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A Calcium Sensor and Its Interacting Protein Kinase Are Global Regulators of Abscisic Acid Signaling in *Arabidopsis*

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Summary

The phytohormone abscisic acid (ABA) triggers an oscillation in the cytosolic Ca^{2+} concentration, which is then perceived by unknown Ca^{2+} binding proteins to initiate a series of signaling cascades that control many physiological processes, including adaptation to environmental stress. We report here that a Ca^{2+} binding protein, SCaBP5, and its interacting protein kinase, PKS3, function as global regulators of ABA responses. *Arabidopsis* mutants with silenced *SCaBP5* or *PKS3* are hypersensitive to ABA in seed germination, seedling growth, stomatal closing, and gene expression. PKS3 physically interacts with the 2C-type protein phosphatase ABI2 (ABA-insensitive 2) and to a lesser extent with the homologous ABI1 (ABA-insensitive 1) protein. Thus, SCaBP5 and PKS3 are part of a calcium-responsive negative regulatory loop controlling ABA sensitivity.

Introduction

The phytohormone abscisic acid (ABA) has a wide range of important roles in plant growth and development including embryo development, seed maturation, dormancy, root and shoot growth, leaf transpiration, and stress tolerance (Koornneef et al., 1998; Leung and Giraudat, 1998; McCourt, 1999). The mechanisms by which plants perceive and transmit ABA signal to cellular machineries to initiate these physiological responses are not well understood. Genetic analysis by using the inhibitory effects of ABA on seed germination has identified several components that are involved in aspects of ABA signaling. The dominant *Arabidopsis abi1-1* and *abi2-1* mutations render plants insensitive to ABA during both seed germination and vegetative growth (Koornneef et al., 1998). In comparison, *abi3*, *abi4*, and *abi5* mutations reduce ABA sensitivity only during seed germination (Koornneef et al., 1998). *ABI1* and *ABI2* encode homologous 2C-type protein phosphatases (Leung et al., 1994, 1997; Meyer et al., 1994), while *ABI3*, *ABI4*, and *ABI5* encode seed-specific transcription factors (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). The *abi1-1* and *abi2-1* mutations disrupt ABA activation of calcium channels (Murata et al., 2001) and reduce ABA-induced cytosolic calcium increases in guard cells (Allen et al., 1999), providing genetic evidence that deregulated Ca^{2+} impairs ABA signaling. Recently, mutations in the α subunit of the heterotrimeric G protein were reported to cause ABA insensitivity in guard cells (Wang et al., 2001). Whether this Gα protein also functions in other aspects of ABA responses such as seed germination, seedling growth, and gene regulation, is not clear.

The fact that ABI1 and ABI2 are protein phosphatases suggests the involvement of protein phosphorylation/dephosphorylation in regulating ABA signal transduction. However, evidence supporting the involvement of protein kinases in ABA signal transduction has been largely circumstantial. In *Vicia faba*, a guard cell-specific protein kinase is activated by ABA and appears to control the activity of plasma membrane anion channels (Li et al., 2000). Transient expression studies in maize protoplasts implicated a role for a calcium-dependent protein kinase (CDPK) in regulating ABA-responsive gene expression (Sheen, 1996). Whether CDPK affects other ABA responses and whether any plant *cdpk* or other protein kinase mutants have altered ABA responses remains to be documented.

In addition to the ABA-insensitive mutants, recent genetic screens have recovered several mutants with enhanced ABA sensitivity. The *era1*, *abh1*, *fry1*, and *sad1* mutations all confer hypersensitivity to ABA in germinating seeds as well as in vegetative tissues. *ERA1* encodes a farnesyl transferase that may modify an ABA signaling component (Cutler et al., 1996). *FRY1* encodes an inositol phosphatase that functions in the degradation of the second messenger IP_3 (Xiong et al., 2001b). *ABH1* and *SAD1* encode an mRNA cap binding protein and an Sm-like protein, respectively, that are predicted to function in aspects of RNA processing (Hugouvieux et al., 2001; Xiong et al., 2001a). ABH1 and SAD1 affect ABA sensitivity, likely by controlling the transcript turnover of an early ABA signaling component(s). Therefore, the roles of ERA1, ABH1, and SAD1 in ABA signaling are probably indirect.

It is known that Ca^{2+} mediates early ABA signaling (reviewed by Leung and Giraudat, 1998; Schroeder et al., 2001; MacRobbie, 1998). ABA responses in guard cells require a specific cytosolic calcium signature. Guard cell cytosolic calcium oscillations with defined frequency and amplitude result in stomatal closing (Allen et al., 2001). In contrast, steady and sustained cytosolic calcium increases without oscillations fail to confer stomatal closure (Allen et al., 2000). These elegant studies suggest that excessive, nonoscillating cytosolic calcium may interfere with or inhibit ABA responses. Hence, in addition to calcium sensors as positive regulators of downstream ABA signaling, there may exist calcium-sensing systems that negatively regulate ABA responses. Furthermore, the presumed negative calcium sensor(s) may be part of the negative regulation of cytosolic calcium required to generate calcium oscillations.

We report here the identification of a calcium sensor, SCaBP5, and its interacting protein kinase, PKS3, as global negative regulators of ABA responses. *scabp5* and *pks3* mutant plants are hypersensitive to ABA in seed germination and in seedling growth and development. The mutant plants exhibit substantially reduced transpirational water loss and their guard cells show

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enhanced ABA responsiveness. The expression of some ABA-responsive genes is also enhanced in the *scabp5* and *pks3* mutant plants. In yeast two-hybrid as well as in vitro and in vivo protein binding assays, SCaBP5 was found to interact with PKS3, which in turn interacted with ABI2 and to a lesser extent with ABI1. SCaBP5 and PKS3 thus constitute a calcium-sensing system that feedback inhibits ABA responses. SCaBP5 and PKS3 may function together with ABI1 and ABI2 in a regulatory loop to modulate calcium oscillations.

Results

Silencing *SCaBP* and *PKS* Genes by RNA Interference

In *Arabidopsis*, SOS3 is a myristoylated calcium binding protein structurally related to the B subunit of calcineurin and to animal neuronal calcium sensors (Liu and Zhu, 1998). SOS3 functions in the sensing of the calcium signal triggered by salt stress (Liu and Zhu, 1998; Ishitani et al., 2000). Despite its similarity to yeast calcineurin B, SOS3 is unique in its mode of function. Instead of acting as a subunit of protein phosphatase, SOS3 functions by interacting with and activating the protein kinase SOS2 (Halfter et al., 2000; Guo et al., 2001a). The *Arabidopsis* genome encodes a family of at least eight SOS3-like calcium binding proteins (ScaBPs; Guo et al., 2001a). We hypothesized that individual SCaBP members may function as calcium sensors in various other plant processes (e.g., ABA responses) that involve calcium signaling.

To attribute an in planta function to the SCaBP family of calcium sensor proteins, we obtained mutants in each of the corresponding genes by RNA interference (RNAi; Chuang and Meyerowitz, 2000; Smith et al., 2000) using gene-specific sequences. The mutant plants were tested systematically in an assortment of hormone and environmental stress response assays to screen for phenotypic alterations. Knockout mutation in one of the calcium sensor genes, *SCaBP5* (Guo et al., 2001a; also known as *CBL1*; Kudla et al., 1999), was found to specifically alter plant responses to ABA. We also used RNAi to generate mutants in each of the 23 SOS2-like protein kinases (i.e., PKSs), some of which are known to interact with SCaBPs (Guo et al., 2001a). Silencing one of the *PKS* genes, *PKS3*, was also found to specifically change plant responses to ABA.

In wild-type plants, *SCaBP5* and *PKS3* were expressed in every plant part examined, that is, roots, stems, leaves, flowers, and siliques (Figure 1A). Their transcript abundance was not regulated by ABA (Figure 1A). In four randomly chosen, independent *scabp5* and *pks* RNAi lines, *SCaBP5* and *PKS3* were shown to be silenced in the respective lines (Figure 1B). There were no PCR products amplified from RNA samples without reverse transcription (Figure 1B, CK lines), demonstrating that there was no DNA contamination. Control experiments showed that the most closely related genes, *SCaBP1* and *SCaBP6*, were not affected in the *scabp5* mutants (Figures 1C and 1D). Likewise, *PKS2* and *PKS18*, which are most closely related to *PKS3*, were not affected in *pks3* mutants (Figures 1C and 1D).

scabp5 and *pks3* Mutants Are Specifically Hypersensitive to ABA

Twenty-four *scabp5* RNAi lines were tested on Murashige-Skoog (MS) nutrient agar media containing different concentrations of ABA. Among them, 22 lines displayed germination arrest in the presence of 0.2 μM ABA. Sixteen of 24 *pks3* RNAi lines tested were hypersensitive to 0.2 μM ABA. The ABA-hypersensitive phenotype of the *scabp5* and *pks3* RNAi lines cosegregated with the hygromycin resistance marker (data not shown).

