise of the cell wall integrity as seen with protoplasts or the cell wall mutants in previous studies, *ced1* mutant seedlings may not have a global defect in the cell wall structure. Gene expression and immunolocalization assays indicated that *BDG* is expressed exclusively in epidermis and the protein is localized extracellularly (Kurdyukov et al., 2006). Consistent with this pattern of localization, the structural defects of *bdg-5* appear to be very specific and restricted to the cuticles and epidermis, although the mutant does have other developmental defects. The isolation of the *ced1* mutant thus defines a specialized and unexpected structure (i.e., the cuticle) as essential for osmotic stress sensing and response.

### Cutin but Not Wax Is Required for Osmotic Stress Response and Tolerance

The plant cuticle is mainly composed of cutin embedded with intracuticular waxes and covered with epicuticular waxes. Cutin is a polyester of hydroxy and epoxy-hydroxy $C_{16}$ or $C_{18}$ fatty acids and glycerol, while waxes consist of very-long-chain fatty acids with chain length from 24 to 26 carbons along with various derivations and modifications. Although both components work together structurally and functionally to seal the epidermis and prevent water loss (Kosma and Jenks, 2007), our studies revealed that they have very different roles in osmotic stress responses. Unlike *bdg-5* and other cutin mutants, mutants defective in wax biosynthesis are not affected in either the expression of ABA biosynthesis and signaling genes (Figure 6) or osmotic stress resistance (see Supplemental Figure 6 online). These results suggest that the defect in cutin but not wax biosynthesis affects cuticle's ability to respond to osmotic stress. In fact, in several cutin biosynthesis mutants, including *bdg-5*, wax contents actually increased (Schnurr et al., 2004; Kurdyukov et al., 2006; Bessire et al., 2007), perhaps as a result of increased channeling of early common substrates to the wax biosynthesis pathway or the activation of wax biosynthetic genes to compensate for the loss of cutin in cuticles.

### Does Osmotic Stress Regulation of ABA Biosynthesis Contribute to the Altered Disease Resistance in Cutin Biosynthesis Mutants?

The discovery that cutin and cuticles are required for either sensing or responding to osmotic stress is very intriguing. Recently, a role of cutin biosynthesis and cuticle integrity in disease resistance has emerged. In multiple mutant screens for resistance to bacterial or fungal pathogens, several mutants susceptible to bacterial pathogens were found to have defects in cutin biosynthesis or integrity (Xiao et al., 2004; Tang et al., 2007). Interestingly, these mutants are more resistant to the necrotrophic fungus *Botrytis cinerea* (Bessire et al., 2007; Chassot et al., 2007; Tang et al., 2007), although the mechanisms for this immunity are unknown. Cutin monomers or other constituents of the cuticles could elicit reactive oxygen species production (Fauth et al., 1998) and can potentially serve as signals to activate defense responses in the host. It is known that ABA is actively involved in plant resistance to pathogens (Mauch-Mani and Mauch, 2005; Adie et al., 2007; de Torres-Zabala et al., 2007; Fan et al., 2009); whether reduced ABA biosynthesis in cuticle mutants has a direct role in regulating disease resistance will need to be investigated in the future.

### Possible Mechanisms for Cuticle Regulation of ABA Biosynthesis

At present, we can only speculate on why cuticle integrity would be needed for osmotic stress induction of ABA biosynthesis and osmotic stress tolerance. Aside from possible signaling molecules derived from cutin biosynthesis, there may be other epidermal cell wall– or even cuticle-associated proteins that are essential for the sensing of osmotic stress signals. Many wall-associated putative receptors, sensors, and other proteins exist, yet their functions in cell signaling are still largely unknown (Humphrey et al., 2007). An intact cuticle may be needed for the correct localization or function of these signaling molecules. *BDG* or other cutin biosynthesis enzymes themselves may not be such molecules since these diverse biosynthetic enzymes have little structural or functional similarity to signaling molecules that might directly serve a signaling role in osmotic stress response.

Alternatively, the physical integrity of the cuticle may be required for osmotic stress responses. Under normal growth conditions, plant cells are under strong positive pressure from the cell wall (turgor pressure). Under osmotic stress, turgor pressure is reduced due to decreased water potential in the apoplast, and the cell may sense this change in turgor pressure and trigger a signal transduction cascade to activate ABA biosynthesis and other osmotic stress responses. When the cuticle is disrupted and the epidermis cell wall may become less rigid and more flexible, the mutant cell wall may no longer be able to sense changes in the osmotic potential of the cell. This would result in no response or reduced response to osmotic stress as seen in *ced1* and other cutin biosynthesis mutants.

