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The Calcium Sensor Calcineurin B-Like 9 Modulates Abscisic Acid Sensitivity and Biosynthesis in Arabidopsis

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Calcium plays a pivotal role in plant responses to several stimuli, including pathogens, abiotic stresses, and hormones. However, the molecular mechanisms underlying calcium functions are poorly understood. It is hypothesized that calcium serves as second messenger and, in many cases, requires intracellular protein sensors to transduce the signal further downstream in the pathways. The calcineurin B-like proteins (CBLs) represent a unique family of calcium sensors in plant cells. Here, we report our analysis of the *CBL9* member of this gene family. Expression of *CBL9* was inducible by multiple stress signals and abscisic acid (ABA) in young seedlings. When *CBL9* gene function was disrupted in *Arabidopsis thaliana* plants, the responses to ABA were drastically altered. The mutant plants became hypersensitive to ABA in the early developmental stages, including seed germination and post-germination seedling growth. In addition, seed germination in the mutant also showed increased sensitivity to inhibition by osmotic stress conditions produced by high concentrations of salt and mannitol. Further analyses indicated that increased stress sensitivity in the mutant may be a result of both ABA hypersensitivity and increased accumulation of ABA under the stress conditions. The *cbl9* mutant plants showed enhanced expression of genes involved in ABA signaling, such as *ABA-INSENSITIVE 4* and *5*. This study has identified a calcium sensor as a common element in the ABA signaling and stress-induced ABA biosynthesis pathways.

INTRODUCTION

The plant hormone abscisic acid (ABA) regulates many aspects of plant development and physiology, including seed maturation and dormancy, as well as responses to environmental stress conditions, such as drought, salinity, and low temperature (Fedoroff, 2002; Finkelstein et al., 2002; Himmelbach et al., 2003). Inhibition of seed germination provides a useful bioassay for both forward and reverse genetic analyses that has revealed several molecular components in plant ABA signal transduction pathways (Giraudat, 1995). These components range from early signaling intermediates such as G proteins and protein kinases/phosphatases, to late stage transcription factors and RNA metabolic proteins (reviewed in Fedoroff, 2002; Finkelstein et al., 2002). Whereas some components appear to be regulators of multiple ABA responses, few are required for all responses, suggesting that ABA responses in different cell types or at various developmental stages may differ to certain extent. Among the components identified by seed germination genetic screens, ABA INSENSITIVE (ABI1-5) represent protein phosphatases and transcription factors (reviewed in Giraudat, 1995; Finkelstein et al., 2002). The phosphatases ABI1 and ABI2 may function as more general regulators that regulate both seed germination and stomatal movement (Pei et al., 1997; Allen et al., 1999). By contrast, the transcription factors ABI3-5 are highly expressed during seed maturation, under stressed conditions, and during seed germination (Finkelstein et al., 2002). As a result, ABI3-5 specifically function in the ABA-mediated regulation of seed maturation, germination, and early growth of seedlings (Finkelstein et al., 2002; Arroyo et al., 2003). Mutations in these genes resulted in ABA-insensitive seed germination, whereas overexpression of these genes led to ABA hypersensitivity (Lopez-Molina et al., 2001; Finkelstein et al., 2002). These findings indicate that ABI3, 4, and 5 are both required and sufficient for ABA signaling, although they may functionally interact with each other (Finkelstein et al., 2002). More recent studies suggest that ABA stabilizes ABI5 protein that is otherwise rapidly degraded under normal growth conditions. It was therefore concluded that ABI5 may play a key role in the growth arrest caused by ABA at the young seedling stage (Lopez-Molina et al., 2001).

In adult plants, ABA serves as a critical chemical messenger for stress responses. Under abiotic stress conditions, especially

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under drought and high salt stress, ABA biosynthesis is upregulated (Xiong and Zhu, 2003). Several studies have shown that ABA accumulation is required for the development of stress tolerance in plants. As a rapid response, stomatal closure is induced by ABA accumulation. This is followed by a slower response to ABA manifested in the induction of stress genes. Consequently, there is a large overlap of ABA-, drought-, and salt-responsive gene expression in the vegetative tissues of adult plants (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein et al., 2002; Xiong et al., 2002). Furthermore, characterization of stress gene promoters indicates that many gene promoters contain *cis*-acting elements that are separately responsible for abiotic stress and ABA. Because of their ABA-responsive promoter elements, these genes can be activated by stress-induced increase in ABA levels, as part of an ABA biosynthesis-dependent regulatory pathway. In addition, the stress-responsive elements are responsible for stress-responsive expression without the need of ABA biosynthesis, resulting in an ABA-independent regulatory pathway (Shinozaki et al., 1998). However, some apparent ABA-independent pathways may require ABA for full response as a result of cross talk between ABA and stress response pathways (Knight and Knight, 2001; Xiong et al., 2002; Kim et al., 2003). It has been proposed that specific cross talk nodes may upregulate or downregulate the signaling strength and interaction between the different ABA-dependent and ABA-independent stress response pathways (Knight and Knight, 2001; Kim et al., 2003). Calcium represents a prime candidate for such cross talk nodes because it has been shown to serve as a second messenger for both ABA and stress responses.

How could calcium function in transmitting different signals to trigger stimulus-specific responses in a plant cell? If calcium signaling in the cell represents a molecular relay, the components immediately following calcium changes would be sensor molecules that decode the changes in calcium concentration and other parameters that constitute calcium signatures (Sanders et al., 1999; Rudd and Franklin-Tong, 2001). Many calcium binding proteins have been identified that may play the role as calcium sensors in plant cells. These include calmodulin and calmodulin-related proteins (Zielinski, 1998; Snedden and Fromm, 2001; Luan et al., 2002), calcium-dependent protein kinases (Harmon et al., 2000; Sanders et al., 2002), and calcineurin B-like proteins (CBLs) (Luan et al., 2002). In each case, the proteins are encoded by large multigene families, suggesting possible redundancy and/or diversity in their functions. For example, CBL1 plays a role in multiple abiotic stress response pathways (Albrecht et al., 2003; Cheong et al., 2003), whereas CBL4/SOS3 is more specifically involved in mediating ionic stress tolerance (Xiong et al., 2002). CBL-type calcium sensors are small EF-hand–containing proteins that function by interacting and regulating the function of a group of protein kinases called CBL-interacting protein kinase (CIPKs) (reviewed in Luan et al., 2002). Recent studies indicated that several CIPK genes function in stress and ABA responses (Gong et al., 2002a, 2002b; Guo et al., 2002; Kim et al., 2003), implying that some CBL-type calcium sensors must also function in ABA signaling pathways. Here, we report the identification of CBL9 as a regulator for both ABA and stress responses in the early development of *Arabidopsis thaliana* plants. In addition, CBL9 regulates the osmotic stress–induced ABA production, thereby representing a cross talk node in connecting ABA signaling and ABA biosynthesis under stress conditions.

RESULTS

CBL9 Expression Is Induced by Stress and ABA Treatments in Arabidopsis Seedlings

At least 10 genes encode CBL-type calcium sensors in Arabidopsis (Luan et al., 2002; Kolukisaoglu et al., 2004). These proteins contain three typical EF-hand calcium binding motifs that have been shown to bind calcium in vitro (Kudla et al., 1999). Four CBL proteins contain the N-terminal signature domain for myristoylation that often targets proteins to cell membranes (Luan et al., 2002; Kolukisaoglu et al., 2004). This study focuses on one CBL member, *CBL9*, with a putative myristoylation site. To gain first insights into possible functions of CBL9, we initially examined the expression pattern of this gene. These analyses revealed that the *CBL9* gene is expressed ubiquitously in all developmental stages of plants (Figure 1A). More detailed analyses of *CBL9* promoter activity revealed that *CBL9* is highly expressed in the radicles of germinated seedlings (Figure 1B). In rosette plants, the expression in the roots was mostly restricted to the growth zones in the tip but not detected in mature regions, consistent with lower level of mRNA in total root RNA (depicted in Figure 1A). Significant expression of *CBL9* was also detected in mature leaves, flowers, and siliques. High promoter activity was observed in the anthers (Figure 1B). The expression of the *CBL9* gene in seedlings was highly inducible by ABA and to a lesser extent by salt (300 mM NaCl), drought (dehydration), and cold (4°C) (Figure 1C). The induction by ABA and each stress followed a slightly different kinetic pattern. Induction by ABA was more rapid and more robust as compared with the induction by NaCl, cold, and drought.