Four representative T_3 homozygous lines (i.e., *scabp5-9*, *scabp5-12*, *pks3-3*, *pks3-16*) are presented here. When seeds were plated on nutrient agar media without supplementation of ABA, *scabp5* and *pks3* mutant seeds germinated slightly later than wild-type seeds, suggesting that the mutant seeds have increased dormancy (Figure 2G). When seeds were treated at 4°C for 3 days to break dormancy and then incubated at 22°C under continuous light, the *scabp5* and *pks3* mutant seeds germinated and the seedlings grew as well as the wild-type (Figure 2A). With the supplementation of 1 μM ABA in the medium, wild-type seeds germinated 3 days earlier than *scabp5* and *pks3* mutant seeds. After the mutant seeds eventually germinated, the growth and development of the seedlings were arrested by ABA, as evidenced by the lack of root growth and leaf development of the mutant seedlings on the agar surface (Figure 2B). In contrast, the growth and development of wild-type seedlings were not arrested by 1 μM ABA. When the seeds were planted on 0.6% agar media and the seedlings were allowed to grow into the medium, the cotyledons of the *scabp5* and *pks3* mutant seedlings were bleached by 0.1 μM or 0.5 μM ABA (Figures 2E and 2F). In contrast, ABA at a concentration of 0.1 μM or 0.5 μM caused much less cotyledon or leaf bleaching of wild-type seedlings. To separate ABA sensitivity at germination from sensitivity at the seedling stage, 4-day-old seedlings on an MS agar plate were transferred to a plate containing 50 μM ABA. Three weeks later, it was found that whereas the wild-type seedlings were still alive, both *scabp5* and *pks3* seedlings were killed (Figure 2H). When the percentage of seedlings that developed true leaves was scored on vertical agar media supplemented with various concentrations of ABA, dose-response curves were obtained for the wild-type and *scabp5* and *pks3* mutants (Figure 2I). The dose-response curves illustrate clearly that the mutants are hypersensitive to ABA.

In addition to ABA, many other hormonal and environmental stress treatments elicit cytosolic calcium signals in plants (Knight, 2000; Sanders et al., 1999). To determine whether SCaBP5 and PKS3 are specifically involved in ABA-elicited calcium signaling, we tested the RNAi mutants under salt and cold stress and auxin, cytokinin, brassinolide, and gibberellic acid treatments, all of which are known to trigger cytosolic calcium signals. Figure 3A shows that neither the *scabp5* nor the *pks3* mutants were more sensitive to NaCl than were wild-type seedlings. In addition, the mutants were not more sensitive to cold treatment (Figure 3B). To determine whether *scabp5* and *pks3* mutants were altered in their responses to other hormones, the seedlings were assayed for their root growth on media supplemented with epibrassinosteroid, gibberellic acid, indol-3-acetic

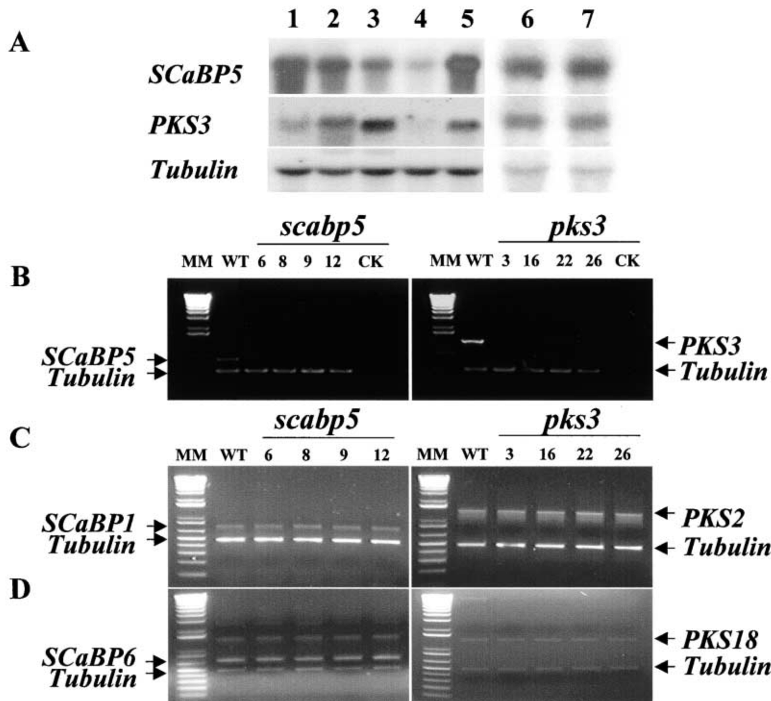

Figure 1. Expression of *PKS3* and *SCaBP5* Genes and Silencing of *PKS3* and *SCaBP5* by RNAi

(A) Expression of *SCaBP5* and *PKS3* in different tissues and in response to ABA. Total RNA was extracted from roots (1); stems (2); leaves (3); flowers (4); siliques (5); wild-type seedlings without ABA treatment (6) or wild-type seedlings with 100 μM ABA treatment (7) for 3 hr. Thirty micrograms of the total RNA was loaded in each lane. A tubulin gene was used as a loading control.

(B) RT-PCR with wild-type and four independent lines of *scabp5* using *SCaBP5*-specific primers (left panel) and with wild-type and four independent lines of *pks3* using *PKS3*-specific primers (right panel).

(C) RT-PCR with wild-type and independent lines of *scabp5* and *pks3* using *SCaBP1* (left panel) and *PKS2* (right panel) gene-specific primers, respectively.

(D) RT-PCR with wild-type and independent lines of *scabp5* and *pks3* using *SCaBP6* (left panel) and *PKS18* (right panel) gene-specific primers, respectively.

In (B)–(D), tubulin primers were used in the PCR reactions as internal controls. MM, molecular size markers; WT, wild-type; numbers refer to independent lines; CK, total RNA samples from *scabp5-6*, *-8*, *-9*, and *-12* or from *pks3-3*, *-16*, *-22*, and *-26* were mixed and used as PCR templates without reverse transcription, as negative controls for DNA contamination.

acid, 2,4-D, or 6-benzyladenine. No significant differences in root growth were found between the RNAi lines and the wild-type seedlings (Figures 3C–3G), indicating that the *scabp5* and *pks3* mutants are not altered in their responses to these hormones. Together, these results suggest that SCaBP5 and PKS3 function specifically in ABA regulation.

ABA-Regulated Gene Expression in *scabp5* and *pks3* Mutants

ABA induces the expression of many plant genes that are also regulated by water deficits (reviewed by Leung and Giraudat, 1998). To test whether the *scabp5* and *pks3* mutant plants show increased sensitivity toward ABA in gene regulation, the mutant seedlings were treated with 100 μM ABA, and ABA-responsive gene expression was determined at various time points after the treatment by using RNA blot analysis. In the wild-type, the stress-responsive genes *COR47*, *COR15A*, *RD29A*, and *RAB18* were all induced by ABA, with a peak of expression at 10 hr after treatment (Figure 3H). In the *scabp5* and *pks3* mutants, expression of these genes also peaked at 10 hr, but the transcript levels of *COR15A*, *COR47*, and *RD29A* but not *RAB18* were substantially higher than in the wild-type and persisted for a longer time. For *COR47* and *COR15A*, significant expression was also detected even prior to ABA treatment in the mutants (Figure 3H).