One intriguing fact about *BDG* is that the gene and protein are mainly expressed in the epidermis (Kurdyukov et al., 2006), while ABA biosynthesis enzymes are found mainly associated with the vascular tissues (Cheng et al., 2002; Gonzalez-Guzman et al., 2002; Tan et al., 2003; Koiwai et al., 2004). It is yet unclear how the signal perceived at the epidermal cells would be transmitted to the vascular tissues. Likely cuticle integrity may impact not only the epidermal cells underneath but also the leaf and other organs of the plant as a whole. In addition to leaf epidermal cells, *BDG* as a putative enzyme in the cutin biosynthesis pathway was expressed in root epidermis and probably pericycle cells where lateral roots emerge (Kurdyukov et al., 2006). Cutin is biochemically similar to the suberin found in root endodermis of most higher plants, and some of their biosynthesis pathways may overlap (Franke et al., 2005), although it is not clear whether *bdg-5* has an altered suberin. Thus, one additional possible connection between cuticle biosynthesis and osmotic stress–induced ABA biosynthesis may have to do with the coupling of suberin integrity and ABA biosynthesis in root vascular tissues. These possibilities would need to be tested in future experiments.

## METHODS

### Plant Materials and Growth Conditions

A firefly LUC reporter gene driven by the drought stress-responsive *NCED3* promoter (−1083 to + 76 bp from the transcription start site) was introduced into *Arabidopsis thaliana* plants in the Columbia *glabrous1* background. Seeds from one homozygous line expressing a single functional copy of the *NCED3*:*LUC* gene (referred to as the wild type) were mutagenized with ethyl methanesulfonate. The *ced1* mutant with altered *NCED3*:*LUC* gene expression was isolated from M2 seedlings with a CCD camera imaging system (Ishitani et al., 1997).

The *att1-1* and *att1-2* mutants were kindly provided by Jian-Min Zhou (National Institute of Biological Sciences, Beijing). The *lacs2-1* mutant was kindly provided by John Browse (Washington State University). *lcr* and *bdg* were kindly provided by Alexander Yephremov (Max Planck Institute for Plant Breeding Research, Germany). The gpat4 gpat8 double mutant and gpat5 were kindly provided by John Ohlrogge (Michigan State University). The At *MYB41* overexpression line was kindly provided by Chiara Tonelli (Università degli Studi di Milano, Italy). The *mah1-1* (SALK_049943), *cer7* (CS8017), and *snrk2.6* (SALK_008068) T-DNA insertion lines were obtained from the ABRC (Columbus, OH). *Arabidopsis* seedlings on MS medium agar plates (1× MS salts, 3% Suc, and 1.5% agar, pH 5.7) were routinely grown under continuous white light (~75 μmol $m^{-2}$ $s^{-1}$) at 23 ± 1°C. Soil-grown plants were kept under a 16-h-light/8-h-dark photoperiod at 23 ± 1°C.

### Physiological Assays

For germination assays, seeds were planted on either a half-strength MS 1.5% agar medium with 3% Suc buffered with 2 mM MES or PEG-infused agar plates without Suc. In each experiment, at least 100 seeds per genotype were stratified at 4°C for 3 d, and radicle emergence was scored as being germinated at the indicated time.

For leaf water loss measurements, fully expanded leaves were removed from 4-week-old plants and incubated under the same conditions used for seedling growth, and each sample (consisting of three to four individual leaves) was weighed at the indicated time. For drought treatment, plants were grown in soil with sufficient water for 3 weeks, and then the water was withheld for durations as indicated.

For ABA quantification, ABA was measured by radioimmunoassay as previously described (Verslues et al., 2007).

### Genetic Mapping and Complementation

The *ced1* mutant was crossed with the Landsberg *erecta* accession, and 734 mutant plants were chosen from the F2 generation by their osmotic stress sensitivity phenotype. Simple sequence length polymorphism markers were designed according to the information in the Cereon *Arabidopsis* Polymorphism Collection and used to analyze recombination events (Jander et al., 2002). The *ced1* mutation was first mapped to chromosome 1 between F23H11 and F5I14. Fine mapping within this chromosomal interval narrowed the *CED1* locus to an ~88-kb region within the BAC clone F1N19. All candidate genes in this region were sequenced from the *ced1* mutant and compared with those in GenBank to find the *ced1* mutation.

For complementation of the *ced1* mutant, an ~4.8-kb genomic fragment that includes 1813 bp upstream of the translation initiation codon and 549 bp downstream of the translation stop codon was amplified (see Supplemental Table 3 online for primer sequences). The amplified fragment was cloned into the pCAMBIA 1380 vector. The construct was transferred into *Agrobacterium tumefaciens* (GV3101 strain), and plants were transformed using the floral dip method (Clough and Bent, 1998).