The *CBL9* Mutant Is Hypersensitive to ABA, Osmotic Stress, and Glucose

The ABA- and stress-inducible expression suggested a possible role of *CBL9* gene in ABA-mediated and stress signal transduction pathways. To further examine the function of the *CBL9* gene, we used a reverse genetics approach (Winkler et al., 1998; Krysan et al., 2002). A T-DNA insertional allele was identified in a collection of T-DNA–transformed Arabidopsis lines. Homozygous mutant lines were established after selfing of the originally identified plant, which was heterozygous for the mutant allele. Sequence analysis identified the insertional site in the 2nd intron after the 618th bp after ATG (Figure 2A). This insertion reduced the expression of *CBL9* to nondetectable levels as indicated by RT-PCR analysis using *CBL9*-specific primers (Figure 2C). We noted that expression of a closely related gene, *CBL1*, was not affected in the *cbl9* mutant (Figure 2C). A genomic DNA fragment of 4.3 kb containing the complete coding region and putative promoter of the *CBL9* gene was used to transform the homozygous mutant plants. Such a genomic fragment restored the expression of *CBL9* in the mutant (Figure 2C).

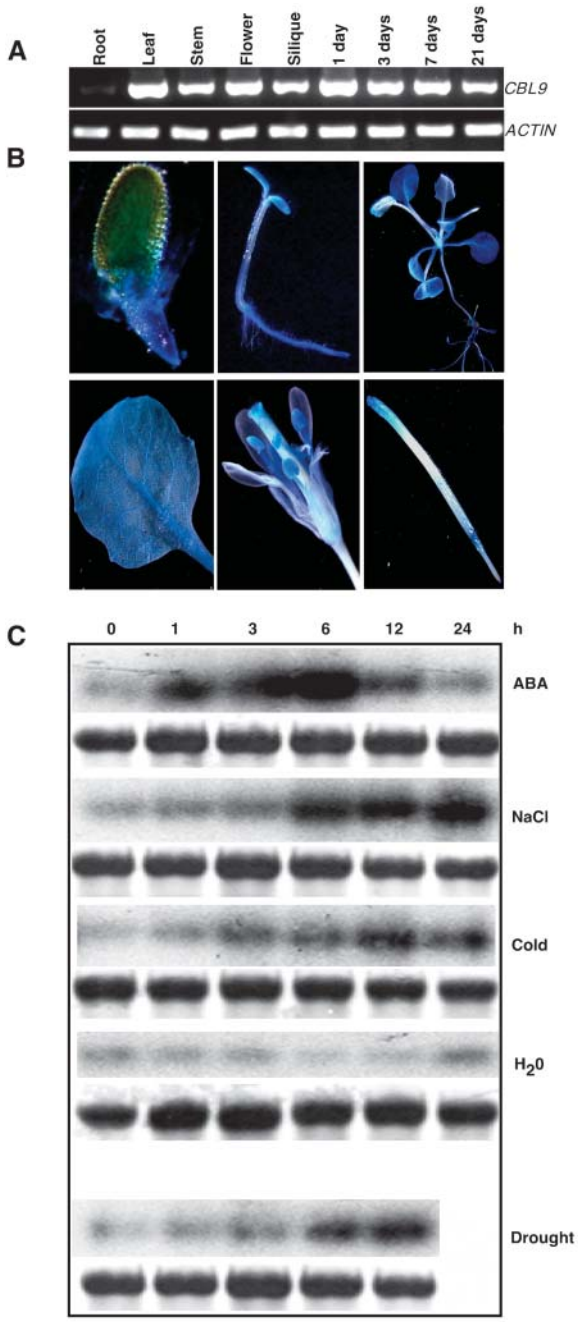

Figure 1. Expression Patterns of the *CBL9* Gene.

(A) RT-PCR analysis of *CBL9* transcripts during seed germination and in different organs of Arabidopsis plants. Total RNA was isolated from various tissues (root, leaf, stem, flower, and silique) of wild-type plants grown under long-day conditions or from germinating seeds and young seedlings (1, 3, 7, and 21 d after sowing). RT-PCR was performed with *CBL9*-specific primers (top gel) or *Actin2*-specific primers (bottom gel).
(B) Histochemical (β-glucuronidase) GUS analysis of *CBL9* promoter-GUS transgenic plants. The top row from left to right depicts a germinating seed, a seedling 2 d after germination, and a 3-week-old plant. The bottom row from left to right shows a leaf of a rosette stage plant, an opened flower, and a silique.

To evaluate the consequences of *CBL9* disruption, we examined the mutant plants under normal growth conditions and found them to be indistinguishable from the wild type (data not shown). Because the *CBL9* gene is regulated by ABA and abiotic stress signals that use calcium in signaling processes, we speculated that CBL9 calcium sensor may mediate calcium function in plant responses to stress and ABA. We tested this hypothesis by several assays. In germination assays for ABA response, the mutant seeds exhibited hypersensitivity to ABA as compared with the wild-type seeds. In addition, mutant seeds and seedlings were hypersensitive to osmotic stress (mannitol), high salt, and glucose. As shown in Figure 3A, the germination and subsequent growth of mutant seedlings were similar to the wild type on the normal medium (MS) but were significantly more inhibited by ABA, glucose, and stress media. Results of more detailed analyses of germination rates under different concentrations of ABA and stress agents are depicted in Figure 3B. Germination in the wild type was not affected by ABA concentration <0.5 μM, but germination of mutant seeds was already significantly inhibited at 0.25 μM ABA. At 0.5 μM ABA, >65% wild-type seeds germinated, whereas only 16% mutant seeds germinated in the 3 d after transferring to 23°C.

At 200 mM NaCl, 70% of wild-type seeds germinated within 3 d, but the germination frequency for the mutant seeds was only 10%. Similarly, germination frequency of wild-type and mutant seeds was 66 and 7%, respectively, on the medium containing 500 mM mannitol. The germination frequency of *cbl9* mutant and the wild type in the 4% glucose medium was significantly different, although such difference was not as large as that under mannitol and salt stress conditions. For example, in the presence of 4% glucose, the 3-d germination frequency was 30 and 12.5% for the wild type and *cbl9* mutant, respectively. The time course of germination was also determined (Figure 3C), and the results further supported the conclusion that mutation of *cbl9* renders the seedlings hypersensitive to ABA, osmotic stress, high salt, and, to a lesser extent, glucose.

Because hyperosmotic stress and high salt induce production of ABA in plants (Leung and Giraudat, 1998; Seo and Koshiba, 2002), *cbl9* mutant sensitivity to these stress conditions could result from increased sensitivity to ABA, increased production of ABA, or both. In other words, altered stress sensitivity may be an ABA-dependent process. To test this possibility, we included an inhibitor for ABA biosynthesis, norflurazon (NF) (Supelco, Bellefonte, PA), in the germination medium. Under normal conditions, 100 μM NF did not affect the germination rate of wild-type or mutant seeds in a 6-d germination assay. On the stress media (containing 250 mM salt or 500 mM mannitol), almost 100% of wild-type seeds germinated in the 6-d incubation. By contrast, only 9 and 20% of the *cbl9* mutant seeds germinated on the salt and mannitol media. In the presence of 100 μM NF, however, the

(C) Four-day-old seedlings grown on MS medium were treated with ABA (100 μM), NaCl (300 mM), cold (4°C), and drought (dehydration) and RNA gel blot analyses performed with *CBL9* gene-specific probe (5′ untranslated region of *CBL9* gene). rRNA on the blot was visualized by staining with methylene blue as an equal loading control.

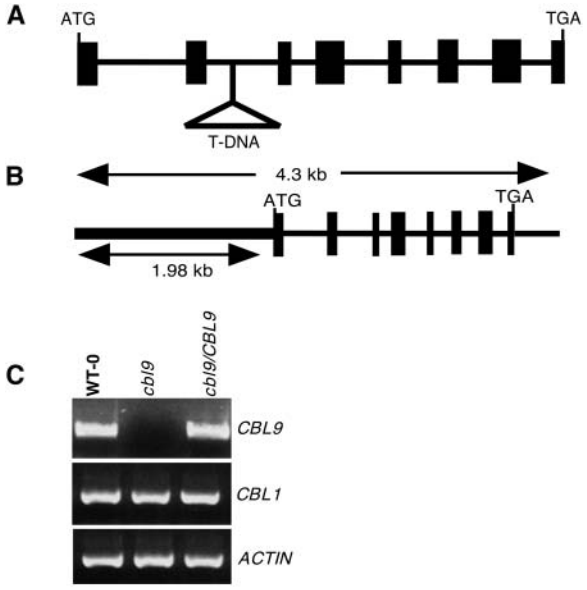

Figure 2. Isolation and Complementation of the *CBL9* T-DNA Insertional Mutant.