SCaBP5 and *PKS3* Modulate ABA Responses in Guard Cells

RNA blot results (Figure 1A) suggest that the *SCaBP5* and *PKS3* genes are expressed ubiquitously. To further examine the tissue expression patterns of *SCaBP5* and *PKS3*, their promoters were each fused with the β-glucuronidase reporter gene (*GUS*) and introduced into wild-type *Arabidopsis* plants. For each gene, GUS expression was determined in ten independent transgenic lines. Both *SCaBP5*::*GUS* and *PKS3*::*GUS* were detected in imbibing seeds, cotyledons, hypocotyls, and roots of young seedlings (data not shown). GUS activity was also detected in the leaves, roots, stems, and floral structures of adult plants (data not shown), which is consistent with the RNA blot data (Figure 1A). Interestingly, strong GUS expression was detected in the guard cells of both *SCaBP5*::*GUS* and *PKS3*::*GUS* transgenic plants (Figure 4A). The expression of *SCaBP5* and *PKS3* genes in guard cells was confirmed by Northern blot analysis on total RNA extracted from guard cell-enriched leaf epidermal strips (data not shown). The strong expression of *SCaBP5* and *PKS3* in guard cells suggests that SCaBP5 and PKS3 may also function in stomatal regulation.

To determine whether the *scabp5* and *pks3* mutations affect the responsiveness of guard cells to ABA, stomatal responses to various concentrations of ABA were tested. When wild-type, *scabp5-12*, and *pks3-3* plants were incubated under light at high humidity for 12 hr to allow stomata to open fully, no significant difference was detected among different plant lines in the size of stomatal pores (WT: 7.58 ± 0.26 μm, three experiments, 147 stomata; *scabp5-12*: 7.80 ± 0.43 μm, three experiments, 176 stomata; and *pks3-3*: 7.45 ± 0.41 μm, three experiments, 196 stomata). However, after a treatment with 1.5 μM ABA for 1.5 hr, stomatal pores were reduced more dramatically in *scabp5-12* and *pks3-3* plants than

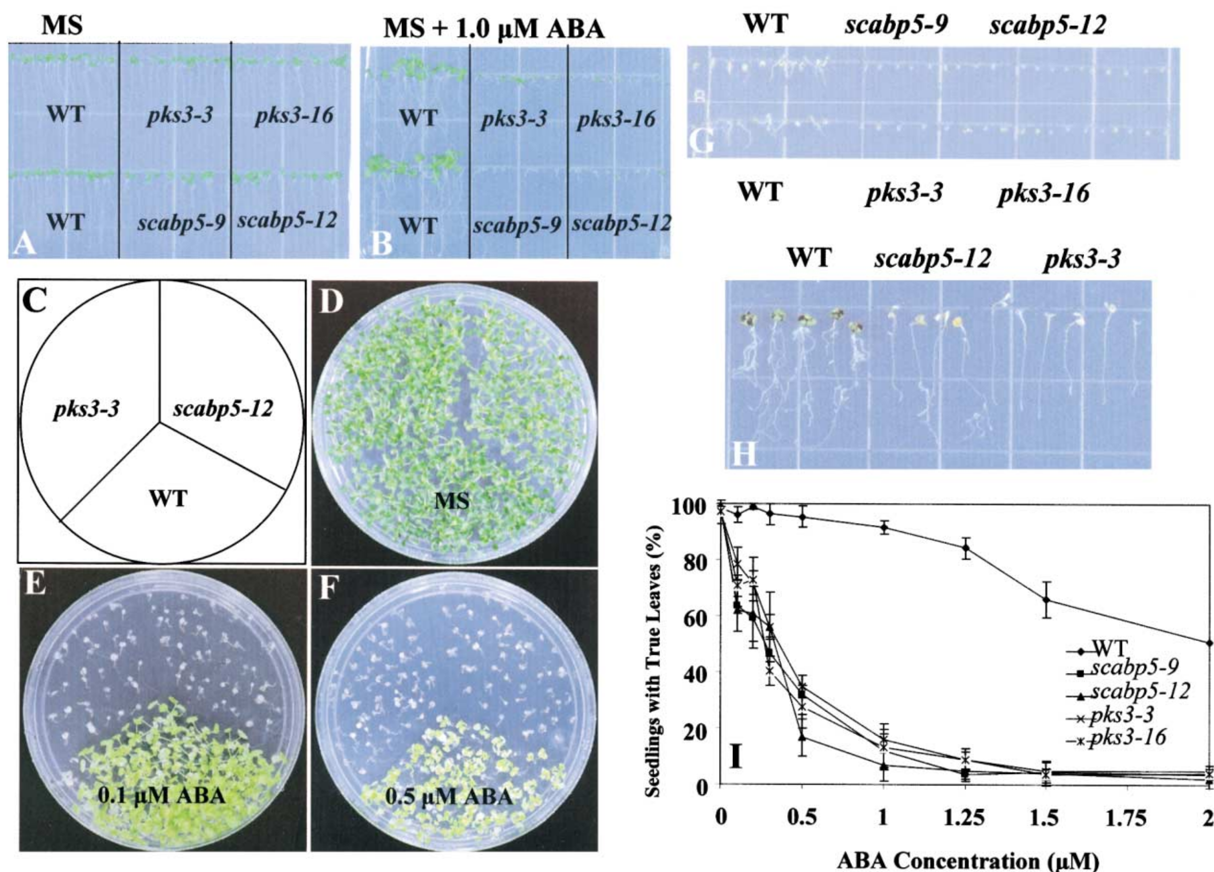

Figure 2. Sensitivity of Wild-Type and *scabp5* and *pks3* Mutants to ABA at Germination and Seedling Stages

(A and B) Germination sensitivity to ABA. Seeds from the wild-type, two representative *scabp5* lines, and two representative *pks3* lines were geminated and allowed to grow on vertical agar media containing MS nutrients (A) or MS nutrients supplemented with 1 μM ABA (B). WT, wild-type. The pictures for (A) and (B) were taken 4 and 18 days, respectively, after seed imbibition.

(C–F) Sensitivity to ABA during seedling development.

(C) Position of wild-type and mutant lines in plates (D)–(F).

(D) Without ABA.

(E) 0.1 μM ABA.

(F) 0.5 μM ABA.

(G) *scabp5* and *pks3* seeds show increased dormancy. Seeds were directly planted on the MS agar plate for germination without cold pretreatment.

(H) Sensitivity of seedlings to ABA. Four-day-old seedlings grown without ABA were transferred to a plate containing 50 μM ABA. The picture was taken 2 weeks after the transfer.

(I) Sensitivity of seedling development to ABA. Shown are ratios of seedlings with true leaves over total number of seeds planted on MS media supplemented with ABA at the indicated concentrations (means ± SD, n = 3).

in wild-type plants (Figure 4B). The stomatal apertures of wild-type plants were decreased to 73% of that without ABA treatment, and in *scabp5-12* and *pks3-3* plants they were decreased to 35% and 37%, respectively (Figure 4B). The stomatal apertures in *scabp5-12* and *pks3-3* were further reduced to 16% and 9%, respectively, by 2.5 hr of ABA treatment, whereas in the wild-type it was only reduced to 52%. These data show that stomatal closure is hypersensitive to ABA in the *scabp5-12* and *pks3-3* mutants, suggesting that both SCaBP5 and PKS3 play important roles in regulating the responses to ABA in guard cells.

To determine whether the increased sensitivity to ABA in guard cells has an impact on plant water relations, we compared the rates of water loss from the rosette leaves of wild-type and *scabp5-9* and *pks3-3* mutants. The results illustrate that both *scabp5-12* and *pks3-3* mutant leaves lost water significantly more slowly than the wild-type leaves (Figure 4C), which is consistent with the ABA-hypersensitive stomatal closure in the mutants.