### RNA Gel Analysis and Real-Time RT-PCR Analysis

Sixteen-day-old seedlings grown on MS media (1× MS salts, 3% Suc, and 0.6% agar, pH 5.7) plates that were placed in a horizontal orientation were used for RNA gel analysis. Total RNA was extracted from the wild type, mutants, and transgenic plants with the Trizol reagent (Invitrogen). Total RNA was separated on a 1.2% formaldehyde-MOPS agarose gel. The blots were probed, washed, and wrapped in plastic wrap to keep them from drying out and then immediately exposed to x-ray film for autoradiography as described (Xiong et al., 2001).

For real-time RT-PCR analysis, 5 μg of total RNA isolated with the RNeasy plant mini kit (Qiagen) was used for the first-strand cDNA synthesized using SuperScript III first-strand synthesis supermix (Invitrogen). The cDNA reaction mixture was diluted three times, and 5 μL was used as a template in a 25-μL PCR reaction. PCR was performed after a preincubation at 95°C for 3 min and followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. All the reactions were performed in the iQ5 real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad). Each experiment was replicated three times. The comparative Ct method was applied. The primers used in this study are listed in Supplemental Table 3 online.

### Microarray Analysis

For Affymetrix GeneChip array analysis, wild-type and *ced1* seedlings were grown on MS plates for 15 d at 22°C with 16 h light and 8 h darkness. Total RNA was extracted using an RNeasy plant mini kit (Qiagen) and used for preparation of biotin-labeled complementary RNA targets. Microarray analysis was performed as described by Breitling et al. (2004). Two biological replicates were used for each genotype. We applied the Robust Multiarray Averaging normalization method for our data sets. The RMA method for computing an expression measure begins by computing background-corrected perfect match intensities for each perfect match cell on every GeneChip. The normalized data were further analyzed, and P values were generated by affymGUI component of Bioconductor in statistics environment R, with the default parameters (Irizarry et al., 2003; Gentleman et al., 2004). We selected lists of genes with statistically significant changes between mutant and wild type under osmotic stress conditions by the RankProd method (nonparamteric method for identifying differentially expressed [up- or downregulated]

genes based on the estimated percentage of false predictions [pfp]) (Hong et al., 2006) (pfp < 0.05). RankProd results were summarized with the script written in PERL.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: BDG/CED1 (At1g64670), NCED3 (At3g14440), ABA1 (At5g67030), AAO3 (At2g27150), ABI1 (At4g26080), ABI2 (At5g57050), RD29A (At5g52310), KIN1 (At5g15960), COR15A (At2g42540), RD22 (At5g25610), RAB18 (At5g66400), P5CS1 (At2g39800), COR414-TM1 (At1g29395), PP2Cs (At3g05640), KIN2 (At5g15970), RD29B (At5g52300), LTP3 (At5g59320), and PKS18 (At5g45820). The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE 25889 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE 25889).

### Supplemental Data

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

**Supplemental Figure 1.** Effect of 5Aza-dC on *NCED3*:*LUC* Expression in Wild-Type Seedlings.

**Supplemental Figure 2.** Expression of Cold Stress-Responsive Genes in *ced1* and Wild-Type Plants.

**Supplemental Figure 3.** Genevestigator Transcriptome Meta-Analysis of Higher Expression Gene in Supplemental Table 1A in Response to ABA and Osmotic Stress Treatment.

**Supplemental Figure 4.** Genevestigator Transcriptome Meta-Analysis of Higher Expression Gene in Supplemental Table 1B in Response to ABA and Osmotic Stress Treatment.

**Supplemental Figure 5.** Positional Cloning of the *ced1* Mutation.

**Supplemental Figure 6.** Sensitivity of Wax Biosynthesis Mutants and AtMYB41-OX Seedlings to Osmotic Stress.

**Supplemental Figure 7.** Leaves from Soil-Grown *ced1* Plants Display Increased Permeability.

**Supplemental Figure 8.** Increased Osmotic Sensitivity May Not Be a Direct Cause of Increased Permeability in Aerial Tissues or Seed Coats.

**Supplemental Table 1.** Functional Catalog of Genes in Supplemental Data Set 1.

**Supplemental Table 2.** Complementation Test.

**Supplemental Table 3.** Primers Used in This Study.

**Supplemental Data Set 1.** List of Genes with Expression Changes in *ced1* from Microarray Analysis.

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