(A) Intron–exon organization of the Arabidopsis *CBL9* gene (coding region) and T-DNA location. Solid boxes and lines indicate exons and introns, respectively. The position of the T-DNA insertion is indicated by a triangle (not to scale).

(B) Genomic DNA fragment used for complementation. A 4.3-kb genomic DNA fragment of the *CBL9* gene including 1.98 kb of the 5′ flanking region upstream from the ATG codon and the complete coding region with 3′ UTR was amplified by PCR and cloned into the pCAMBIA1300 vector for plant transformation.

(C) RT-PCR analysis of *CBL9* transcripts in wild-type (WT-0), mutant (*cbl9*), and complementation transgenic lines (*cbl9*/*CBL9*). Eight independent complementation lines were analyzed with similar results, and results from one representative line are shown. Expression of the closely related *CBL1* gene was also examined. *Actin-2* expression level was analyzed as a quantification control.

germination rate of the mutant seeds under the NaCl and mannitol exposure was restored to 70 and 94%, respectively. These results indicate that osmotic stress and salt exerted their inhibitory effect on mutant seed germination through ABA production. It is possible that the *cbl9* mutant is altered in ABA sensitivity (Figure 4A) and biosynthesis.

In the course of the germination assays, we noted that the post-germination development of *cbl9* mutant seedlings appeared to be more sensitive to ABA and stress media as compared with the wild-type seedlings (Figure 3A). To further corroborate this observation, we germinated the wild-type and mutant seeds on MS medium and subsequently transferred similar seedlings to ABA-containing or stress media after 4 d of growth. True leaf emergence and relative root elongation were then scored and compared between the wild-type and mutant seedlings. Because root growth and leaf emergence were inhibited by different levels of ABA, we used different concentrations of ABA for these assays. Figures 4B and 4C indicate that both root growth and leaf emergence of *cbl9* mutant seedlings were more sensitive to ABA and the other stress conditions than the corresponding wild-type seedlings. These results indicate that CBL9 not only regulates ABA, osmotic, and salt stress responses at the germination stage of Arabidopsis but also affects post-germination developmental processes.

Mutation of CBL9 Affects ABA- and Drought-Induced Gene Expression

The germination and post-germination assays revealed an increased sensitivity of *cbl9* mutant under ABA exposure and osmotic stress conditions. The specific and dramatic induction of stress gene expression represents prominent response to ABA exposure and abiotic stress stimuli at the molecular level. Therefore, stress genes such as *RD29A*, *RD29B*, *RD22*, *KIN1*, and *RAB18* and *COR47* serve as convenient markers for monitoring the ABA and stress response pathways in plants (Gilmour et al., 1992; Kurkela and Borg-Franck, 1992; Lang and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Tahtiharju et al., 1997; Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Kim et al., 2003). The expression of *RD29A* and *KIN1* genes has been shown to be responsive to multiple stress signals (drought, high salt, and cold) and ABA, whereas the expression of *RD29B* and *RD22* responds only to drought, salt, and ABA (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinozaki and Yamaguchi-Shinozaki, 2000; Kim, et al., 2003). *RAB18* and *COR47* genes are mainly responsive to ABA and osmotic stress (Gilmour et al., 1992; Lang and Palva, 1992). Using these gene markers, we compared the *cbl9* mutant and the wild-type plants in their patterns of stress gene expression.

Upon ABA treatment, *RD29A*/*B* and *KIN1* were induced in both the wild-type and *cbl9* mutant seedlings (Figures 5A and 5B). However, ABA-mediated induction of these genes in the *cbl9* mutant plants was significantly stronger than the induction in wild-type plants. This was observed for all gene markers, although the extent and kinetics of induction were different among different markers. A similar hyperinduction of stress marker genes was apparent under drought conditions (Figures 5C and 5D). Therefore, CBL9 appears to act as a negative regulator in ABA and drought-responsive gene expression, consistent with the results from seed germination assays.

In some cases, levels of stress gene expression correlates with stress tolerance in plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998). But in other cases, such modification of stress gene expression appears to be insufficient to induce tolerance changes at the whole plant level (Kim et al., 2003). We examined stress tolerance in *cbl9* mutant plants and found that it is similar to the tolerance of wild-type plants (data not shown). In addition, stomatal response to ABA was not altered in the *cbl9* mutant (data not shown), suggesting that the *CBL9* gene may play an indispensable role in ABA and stress response during seed germination and early development of the plants. Although changes in later developmental stages of the mutant were not observed, there may be additional phenotypic changes under specific conditions not examined in this study.

To determine whether the phenotypic changes in the *cbl9* mutant were caused by the disruption of *CBL9* gene, we examined the germination and gene expression pattern in

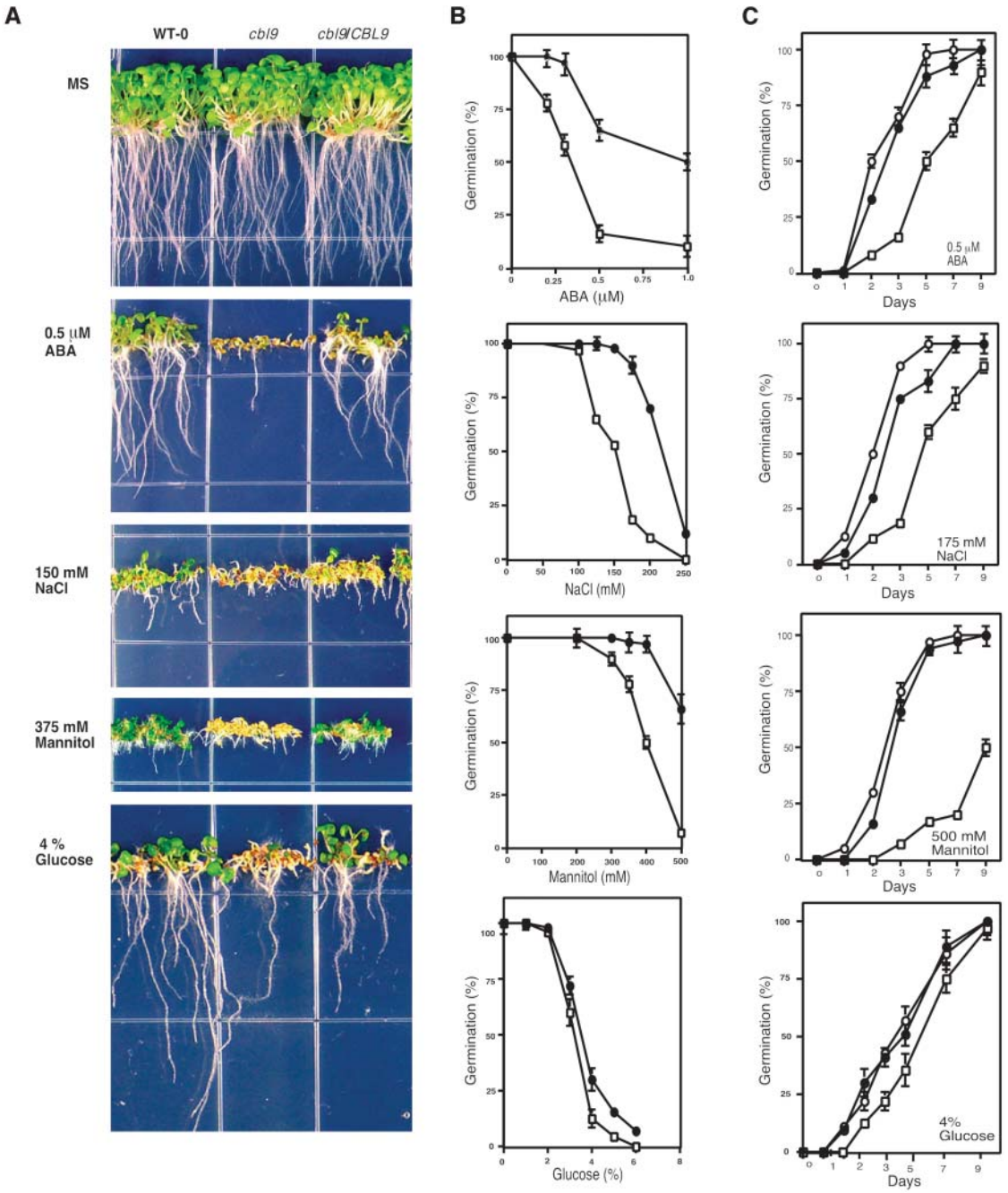

Figure 3. Germination of *cbl9* Mutant Seeds Is Hypersensitive to ABA, Glucose, and Osmotic Stress Conditions.