PKS kinases contain a FISL motif in their regulatory domains that mediates interaction with specific SCaBPs (Guo et al., 2001a). The deletion of the FISL motif in SOS2 results in a constitutively active kinase that is independent of SOS3 (Guo et al., 2001a). Deletion of the FISL motif in PKS3 rendered the kinase independent of SCaBP5 (see below). We expressed this deletion mutant (i.e., PKS3ΔF) ectopically in wild-type *Arabidopsis*

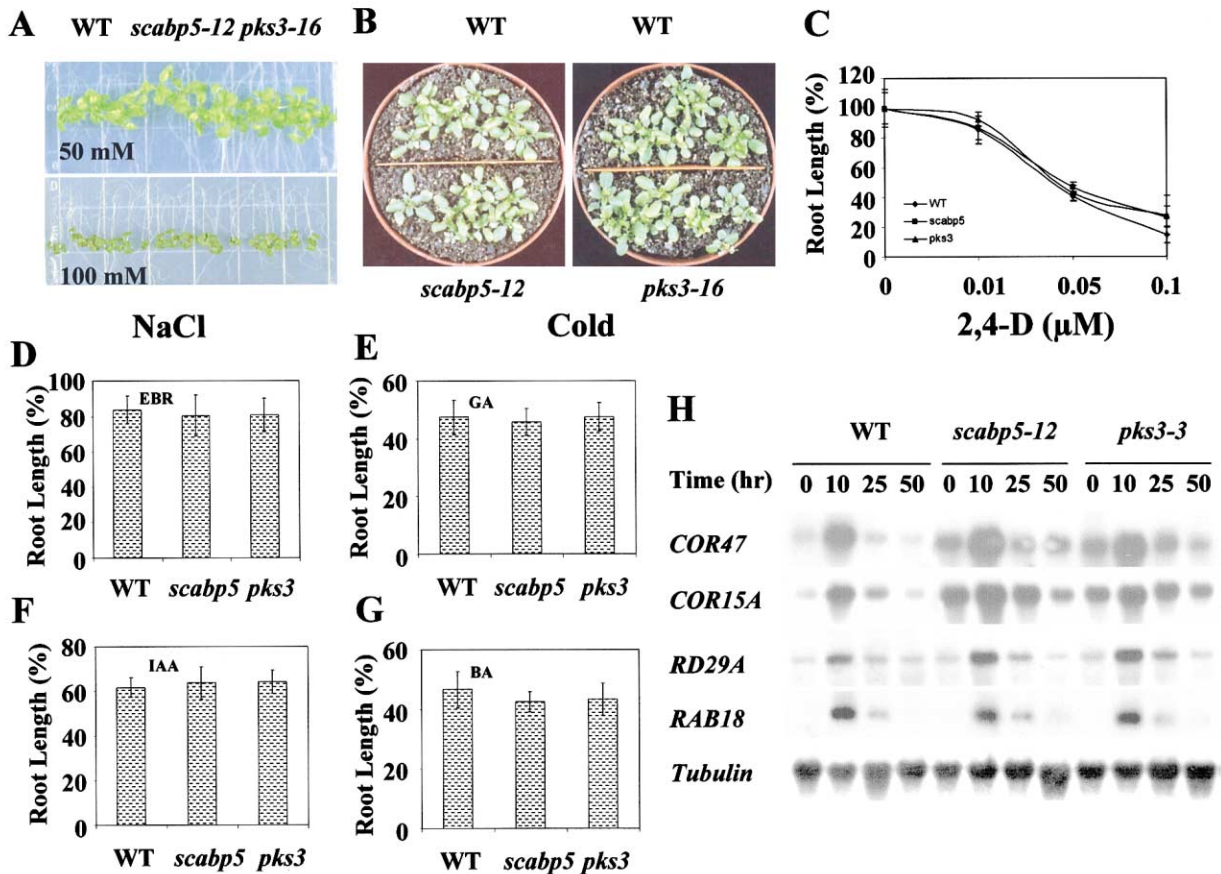

Figure 3. The Stress and Hormone Responses of *scabp5* and *pks3* Mutants and Expression of ABA-Responsive Genes in Wild-Type, *scabp5*, and *pks3* Seedlings

(A) Growth of wild-type, *scabp5*, and *pks3* seedlings on an MS agar medium containing 50 mM or 100 mM NaCl.

(B) Growth of 2-week-old wild-type, *scabp5*, and *pks3* plants after treatment at 4°C for 2 weeks.

(C–G) Relative root growth of wild-type, *scabp5*, and *pks3* seedlings in response to 2,4-D, epibrassinosteroid (EBR; 2 μM), gibberellic acid (GA; 1 μM), indole-3-acetic acid (IAA; 0.01 μM), and cytokinin (BA; 0.1 μM). Inhibition of root growth is relative to the mean growth in the absence of hormones.

(H) Transcript levels of ABA-responsive genes in wild-type, *scabp5-12*, and *pks3-3* seedlings at 0 (control), 10, 25, and 50 hr after treatment with 100 μM ABA.

plants. Figure 4D shows that the transgenic plants had reduced responses to ABA in stomatal closing assays. In response to a 2 hr treatment with 2.5 μM ABA, the aperture of wild-type stomata was reduced by approximately 80%, whereas that of the transgenic plants expressing PKS3ΔF was much less reduced or not reduced significantly. These results suggest that PKS3ΔF confers insensitivity to ABA.

PKS3 Interacts with SCaBP5 and ABI2/1

Because SOS3 is known to physically interact with SOS2 (Halfter et al., 2000), we tested whether SCaBP5 might interact with PKS3. Figure 5A shows that SCaBP5 interacted strongly with PKS3 in yeast two-hybrid assays, as indicated by the strong β-galactosidase activity generated when the PKS3 bait and SCaBP5 prey were combined. PKS3 or SCaBP5 by itself did not activate β-galactosidase reporter expression. To test whether SCaBP5 can bind to PKS3 in vitro, [^{35}S]methionine-labeled SCaBP5 protein was incubated together with PKS3-GST, or with RB-GST or ABI2-GST as controls. The GST-fusion proteins were sedimented by the addition of glutathione-Sepharose beads and extensively washed. Gel electrophoresis analysis revealed that the [^{35}S]methionine-labeled SCaBP5 protein was pulled down by PKS3-GST but not by RB-GST or ABI2-GST (Figure 5C). These results demonstrate that SCaBP5 does bind to PKS3.

Protein kinases are often found in complexes together with specific protein phosphatases (Stone et al., 1994; Williams et al., 1997; Westphal et al., 1998). Because the protein phosphatases ABI1 and ABI2 also function in controlling ABA sensitivity in seed germination, leaf transpiration, and gene regulation, we tested whether PKS3 might interact with ABI1 and/or ABI2. Remarkably, we found that ABI2 interacted very strongly with PKS3

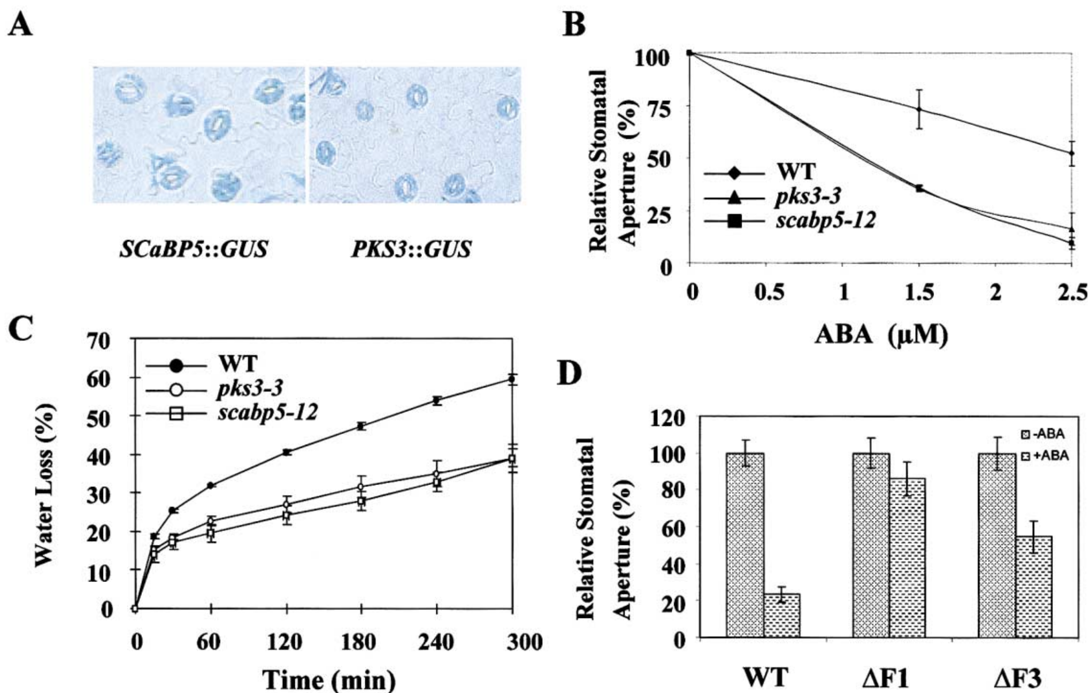

Figure 4. SCaBP5 and PKS3 Mediate ABA Signaling in Guard Cells

(A) *SCaBP5* and *PKS3* are expressed in guard cells. Shown are pictures of GUS staining of transgenic plants expressing *SCaBP5::GUS* or *PKS3::GUS* construct.