(A) Inhibition of germination and young seedling growth in the *cbl9* mutant, wild-type, and complemented (*cbl9*/*CBL9*) plants. Wild-type (WT-0), *cbl9* mutant, and complemented seeds on MS agar medium or MS medium containing 0.5 μM ABA, 150 mM NaCl, or 375 mM mannitol and 4% glucose were incubated at 4°C for 6 d before transfer to 23°C for germination. The photograph was taken on day 10 after transfer to 23°C.

(B) Germination frequency of the wild-type and *cbl9* mutant seeds at 3 d after transfer to 23°C in the presence of different concentrations of ABA, NaCl, or mannitol and glucose. The wild type is indicated as closed circles and mutants as open squares.

(C) Germination time course (days after incubation at 23°C) on medium containing 0.5 μM ABA, 175 mM NaCl, or 500 mM mannitol and 4% glucose. The wild type is indicated as closed circles, mutant as open squares, and complemented line (*cbl9*/*CBL9*) as open circles. Results in **(B)** and **(C)** are presented as average values and standard errors from three experiments.

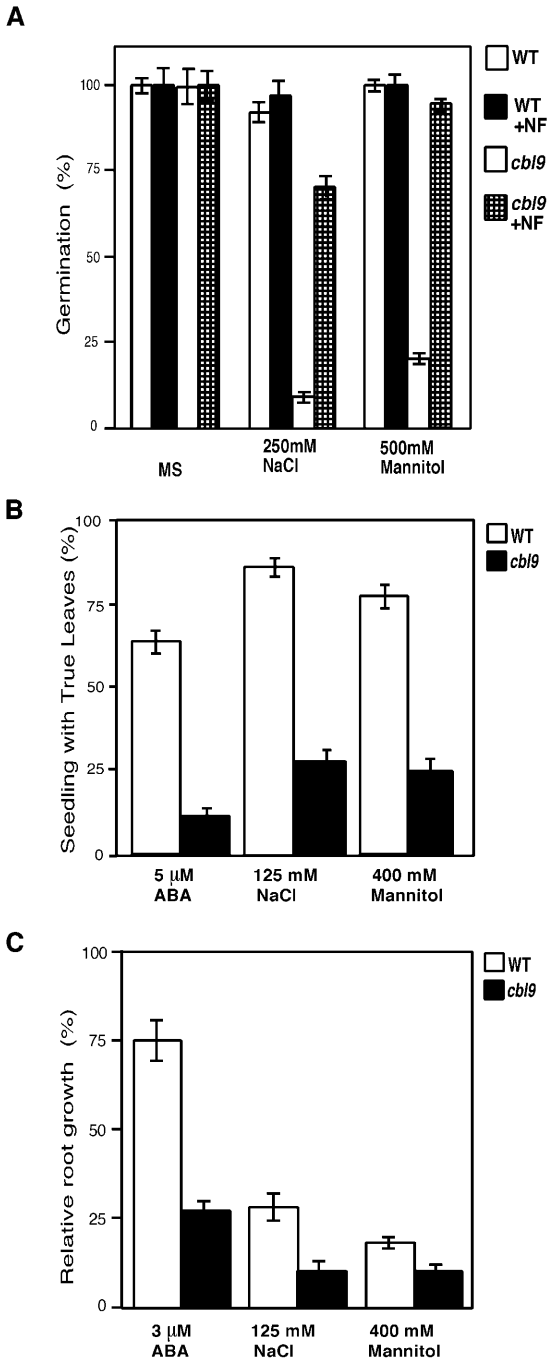

Figure 4. Effect of the ABA Biosynthesis Inhibitor NF on the Germination and Sensitivity of Seedling Development to ABA and Osmotic Stress.

(A) Germination of *cbl9* mutant seeds and the wild type on medium containing 100 μM NF and 250 mM NaCl or 500 mM mannitol. Seed germination rate was scored at 6 d after transfer to 23°C growth chamber.

(B) Sensitivity of seedling development to ABA and osmotic stress. Ratio of seedlings with true leaves over total number of seedlings transferred onto the MS medium supplemented with defined concentration of ABA (5 μM), NaCl (125 mM), or mannitol (400 mM) was measured after 2 weeks.

(C) Sensitivity of root growth to ABA and osmotic stress after germination. Four-day-old seedlings of *cbl9* mutant and WT-0 were transferred from MS medium to MS medium supplemented with ABA, NaCl, and mannitol. The relative root length (ratio of average root elongation on ABA or stress containing media over average root elongation on MS medium) was measured 2 weeks after transfer. Results in **(A)** to **(C)** are presented as average values and standard errors from three experiments.

complemented lines as compared with the wild-type and mutant plants. We studied eight independent complemented lines and found that all of them displayed phenotypes similar to the wild type. Figure 3A illustrates the results with one of the eight representative lines in the germination assays. Figure 3C shows detailed analysis of germination rate and time course of germination in the wild-type, mutant, and one complemented line. In all these assays, the ABA sensitivity of complemented lines was similar to the wild type. The stress gene expression in the complemented lines was also largely restored to wild-type levels (Figure 5). Taken together, these results from the complementation analysis confirmed that the observed mutant phenotypes resulted from disruption of *CBL9* expression.

CBL9 Plays a Role in the Regulation of ABA Biosynthesis

The germination of *cbl9* mutant seeds was not only hypersensitive to ABA but also to osmotic stress caused by mannitol and salt that often activate ABA biosynthesis. Addition of an ABA biosynthesis inhibitor rescued stress hypersensitivity in the *cbl9* mutant, suggesting that osmotic stress response in the mutant is an ABA-dependent phenomenon. The stress hypersensitivity can be caused by ABA hypersensitivity, elevated ABA biosynthesis, or both. Because we had already established that mutation of *cbl9* renders plants hypersensitive to ABA, we went further to test if biosynthesis of ABA was also changed in the *cbl9* mutant. ABA content was determined in seedlings grown on MS medium and MS medium supplemented 150 mM NaCl, 375 mM mannitol, or 4% glucose. We measured ABA contents of the wild type, the mutant, and a complemented line at two developmental stages, 4 d and 10 d after transfer to 23°C (Figures 6A and 6B). At both developmental stages, osmotic stress increased the levels of ABA in all plants. Interestingly, mutant seedlings contained much higher levels of ABA than the wild-type and complemented lines under salt and mannitol treatments. The ABA levels in plants grown on the MS or MS-glucose medium were comparable. These data indicated that CBL9 not only plays a role in the regulation of ABA sensitivity but also in the biosynthesis of ABA under osmotic stress conditions. Therefore, the hypersensitive response to osmotic stress conditions in the mutant could be a result of both altered ABA sensitivity and biosynthesis.

Increased Expression of ABA Signaling Genes in the *cbl9* Mutant

As discussed earlier, several regulatory factors in ABA responses during seed germination and early seedling development have been identified by genetic screens. These factors include ABI1 and ABI2, two protein phosphatases that serve as negative

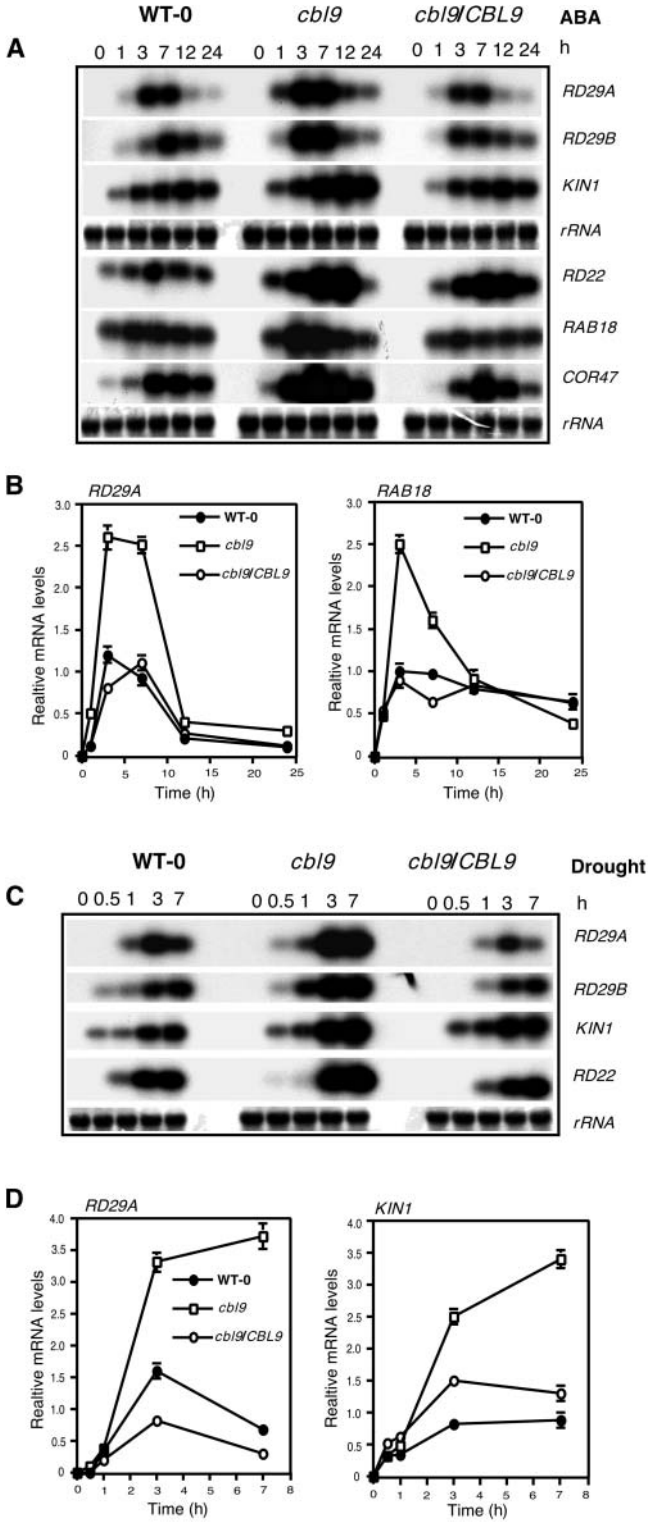

Figure 5. Expression of Stress Responsive Gene Markers in Wild-Type (WT-0), *cbl9* Mutant, and *cbl9/CBL9* Seedlings after ABA Exposure and Drought Treatment.

(A) Four-day-old seedlings grown on MS medium were treated with ABA (100 μM), and RNA gel blot analyses were performed with respective stress marker gene probes. rRNA on the blot was visualized by staining with methylene blue as an equal loading control.

(B) Relative levels of *RD29A* and *RAB18* transcripts in **(A)**.

(C) RNA gel blot analyses with RNA isolated from drought treated plants.

(D) Relative levels of *RD29A* and *KIN1* transcripts in **(C)**.

regulators of ABA responses, and ABI3, ABI4, and ABI5, transcription factors that function as positive regulators of ABA responses. In many cases, these signaling genes are regulated by stress conditions and ABA, and such regulation may play a role in ABA sensitivity (Lu and Fedoroff, 2000; Finkelstein et al., 2002; Lu et al., 2002). Because *cbl9* mutants display strong ABA hypersensitivity in early seedling development, we tested the possibility that the expression of ABA signaling genes may have been affected in the *cbl9* mutant. We examined the expression levels of these genes under ABA treatment. The RNA gel blot results revealed significant changes in the expression levels of ABI3, ABI4, and ABI5 in *cbl9* mutant plants in response to ABA (Figure 7). In the presence of ABA in the medium, the mRNA levels of ABI4 and ABI5 genes were three to four times higher in the *cbl9* mutant seedlings as compared with the wild type. The expression of ABI4 and ABI5 under control conditions (without ABA) was also slightly enhanced in the mutant (Figure 7). We did not detect discernable changes in the expression of *ABI1* and *ABI2* (data not shown). Because overexpression of any member of ABI3-5 causes ABA hypersensitivity (Lopez-Molina et al., 2001; Finkelstein et al., 2002), elevated levels of *ABI3-5* genes in the *cbl9* mutant may be linked to the ABA hypersensitive response in this mutant.

Our study indicated that stress hypersensitive response in the germination in the *cbl9* mutant may result from ABA hypersensitivity and elevated level of ABA biosynthesis in the mutant. If this was the case, the expression of ABA signaling genes, such as *ABI3-5*, in the *cbl9* mutant should be hypersensitive to osmotic stress conditions as well. We therefore examined the mRNA levels of *ABI1-5* and found that expression of *ABI3-5* genes was strongly elevated in the *cbl9* mutant under stress conditions (Figures 8A and 8B). Especially the *ABI5* gene exhibited a dramatic increase in the detectable mRNA amounts. We noted that *cbl9* mutant plants grown on the MS medium did not show significant increase in *ABI4* and *ABI5* mRNA levels. We did not detect discernable changes in the expression of the *ABI1* and *ABI2* genes in the mutant plants under either control or stress conditions (data not shown). The expression pattern of *ABI* genes in the complemented line matched the pattern in the wild-type plants, again confirming that changes in gene expression in the mutant resulted from the disruption of the *CBL9* gene.

DISCUSSION

Many studies implicate Ca^{2+} as a second messenger in abiotic stress and ABA responses (Knight et al., 1996, 1997; Sanders et al., 1999, 2002; Knight and Knight, 2000; Rudd and Franklin-Tong, 2001). There are several lines of evidence supporting this conclusion. First, elevation of cellular Ca^{2+} is a rapid response to ABA, cold, drought, and salinity (Knight et al., 1996, 1997). Second, Ca^{2+} elevation is required for the expression of some

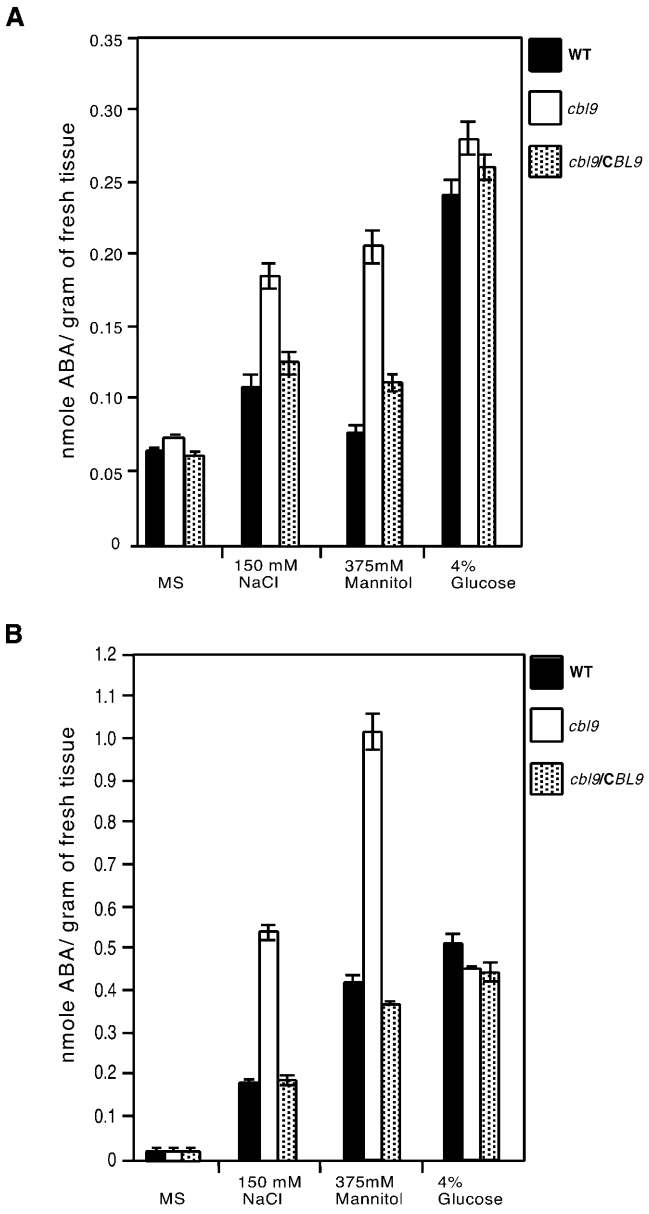

Figure 6. ABA Levels in Wild-Type, *cbl9* Mutant, and Complemented (*cbl9*/*CBL9*) Plants with or without Stress.