(B) Stomatal closing in *scabp5-12* and *pks3-3* mutants is more sensitive to ABA. Relative stomatal aperture in response to ABA treatments at the indicated concentrations is shown. Data represent means ± SD from three independent experiments. At least 100 stomates for each individual line were measured.

(C) Leaf transpirational water loss in wild-type (black circles), *scabp5-12* (open circles), and *pks3-3* (open squares) rosettes at indicated times after detachment from roots (means ± SE, n = 3).

(D) Stomates in transgenic plants overexpressing PKS3ΔF are less sensitive to ABA. Relative stomatal aperture in response to 2.5 μM ABA treatment for 2 hr is shown for two independent lines, ΔF1 and ΔF3. Data represent means ± SD from three independent experiments, each with a measurement of at least 100 stomates for each individual line.

in the yeast two-hybrid system (Figure 5A). ABI1 also interacted with PKS3 (Figures 5A and 5B), but the interaction was considerably weaker than that of ABI2 (Figure 5B). We then performed a pull-down assay to determine whether PKS3 interacts with ABI2 or ABI1 in vitro. The result shows that PKS3 was capable of binding to ABI2-GST or ABI1-GST but not RB-GST (Figure 5C).

To test whether SCaBP5 and PKS3 can also interact in vivo, Myc-tagged *PKS3* (*PKS3-Myc*) and HA-tagged *SCaBP5* (*SCaBP5-HA*) were transfected into *Arabidopsis* protoplasts either separately or together. PKS3-Myc and SCaBP5-HA were then precipitated with anti-Myc and anti-HA antibodies, respectively. Figure 5D shows that SCaBP5-HA was detected in the proteins precipitated by anti-Myc only from protoplasts cotransfected with both PKS3-Myc and SCaBP5-HA but not from protoplasts transfected only with PKS3-Myc. The results demonstrate that PKS3 and SCaBP5 could be coimmunoprecipitated. Control experiments show that PKS3 did not coimmunoprecipitate with SCaBP1 from protoplasts cotransfected with PKS3-Myc and SCaBP1-HA (Figure 5D). Similarly, we found that PKS3-Myc but not PKS5-Myc was coprecipitated by anti-SCaBP5-HA (Figure 5D). These results suggest that SCaBP5 and PKS3 interact specifically in vivo in plant cells.

Experiments were also performed to determine whether ABI2 is a substrate for phosphorylation by PKS3, or whether autophosphorylated PKS3 is a substrate for dephosphorylation by ABI2. However, the results suggest that ABI2 could not dephosphorylate PKS3, nor could PKS3 phosphorylate ABI2 (data not shown).

The *abi1-1* and *abi2-1* Mutations Suppress the ABA-Hypersensitive Phenotypes of *scabp5* and *pks3*

To determine the genetic relationship between PKS3 and ABI2/1, the dominant mutant *pks3-3* was crossed with the dominant *abi2-1* and *abi1-1* mutants, and the resulting F_1 seeds were tested in a seed germination assay in the presence of 0.2 μM or 0.5 μM ABA. The results show that the F_1 seeds behaved like the respective *abi* mutants (Figures 6A and 6B). Crosses were also performed between *abi2-1* or *abi1-1* and *scabp5-12*, and between *pks3-3* and *scabp5-12*. F_1 seeds from the cross between *scabp5-12* and *abi2-1* or *abi1-1* behaved like the *abi* mutants when they were germinated in a

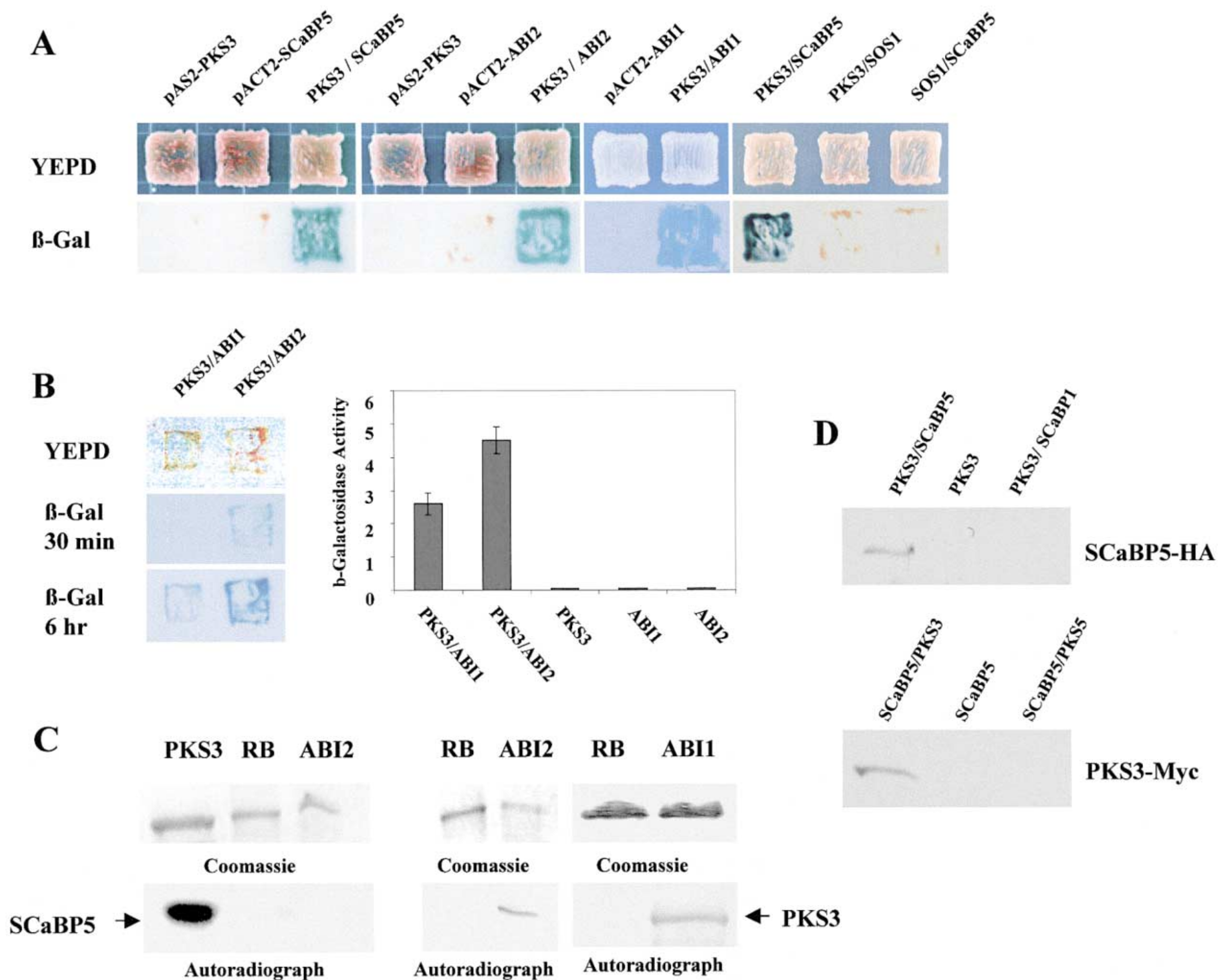

Figure 5. Interactions between SCaBP5 and PKS3, between PKS3 and ABI2, and between PKS3 and ABI1

(A) Interactions between SCaBP5 and PKS3, between PKS3 and ABI2, and between PKS3 and ABI1 in the yeast two-hybrid system. Yeast strains containing the pAS-PKS3 bait and the pACT-SCaBP5, pACT-ABI2, or pACT-ABI1 prey were assayed for *LacZ* expression. The pAS-SOS1 and pACT-SCaBP5, and pAS-PKS3 and pACT-SOS1 combinations were used as negative controls. Yeast grown on YEPD media is shown in the upper panel. β-galactosidase filter assays are shown in the lower panel.

(B) The interaction between PKS3 and ABI2 is stronger than that between PKS3 and ABI1. Shown on the left are β-galactosidase filter assays at different time points after the color reaction was started. On the right is a quantitative measurement of β-galactosidase activity of the yeast strains in liquid culture.

(C) In vitro binding assays. [^{35}S]methionine-labeled SCaBP5 protein was pulled down by PKS3-GST but not by RB-GST or ABI2-GST. [^{35}S]methionine-labeled PKS3 protein was pulled down by ABI2-GST and ABI1-GST. RB-GST was used as a negative control.

(D) PKS3 interacts with SCaBP5 in an in vivo assay. PKS3-Myc and SCaBP5-HA were transiently coexpressed or individually expressed in the wild-type protoplasts, and then PKS3-Myc and SCaBP5-HA were precipitated with anti-Myc or anti-HA, respectively. The precipitated PKS3-Myc samples were subjected to an anti-HA Western blot (upper panel) and the precipitated ScaBP5-HA samples were subjected to an anti-Myc Western blot (lower panel). PKS3 did not coimmunoprecipitate with SCaBP1 (upper panel), and SCaBP5 did not coimmunoprecipitate with PKS5 (lower panel).

medium containing 0.5 μM ABA (Figures 6A and 6B). These results show that the *abi1-1* and *abi2-1* mutations suppress the *scabp5* and *pks3* mutant phenotype. There was no additive effect in the double mutant *pks3scabp5*, as compared to *scabp5* and *pks3* single mutants (Figures 6C and 6D), supporting the hypothesis that *PKS3* and *SCaBP5* function in the same pathway.

Regulation of PKS3 Activity by SCaBP5, Calcium, and ABA

SOS2 phosphorylation of peptide substrates depends on the presence of SOS3 and Ca^{2+} (Halfter et al., 2000; Ishitani et al., 2000). In this study, however, PKS3 itself was found to be capable of phosphorylating the synthetic peptide p3 (ALARAASAAALARRR). SCaBP5 did not affect the capacity of PKS3 to phosphorylate p3 when there was no calcium in the reaction medium (Figure 7). Intriguingly, PKS3 phosphorylation of p3 (Figure 7C) was greatly repressed by high concentrations of Ca^{2+}. The p3 phosphorylation activity was decreased to one third of the original level by the addition of 50 μM Ca^{2+}. Interestingly, this decrease was relieved by adding SCaBP5, suggesting that SCaBP5 may function in maintaining PKS3 activity in the presence of Ca^{2+}. Calcium

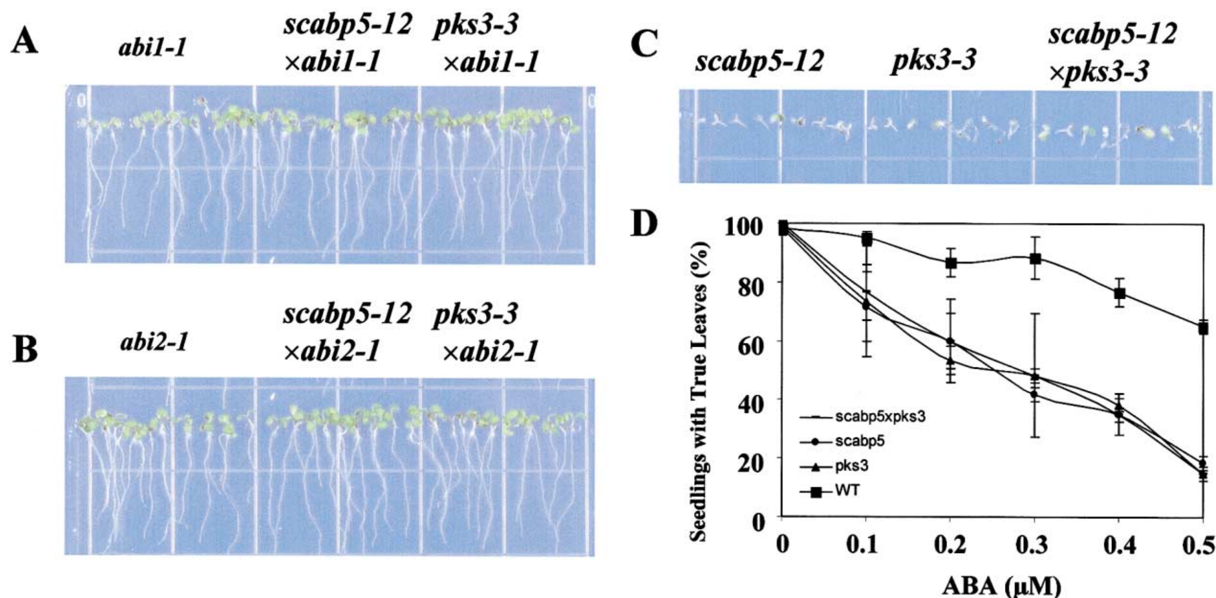

Figure 6. Suppression of *scabp5* and *pks3* Mutant Phenotypes by the *abi1-1* and *abi2-1* Mutations

(A) The *abi1-1* mutation suppresses the ABA-hypersensitive phenotype of *scabp5* and *pks3* mutants in the presence of 0.5 μM ABA.
(B) The *abi2-1* mutation suppresses the ABA-hypersensitive phenotype of *scabp5* and *pks3* mutants in the presence of 0.5 μM ABA.
(C and D) The phenotypes of *scabp5* and *pks3* mutations are not additive. F_1 seeds from the crosses between the respective mutants were planted on MS agar plates supplemented either with 0.5 μM ABA and allowed to grow for 2 weeks before the pictures were taken (C), or with different concentrations of ABA and scored for leaf development (D). Data in (D) are ratios of seedlings with true leaves over total number of seeds planted (means ± SD, n = 3).

or SCaBP5 did not have any significant effect on the autophosphorylation of PKS3 (Figures 7A and 7B). In contrast to that of wild-type PKS3, the peptide phosphorylation activity of PKS3ΔF was not reduced by calcium, and was not influenced by the presence of SCaBP5 (Figure 7D).

We tested potential in vivo regulation of PKS3 activity in response to ABA. PKS3-Myc immunoprecipitated from transfected protoplasts was used to phosphorylate the peptide substrate p3. There was a constitutive level of PKS3 activity in the absence of ABA treatment (Figure 7E). In response to ABA treatment, a transient decrease in PKS3 activity was consistently observed (Figure 7E). The decrease was evident within the first 20 min, and the activity recovered by 30 min of ABA treatment. No such transient decrease in PKS3 activity was detected in response to cold treatment at 4°C, even though cold is known to also elicit a cytosolic calcium signal (Figure 7F). These results indicate that PKS3 activity in vivo may be regulated specifically by ABA.

Discussion

In the present study, we identified two components of ABA signal transduction: a calcium sensor protein, SCaBP5, and a protein kinase, PKS3. *Arabidopsis* mutants with either gene knocked out by RNAi exhibited increased sensitivity to ABA during seed germination, vegetative growth, stomatal closure, and gene expression. The results show that SCaBP5 and PKS3 are global regulators of ABA responses. SCaBP5 and PKS3 physically interact with each other and the *scabp5* and *pks3* mutants had identical ABA response phenotypes that are not additive in their double mutant. Thus, SCaBP5 and PKS3 act together in a common pathway. PKS3 is constitutively active but its activity is repressed by Ca^{2+}. In the presence of Ca^{2+}, SCaBP5 appears to reactivate PKS3 (Figure 7C).

The 2C-type protein phosphatases ABI1 and ABI2 are the best-characterized global regulators of ABA signaling (Koornneef et al., 1998; Leung and Giraudat, 1998; McCourt, 1999). PKS3 interacted strongly with ABI2 and relatively weakly with ABI1 (Figure 5). The interaction appears not to cause a direct phosphorylation of ABI2 by PKS3 or dephosphorylation of autophosphorylated PKS3 by ABI2 (data not shown). It is likely that PKS3 and ABI2 control the phosphorylation status of a common target protein. Although it is difficult to interpret the epistatic relationship between ABI2/1 and PKS3 using the dominant *abi2-1* or *abi1-1* mutants, it is clear that PKS3 and SCaBP5 act in the same pathway with *ABI2/1*, since both *abi2-1* and *abi1-1* could suppress the ABA-hypersensitive phenotype of *pks3* and *scabp5* in seed germination and seedling growth. The *abi* mutations may suppress the *scabp5* and *pks3* mutations through modulation of the initial ABA-induced Ca^{2+} influx (Murata et al., 2001).