ABA level was determined in 4-d-old **(A)** and 10-d-old **(B)** seedlings grown on MS medium alone and MS medium supplemented with 150 mM NaCl, 375 mM mannitol, and 4% glucose. Results shown are average of two independent experiments.

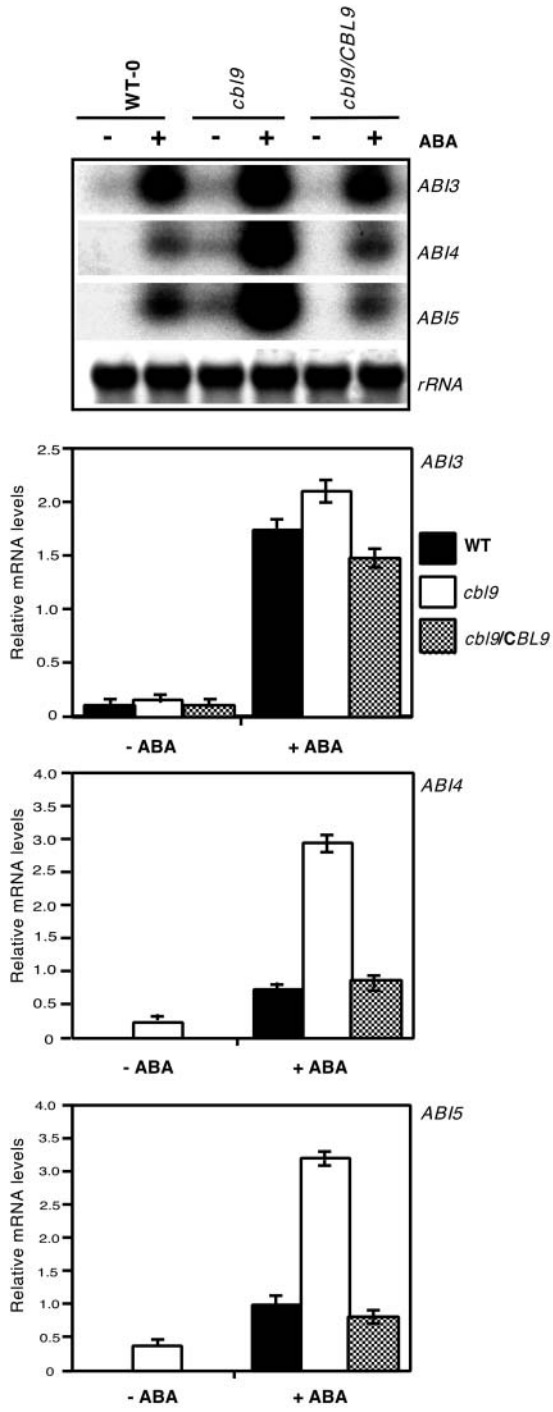

Figure 7. Regulation of ABI Transcripts upon ABA Treatment.

Seeds of the wild type, *cbl9* mutant, and *cbl9*/*CBL9* were plated on MS medium with (+) or without (–) 0.7 μM ABA and stratified at 4°C for 5 d. RNA was isolated from these seedlings after 3 d of growth at 23°C. RNA gel blot analyses using *ABI3*, *ABI4*, and *ABI5* probe. A single representative rRNA on the membrane was visualized by staining with methylene blue and was shown as loading control. Graphs representing relative expression levels of *ABI3*, *ABAI4*, and *ABI5* in wild-type (WT-0), *cbl9* mutant, and complemented seeds grown on MS medium with or without 0.7 μM ABA.

stress-induced genes in plants (Knight et al., 1996). Third, Ca^{2+} elevation is sufficient for activation of a stress gene promoter (Sheen, 1996). It is generally believed that Ca^{2+} transmits the stress signal downstream in the pathway by interacting with protein sensors. However, little is known on how these calcium sensors might function in different signaling processes. Here, we provide evidence that a CBL-type calcium sensor, CBL9, regulates ABA signaling and stress-induced ABA biosynthesis

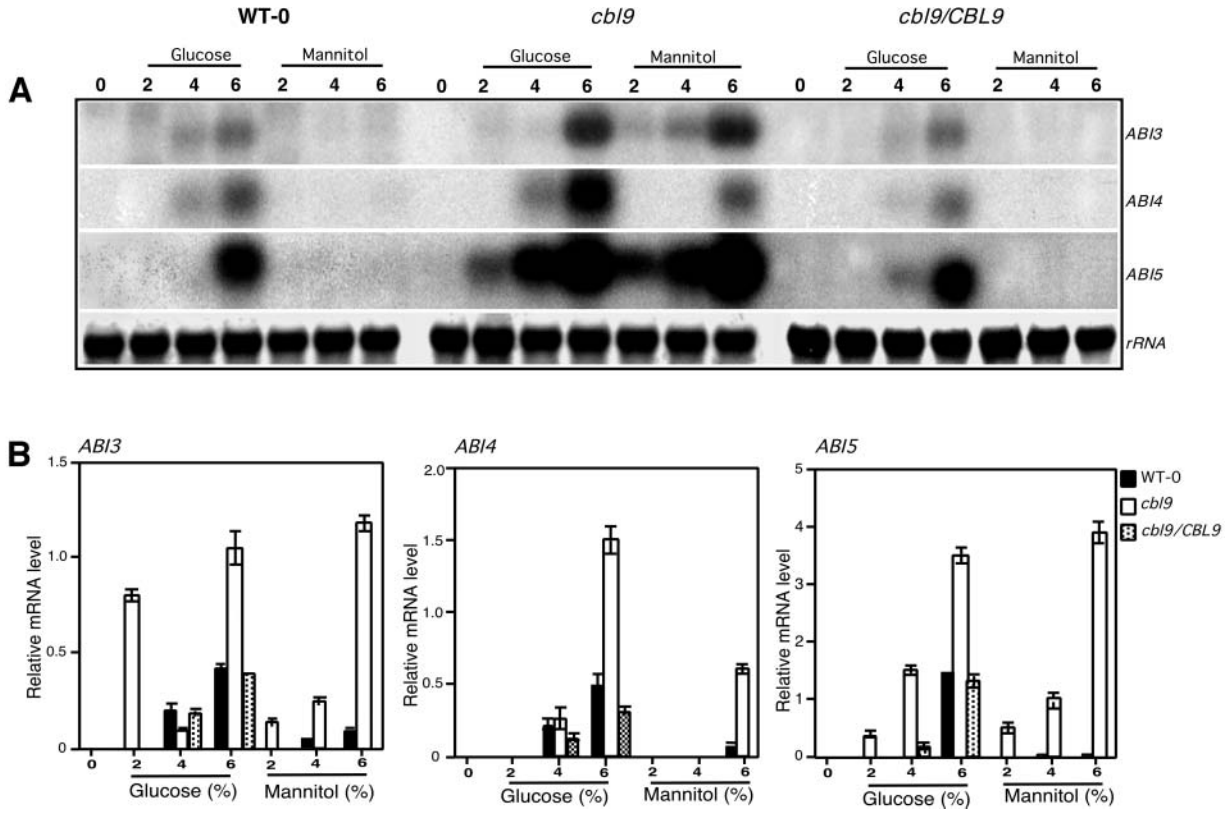

Figure 8. Regulation of ABI Transcripts in WT-0, *cbl9*, and *cbl9/CBL9* under Glucose and Mannitol Stress Condition.

(A) Plants were grown on MS plates without (0) and with 2, 4, and 6% glucose or 2, 4, and 6% mannitol for 14 d under a 16/8-h photoperiod. RNA gel blot analyses were performed three times with consistent results.

(B) Graphs representing relative expression levels of *ABI3*, *ABI4*, and *ABI5* upon glucose and mannitol stress in wild-type (WT-0), *cbl9* mutant, and complemented (*cbl9*/*CBL9*) seedlings.

pathways. In contrast with the conventional paradigm that calcium sensors should be required or serve as positive regulators of stress and ABA responses, we found that CBL9 serves as a negative regulator in these processes, and its normal function appears to desensitize ABA and stress responses. This study thus provides a novel role of calcium signaling in the regulation of these cellular processes.