Unlike the calcium sensor SOS3, which is a positive regulator of salt stress responses (Liu and Zhu, 1998), SCaBP5 is a negative regulator of ABA signaling. Previous studies suggested that Ca^{2+} is a second messenger mediating many cellular responses including responses to ABA (Sanders et al., 1999; Knight, 2000; Leung and Giraudat, 1998; MacRobbie, 1998). Recent studies both in plants and in other eukaryotes demonstrated that the

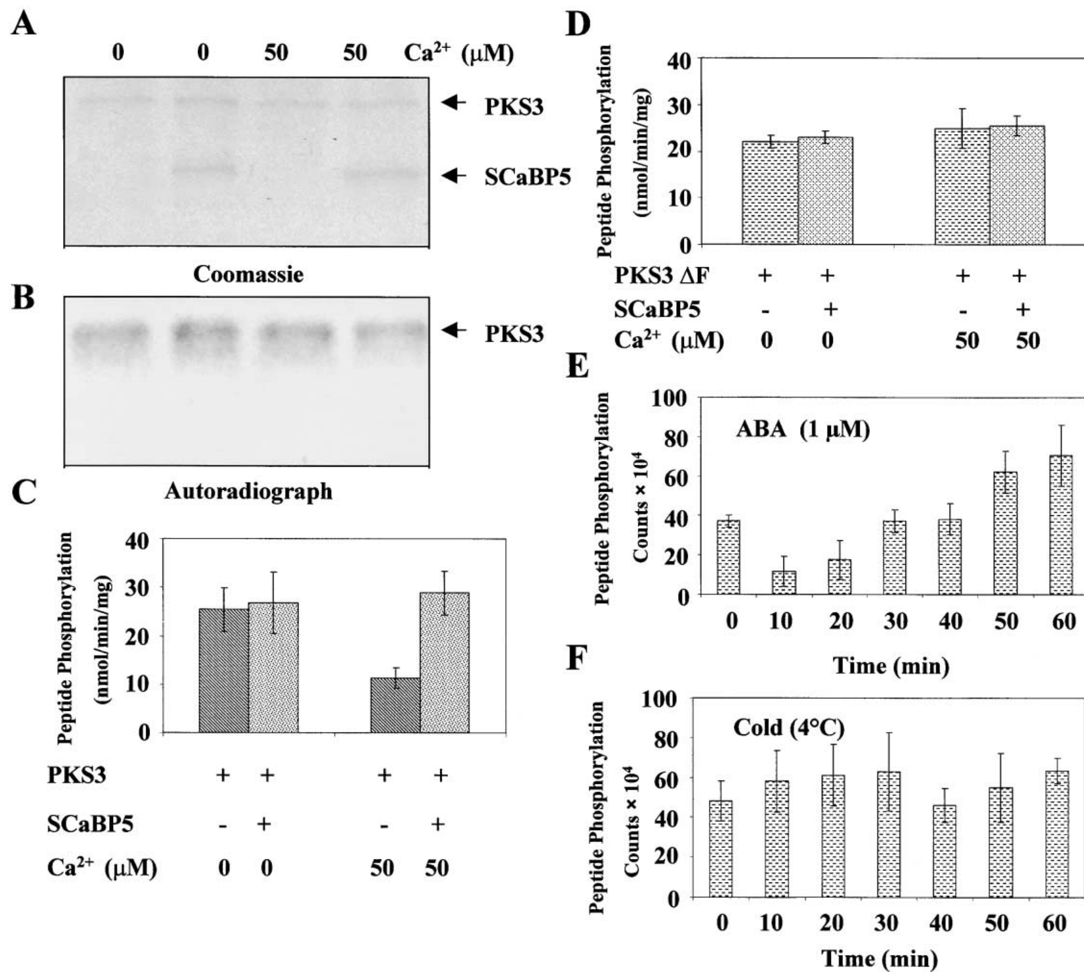

Figure 7. SCaBP5 Activates PKS3 at High Calcium Concentrations

(A) Coomassie blue-stained SDS-PAGE gel.

(B) Autophosphorylation of PKS3.

(C) p3 peptide phosphorylation activity of PKS3 and the effects of calcium and/or SCaBP5.

(D) Effect of Ca^{2+} and/or SCaBP5 on p3 peptide phosphorylation activity of PKS3ΔF.

(E) Effect of ABA on p3 peptide phosphorylation activity of immunoprecipitated PKS3-Myc prepared from transfected protoplasts that had been treated with 1.0 μM ABA for the indicated time.

(F) Effect of cold on p3 peptide phosphorylation activity of immunoprecipitated PKS3-Myc prepared from transfected protoplasts that had been treated at 4°C for the indicated time. Error bars in (C)–(F) indicate SD (n = 3).

role of Ca^{2+} as a second messenger not only depends on its amplitude but also its oscillations, and oscillation parameters encode signaling specificity (Dolmetsch et al., 1998; Allen et al., 2001). Consistent with these notions, steady increases in cytosolic Ca^{2+} without oscillations impair the role of Ca^{2+} in ABA signaling (Allen et al., 2000, 2001). There must be mechanisms that downregulate the Ca^{2+} transients in order to generate Ca^{2+} oscillations. This can be accomplished by turning off Ca^{2+} influx or turning on Ca^{2+} efflux. In animal cells, experimental evidence has shown that increased Ca^{2+} concentration can feedback repress Ca^{2+} channels through the binding of calmodulin to the Ca^{2+} channel proteins (Scott et al., 1999; DeMaria et al., 2001; Catterall, 2000). Therefore, Ca^{2+} binding proteins can be not only positive regulators but also negative regulators in Ca^{2+} signaling. For example, the *Arabidopsis* Ca^{2+} binding protein SUB1 was demonstrated to be a negative regulator of blue/far red light signaling in seedlings (Guo et al., 2001b). Our data presented in this study provide strong genetic and biochemical evidence that SCaBP5 acts as a negative regulator that specifically modulates ABA signal transduction, since *scabp5* (and *pks3*) mutants did not show altered responses to other plant hormones and stresses.

PKS3 has a constitutive activity that appears to repress ABA signaling, as *pks3* knockout mutants showed increased sensitivity to ABA. Consistent with this notion, ectopic expression of the dominant positive PKS3 mutant with the deletion of the FISL motif conferred insensitivity to ABA in stomatal regulation. Interestingly, ABA appears to be able to relieve the negative effect of PKS3, since ABA treatment transiently repressed PKS3 activity, probably as a result of increased cytosolic Ca^{2+}. Upon ABA treatment, it is known that cells experience a transient increase in cytosolic Ca^{2+}. In addition to

activating as yet unknown calcium sensors that are positive regulators in downstream ABA responses, this increase in Ca^{2+} may also be perceived by SCaBP5. SCaBP5, like SOS3, has a conserved myristoylation motif (Guo et al., 2001a), which may help tether SCaBP5 and its interacting partner (e.g., PKS3) to special membrane patches where Ca^{2+} channels are localized. N-myristoylation has been shown to be required for SOS3 function in salt tolerance (Ishitani et al., 2000). Our data suggest the following chain of events. When treated with ABA, the increased cytosolic Ca^{2+} suppresses the activity of PKS3 and therefore relieves PKS3 repression on ABA signaling. At high concentrations of Ca^{2+}, SCaBP5 may become active and cause the reactivation of PKS3, which then manifests its negative regulation on ABA signaling once the signal transduction has been initiated. This feedback circuitry may also be involved in the generation of Ca^{2+} oscillation if SCaBP5-PKS3 and ABI1/2 work together to control Ca^{2+} influx through regulation on Ca^{2+} channels or transporters. Patch clamp experiments have shown that the *abi1-1* and *abi2-1* mutations disrupt ABA activation of calcium channels (Murata et al., 2001). It is possible that SCaBP5 and PKS3 may be involved in negatively regulating the ABA-activated channels. Recently, we have found that other calcium sensors in the SCaBP family and other protein kinases in the PKS family are involved in the activity regulation of membrane transporters. For example, SOS3 and SOS2 are required for the activation of the plasma membrane Na^+/H^+ antiporter, SOS1 (Qiu et al., 2002). PKS5 phosphorylates and inactivates plasma membrane H^+-ATPases (Y.G. and J.-K.Z., unpublished data).