CBL9 Desensitizes ABA Effects in Seed Germination and Reduces ABA-Induced Gene Expression in Vegetative Tissues

Although several studies have shown that calcium is required for ABA responses in the stomatal guard cells (Allen et al., 1999), little is known about its role in seed germination in dicot plants. During seed germination in monocots, calcium serves as a second messenger for gibberellic acid (GA) that promotes germination by stimulating α-amylase production in the aleurone cells (Gilroy, 1996). In the aleurone model, ABA antagonizes GA function, thereby inhibiting germination processes. Unlike the situation in the guard cells, a potential role has not been assigned for calcium in ABA response in aleurone cells (Kuo et al., 1996). Although less is known about GA and ABA interaction in seed germination of dicot plants, such as Arabidopsis, several studies have revealed a similar antagonistic relationship between ABA and GA. However, the role of calcium has not been addressed in GA or ABA signaling during seed germination in dicot plants. Our results in this study provide new information regarding calcium signaling in dicot seed germination processes. Although we cannot exclude the possibility that calcium could potentially serve as a positive regulator for ABA responses, our finding places a calcium sensor (CBL9) as a negative regulator of ABA responses during seed germination in Arabidopsis.

A negative role for CBL9 in ABA inhibition of seed germination naturally fits to the ABA and GA antagonism. If, like in monocot seeds, calcium functions as a downstream messenger that is essential for GA responses leading to seed germination, it appears conceivable for calcium to serve as a negative regulator for ABA signaling that antagonizes GA action and results in seed dormancy. Therefore, calcium may function as a cross talk node between ABA and GA signaling pathways in these processes. Calcium could activate downstream components in the GA pathway but inhibit downstream components in the ABA signaling network. The CBL9 protein would thereby represent the calcium sensor that imposes a negative role toward the ABA response. Some potential downstream targets for CBL9 function

may include ABI signaling proteins. Under ABA application and glucose and osmotic stress conditions, expression levels of *ABI3-5* mRNA were elevated in the *cbl9* mutant. At the young seedling stage, expression of at least *ABI4* and *ABI5* genes was upregulated in the *cbl9* mutant even without ABA or stress treatments. This might indicate that CBL9 protein normally functions as an upstream suppressor of these ABI genes. Elevated levels of *ABI3-5* have been shown to be sufficient for ABA hypersensitive response in transgenic plants (Finkelstein et al., 2002). This might suggest that upregulation of these genes may lead to the ABA hypersensitivity observed in the *cbl9* mutant. However, it might be equally tenable that the upregulation of the *ABI* genes in the mutant represents a consequence of ABA hypersensitivity in the *cbl9* mutant. In addition, increased endogenous ABA levels in the *cbl9* mutant might contribute to increased expression of *ABI* genes under stress conditions. Further work is necessary to dissect the functional relationship between CBL9 and ABI proteins in the regulation of ABA signaling.

Unlike the germination process, ABA-responsive gene expression in vegetative tissues has been shown to require calcium (Sheen, 1996; Knight et al., 1997). In one study, elevation of calcium is sufficient to trigger ABA-responsive gene expression (Sheen, 1996). In that case, two calcium-dependent protein kinases may function as calcium sensors and activate their downstream targets. How might CBL9, also a calcium sensor, function as a negative regulator to suppress ABA activation of gene expression? We speculate that CBL9-mediated processes may function as a feedback mechanism to downregulate the signaling strength and/or duration when calcium reaches high levels in the cell. This mechanism would desensitize the cell to ABA signals when ABA becomes a constant presence under stress conditions. Such a mechanism could also terminate this signaling pathway when ABA is removed. In either case, disruption of *CBL9* gene function would lead to the ABA hypersensitive phenotype observed in the mutant plants.

Although CBL9 functions as a negative regulator of ABA response in both seed germination and gene expression regulation in vegetative tissues, the molecular context for CBL9 action may be distinct. In our previous study on the CIPK3 (Kim et al., 2003), we observed that the mutant disrupted in CIPK3 function exhibited similar ABA-hypersensitive responses in the germination assays. Because both CBL9 and CIPK3 are preferentially expressed in germinating seeds, it is conceivable that CBL9 may interact with CIPK3 in the regulation of ABA sensitivity during seed germination. However, the ABA-responsive gene expression in the *cipk3* mutant plants showed a distinct pattern from that of the *cbl9* mutant, suggesting that CBL9 may also interact with other CIPK(s) in the regulation of ABA response in adult plants.

A Calcium Sensor for the Modulation of ABA Biosynthesis

In response to certain abiotic stress conditions, especially osmotic stress, plants accumulate ABA that facilitates the development of stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002). Despite the importance of stress-induced ABA in stress physiology, little is known about the signaling mechanisms that regulate ABA biosynthesis under stress conditions.

Several studies have established that ABA biosynthesis genes are positively regulated by both exogenous ABA and stress conditions such as drought or salinity. Such regulation may be largely achieved by increased transcriptional activity of genes encoding ABA biosynthetic enzymes (Bray, 2002; Seo and Koshiba, 2002; Xiong and Zhu, 2003). With only few exceptions, expression of many ABA biosynthesis genes, including *ABA1*, *ABA3*, *AAO3*, and *NCED3*, is upregulated by ABA exposure and osmotic stress in Arabidopsis. Consequently, a positive regulatory loop has been proposed to explain increased ABA levels in plants under stress conditions (Xiong and Zhu, 2003). In this scenario, the stress signal would upregulate the expression of ABA biosynthesis genes leading to ABA accumulation in the plants. Higher ABA levels would further activate the ABA biosynthesis genes and ABA production in the plants. However, these processes require a balanced signal transmission. Our study is consistent with this principle and illustrates CBL9 as a negative regulator of this positive feedback loop. The CBL9 protein appears to function as a negative regulator of ABA signaling that leads to inhibition of seed germination and ABA biosynthesis. When CBL9 function is disrupted, the *cbl9* mutant displayed ABA hypersensitivity in seed germination and increased ABA biosynthesis under stress conditions. Thus, CBL9 is located in an ABA signaling branch that controls both seed germination and ABA biosynthesis.

Functional Specificity of CBL Calcium Sensors in Diverse Signaling Processes

This and other studies (Albrecht et al., 2003; Cheong et al., 2003; Kim et al., 2003) have revealed a complex molecular network composed of CBL calcium sensors and their target kinases that function in a diverse array of signaling processes. Perhaps the most striking finding is the functional specificity of single components of the CBL-CIPK network. A good example has been set by studies on CBL9 (this report) and CBL1 (Albrecht et al., 2003; Cheong et al., 2003). These studies demonstrate that highly similar calcium sensors can fulfill very distinct functions. At the level of amino acid sequence, CBL1 and CBL9 are ~90% identical (Kolukisaoglu et al., 2004). Both *CBL1* and *CBL9* genes are expressed in most plant tissues. CBL1 and CBL9 also share a large fraction of CIPK target proteins. With these common properties, they are expected to function in similar processes. However, the genetics analysis using loss-of-function and gain-of-function mutants and transgenic plants showed that CBL1 and CBL9 have distinct functions. Disruption of the *CBL1* gene resulted in changes in stress responses, including reduced level of stress gene expression in response to drought and hypersensitivity to drought stress at whole plant level (Albrecht et al., 2003; Cheong et al., 2003). Interestingly, disruption of CBL9 resulted in opposite changes in drought-induced gene expression, and such changes did not cause a whole-plant phenotype (this study). Whereas *cbl9* mutants showed strong ABA hypersensitive phenotype, the *cbl1* mutant was not altered in its ABA responsiveness (Albrecht et al., 2003; Cheong et al., 2003). Another report (Guo et al., 2002) indicated that *CBL1* gene

silencing by an RNA interference procedure caused ABA hypersensitivity. Because the coding region of *CBL1* shares high homology with *CBL9* in the nucleotide sequence, the RNA interference construct using the entire *CBL1* coding region (Guo et al., 2002) might have suppressed the expression of both *CBL1* and *CBL9* genes leading to the observed ABA hypersensitive response. Analyses of individual knockout mutants of *CBL1* and *CBL9* clearly demonstrated high specificity of their functions in stress and ABA responses. Regarding the opposite function of CBL1 and CBL9 in drought-responsive gene expression, we speculate that CBL1 and CBL9 may antagonize each other's function because of shared CIPK targets (Cheong et al., 2003; Kolukisaoglu et al., 2004). For example, if CBL1 functions as a positive regulator of drought-induced gene expression by interacting with CIPKn that also interacts with CBL9, CBL1 and CBL9 would compete for the same CIPK target(s). Disruption of CBL9 would lead to more effective CBL1 function because of removal of competition from CBL9. Conversely, the similarity in the amino acid sequence, expression pattern, and interactive CIPKs may suggest that CBL1 and CBL9 share common functions. Such functional redundancy may be one of the reasons for the limited phenotypic changes observed in the *cbl9* mutant despite a rather ubiquitous expression pattern of the *CBL9* gene. Further analysis of double mutants disrupted in *CBL9* and *CBL1* genes will help test this hypothesis.