Experimental Procedures

Plasmid Constructs and Plant Transformation

Gene-specific cDNA fragments of *SCaBP5* (GenBank accession number AF076251) and *PKS3* (GenBank accession number AF-339144) were amplified by polymerase chain reaction (PCR) using the following primer pairs: for *SCaBP5*, forward primer 5′-CGGGATCCATTTAAATTTCGAGGACACGAAG-3′ and reverse primer 5′-GGACTAGTGGCGCGCCATCTTGATTCACGTCTG-3′; for *PKS3*, forward primer 5′-CGGGATCCATTTAAATGGCCTACAAGAGGAGGTG-3′ and reverse primer 5′-GGACTAGTGGCGCGCCTCAGTGCCAAGCTAATAC-3′. The forward primers contain BamHI and SwaI restriction sites and reverse primers contain SpeI and AscI restriction sites, which are underlined. The PCR fragments of *SCaBP5* and *PKS3* were first cloned into the pFGC1008 vector (http://ag.arizona.edu/chromatin/fgc1008.html) between the SwaI and AscI sites in the antisense orientation. The sense fragments were then inserted between the BamHI and SpeI sites. The RNAi constructs of *SCaBP5* and *PKS3* were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type *Arabidopsis* (Columbia ecotype) plants by floral infiltration.

To make the SCaBP5-HA and PKS3-Myc transient expression vectors, HA tag and c-Myc tag were respectively fused to the 3′ end of the *SCaBP5* and *PKS5* coding region and were cloned into the plasmid pGFP-JS (kindly provided by Dr. J. Sheen, Massachusetts General Hospital) between the NcoI and BamHI sites to replace the GFP fragment and under the control of a double 35S promoter.

Northern Blot and RT-PCR Analysis

For tissue-specific Northern blot analysis, total RNA was extracted from leaves, stems, roots, flowers, and siliques. For ABA-regulated gene expression, 2-week-old seedlings were sprayed with 100 μM ABA. The plants were harvested at 0, 10, 25, and 50 hr after the treatment. To isolate RNA from guard cell-enriched epidermal strips, the method of Hugouvieux et al. (2001) was followed. Thirty micrograms of total RNA was used for RNA gel blot analysis. For reverse transcriptase (RT)-PCR analysis, the first strand cDNAs were synthesized from total RNA samples from wild-type, *scabp5-9*, *scabp5-12*, *pks3-3*, and *pks3-16* plants. RT-PCR was performed by using the reverse primers of *SCaBP5* and *PKS3* for RNAi constructs; forward primers are 5′-ATGGAGAAGAAAGGATCTGTG-3′ for *PKS3*, and 5′-ATGGGCTGCTTCCACTCAAAGG-3′ for *SCaBP5*. The forward primer sequences are not present in the gene-specific *SCaBP5* and *PKS3* cDNA fragments in the RNAi constructs, and therefore only amplify respective endogenous gene transcripts.

ABA Sensitivity Assays at Seed Germination

Seeds of the wild-type, RNAi mutants, and those resulting from various crosses were surface sterilized in 7% (w/v) hypochlorite and 0.01% (w/v) Triton X-100, and then rinsed five times with sterile water. The seeds were sown on Murashige-Skoog nutrient media containing 0.6% or 1.2% (w/v) agar and different concentrations of ABA (mixed isomers; Sigma). The seeds were stratified at 4°C for 3 days, and then transferred to 22°C under continuous light for germination and growth.

Water Loss Measurement

Rosette leaves of wild-type and mutant plants were detached from their roots and placed in weighing dishes and incubated on the lab bench. Loss in fresh weight was monitored at the indicated times.

Epidermal Strip Bioassay

The sensitivity of guard cells to ABA as measured by the degree of stomatal closure was assayed as described by Zhang et al. (2001) with slight modifications. Four-week-old plants were incubated for 12–16 hr under light at high humidity in a growth chamber. The leaves were then incubated for 2 hr in a stomatal opening solution (10 mM MES-Tris [pH 6.15], 50 mM KCl) at 22°C under a photon flux density of 0.20–0.30 mmol $m^{-2} s^{-1}$ to open the stomata. Epidermal strips from the leaves were subsequently examined under a microscope to determine the aperture of the stomatal pores at 0, 1.5, and 2.5 hr after treatment with 1.5 μM or 2.5 μM ABA.

Yeast Two-Hybrid Interaction and In Vitro Protein Binding Assays

Yeast two-hybrid interaction assays were performed as described (Halfter et al., 2000; Guo et al., 2001a). To produce bacterially expressed GST-PKS3, GST-SCaBP5, GST-ABI2, and GST-ABI1, the coding regions of *PKS3*, *SCaBP5*, *ABI2*, and *ABI1* cDNAs were cloned in-frame into the BamHI-EcoRI sites of pGEX-2TK. The constructs were introduced into *E. coli* BL21 DE3 cells. Radiolabeled SCaBP5 and PKS3 proteins were produced from pET14b-SCaBP5 and pET14b-PKS3 by using an in vitro transcription and translation assay kit (TNT Coupled Reticulocyte Lysate system; Promega) with [^{35}S]methionine as the sole source of methionine, following the manufacturer's instructions. Protein pull-down assays were performed as described (Halfter et al., 2000).

Genetic Analysis

To obtain double mutants, *abi2-1* and *scabp5-12*, *abi1-1* and *scabp5-12*, *abi2-1* and *pks3-3*, *abi1-1* and *pks3-3*, and *scabp5-12* and *pks3-3*, were crossed and the resulting F_1 progeny were assayed for ABA sensitivity at seed germination and seedling growth stages.

Histochemical Detection of GUS Activity

For *SCaBP5*::*GUS* and *PKS3*::*GUS* constructs, a 1657 bp *SCaBP5* promoter fragment and a 1543 bp *PKS3* promoter fragment were obtained by PCR. The promoter fragments were then cloned into pBI101 plasmid between the HindIII and XbaI sites, respectively. Ten independent transgenic lines from each construct were tested for GUS activity. Tissues were incubated overnight in a GUS staining buffer (3 mM x-Gluc, 0.1 M sodium phosphate buffer [pH 7], 0.1% Triton X-100, 8 mM β-mercaptoethanol) at 37°C in the dark. The staining was terminated by replacing the staining solution with 70% EtOH solution and samples were stored at 4°C until observation under a microscope.

Site-Directed Mutagenesis and Expression of PKS3ΔF In Planta

The FISL motif deletion mutation of the PKS3 (i.e., PKS3ΔF) was generated using oligonucleotide-directed in vitro mutagenesis. The primers were as follows: pPKS3ΔF-forward: 5′-GTGTATCAACGGA GAGGAGAAGGAGGAGATGAGGTTTACATC-3′; PPKS3ΔF-reverse: 5′-TCTCCTCTCCGTTGATACACCGCTTCTTTTCCTTCTCTGC-3′. Mutagenesis reactions were carried out on pGEX-PKS3 plasmid DNA using an enzyme mix of *LA Taq* (TaKaRa Shuzo Ltd.) and *Pfu* Turbo DNA polymerase (Stratagene). The PCR product was gel purified, and treated with DpnI to digest the parental supercoiled double-stranded DNA. The digested PCR product was transformed into DH5α competent cells. The mutation was confirmed by DNA sequencing.

To make the *PKS3ΔF* construct for plant transformation, a PCR reaction was carried out using the primer pair 5′-TCCCCCGGGATG GAGAAGAAAGGATCTGTGTTG-3′ (forward; SmaI site underlined)- and 5′-CGGGGTACCTCAGTGCCAAGCTAATACAAAGTC-3′ (reverse; KpnI site underlined) on the PKS3ΔF cDNA template. The PCR product was purified, digested, and cloned into the binary vector pBIB, under control of the supermas promoter (Narasimhulu et al., 1996). The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and transformed into *Arabidopsis* by floral infiltration.

Protoplast Transient Expression Assays

The protoplast transient expression assay was performed as described by Sheen (2001). After 10–12 hr of incubation, the protoplasts were either not treated, treated with 1 μM ABA, or treated at 4°C and harvested at the indicated time points. The protein extracts were prepared using 10^5 protoplasts per 100 μl extraction buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 2 μM leupeptin, 2 μM pepstatin). Two microliters of anti-Myc (Sigma) or anti-HA (Sigma) was used for immunoprecipitation of proteins from the extracts. Western blot was performed by standard methods. Both anti-Myc and anti-HA were used at 1:100 dilution.

Kinase Activity Assay

GST-PKS3 or immunoprecipitated PKS3-Myc phosphorylation on custom synthesized peptide p3 (ALARAASAAALARRR; Research Genetics; Halfter et al., 2000) was performed as described by Guo et al. (2001a). The kinase buffer contains 20 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 10 μM ATP, 1 mM DTT. The phosphorylated peptide was quantified by phosphorimaging (Molecular Dynamics).

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