The specific functions of different CBLs may result from several factors, including the temporal and spatial pattern of their expression and subcellular localization, their calcium binding affinity, and the repertoire of interactive CIPKs. In addition, each calcium sensor may have a unique capability of interpreting calcium signatures. For example, CBL1 and CBL9 may recognize different calcium signatures and thereby function in different signaling pathways. Clearly it deserves much future effort to dissect the mechanism underlying the connection between the specific function of a calcium sensor and the calcium signature it decodes. In this regard, studies on CBL1 and CBL9 reveal the complexity and fine-tuning of the CBL-CIPK signaling network that may provide a critical model system for studying the decoding mechanism of calcium signals in plants (Luan et al., 2002).

METHODS

Plant Materials, Stress Treatments, and RNA Analysis

Arabidopsis thaliana plants (ecotype Columbia) were grown in the greenhouse under long-day conditions (16-h-light/8-h-dark cycle) to the flowering stage for plant transformation. For RNA analysis, 4-d-old seedlings grown on MS medium (Sigma, St. Louis, MO) were treated under different stress conditions. Seeds were treated with isopropanol for 5 min and with 50% bleach for 15 min, washed five times with sterile water, and plated on MS medium solidified with 0.8% agar.

For ABA treatment, 100 μM (±)-*cis*, *trans*-ABA solution in water was sprayed onto the 4-d-old seedlings grown on MS plates to ensure total coverage of the foliage area. Seedlings treated with ABA were incubated at room temperature under white light. In parallel experiments, water was sprayed as control. To perform drought treatments, 4-d-old seedlings grown in MS medium were exposed in the laminar flow hood for dehydration as described previously (Kim et al., 2003). For RNA gel blot analysis, total RNA (10 μg) isolated with Tripure isolation reagent (Roche Diagnostics, Indianapolis, IN) was separated by electrophoresis on a 1.5% agarose gel and transferred to GeneScreen Plus nylon membranes. The membranes were prehybridized and hybridized at 65°C as described (Pandey et al., 2002) with ^{32}P-labeled probe specific for each gene marker. The membranes were autoradiographed with Kodak XAR film (Rochester, NY). Membrane-bound 23s rRNA was stained with methylene blue and used as a quantification control. The mRNA was quantified using a Storm Bio-Imaging Analyzer (Molecular Dynamics, Sunnyvale, CA). All RNA gel blot experiments were repeated three times using new plant materials, and results from one representative experiment are shown.

RT-PCR Analysis of *CBL9* Gene Expression

To examine the expression of *CBL9* by RT-PCR, DNase I treated, total RNA (5 μg) was denatured and subjected to reverse transcription reaction using Superscript II (200 units per reaction; Invitrogen, San Diego, CA) at 42°C for 50 min followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. PCR amplification was performed with initial denaturation at 94°C for 3 min followed by 25 cycles of incubations at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 10 min using *CBL9*-specific forward (5′-GATGATGGGGAGTGAGTAATATCAGAA-3′) and reverse (5′-GTCCACCTCCGAGTTAAATACGAAACT-3′) primers. Expression levels of *Actin-2* were monitored with forward (5′-GGAAAGGATCTGTACGGTAAC-3′) and reverse (5′-TGTGAACGATTCCTGGAC-3′) primers to serve as a quantifying control. The *CBL1* gene-specific forward (5′-GGGCTCGGGTATAAGGTTTCATCGTCT-3′) and reverse (5′-GCAATCTCATCGACCTCCGAATGGAAG-3′) primers were used to detect *CBL1* transcript level. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Isolation and Complementation of the *cbl9* T-DNA Insertional Mutant

The *cbl9* mutant was isolated from the T-DNA–transformed Arabidopsis collection from Syngenta Research and Technology (San Diego, CA). The insertion in the gene was identified by using T-DNA left border primer, LB-1 (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCCT-3′) and *CBL9* gene-specific forward (5′-CTCTTGTCATCCGTAAATGGGTTGTTTC-3′) and reverse (5′-GCTTGTCTTGTCTCTTTCACGTCGCAATCTCG-3′) primers. The T-DNA insertion in the mutant (*cbl9*) was confirmed by PCR and DNA gel blot analysis, and its exact position was determined by sequencing. After selfing of heterozygous plants, homozygous *cbl9* mutant was identified by PCR and analyzed further by DNA sequencing to confirm the insertion of the T-DNA in the gene.

For complementation of the *cbl9* mutant, a 4.3-kb fragment including the *CBL9* coding region and 1.98 kb of the 5′ flanking DNA upstream of the starting codon was amplified by PCR from Arabidopsis genomic DNA with forward (5′-AAATCTAGACATGCATGATTTGGCCATGTCTTATGG-3′) and reverse (5′-AAAGGATCCGCTACTGGGATCATCATTGGTCTGT-3′) primers. The PCR product was cloned into the binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia) using *Xba*I and *Bam*HI restriction sites (underlined in the primer sequences). The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into *cbl9* mutant plants by the floral dip method (Clough and Bent, 1998). Transgenic seeds were plated on half-strength MS medium containing 0.8% (w/v) agar, 112 mg/L of Gamborg's B5 vitamin mixture, and 15 μg/mL of hygromycin. Resistant seedlings were transplanted to soil and grown in the greenhouse to produce seeds. The complemented lines (*cbl9*/*CBL9*) were used for germination assays or RNA gel blot analyses. All of the PCR procedures were performed using Pfu DNA

polymerase (Stratagene, La Jolla, CA) to enhance fidelity. All constructs were verified by DNA sequencing.

Germination Assay

Approximately 100 seeds each from the wild type, *cbl9* mutant, and *cbl9*/*CBL9* complemented lines were planted in triplicate on MS medium with different concentrations of ABA, NaCl, mannitol, or glucose and incubated at 4°C for 6 d before being placed at 23°C under long-day conditions. Germination (emergence of radicles) was scored daily for 9 d. The vertical germination and growth assays shown in Figure 3 were performed in a similar manner except that the plates were placed vertically on a rack. Plant growth was monitored and photographed after 9 d.

Analysis of *CBL9* Promoter-GUS Expression in Transgenic Plants

To generate the *CBL9* promoter-GUS construct, the 5′ flanking DNA of the *CBL9* coding region was amplified with forward (5′-AAAGTCGACTGACCATTTAATTTGGAATTCTAA-3′) and reverse (5′-AAATCTAGATTACGGATGACAAGAGTAAAACG-3′) primers. The 1575-bp PCR fragment was cloned into *Sal*I-*Xba*I sites (underlined in the primer sequences) in the pBI101.1 vector (Clontech, Palo Alto, CA). The construct was transformed into wild-type plants and transformants were selected on 50 μg/mL of kanamycin. T1 transgenic seedlings were stained with 5-bromo-4-chloro-3-indolyl-D-glucuronide for 12 h followed by incubation in 80% ethanol to remove chlorophyll (Jefferson et al., 1987).

ABA Measurement

Seedlings were grown on MS medium with or without NaCl (150 mM), mannitol (375 mM), and glucose (4%) for 4 or 10 d at 23°C under long-day conditions in a growth chamber. Plant tissues were then harvested and immediately frozen in liquid nitrogen and ground to powder. ABA was extracted as described by Xiong et al. (2001).

One gram of powdered tissue was suspended in 15 mL of extraction buffer containing 80% methanol, 100 mg/L of butylated hydroxyoluene, and 0.5 g/L of citric acid monohydrate. The suspension was stirred overnight at 4°C and centrifuged at 1000*g* for 20 min. ABA concentration in the solution was determined using the Phytodetek ABA immunoassay kit (Agdia, Elkhart, IN).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AF411958.

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