

Identification of Two Protein Kinases Required for Abscisic Acid Regulation of Seed Germination, Root Growth, and Gene Expression in *Arabidopsis*^W

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Abscisic acid (ABA) is an important phytohormone regulating various plant processes, including seed germination. Although phosphorylation has been suggested to be important, the protein kinases required for ABA signaling during seed germination and seedling growth remain elusive. Here, we show that two protein kinases, SNF1-RELATED PROTEIN KINASE2.2 (SnRK2.2) and SnRK2.3, control responses to ABA in seed germination, dormancy, and seedling growth in *Arabidopsis thaliana*. A *snrk2.2 snrk2.3* double mutant, but not *snrk2.2* or *snrk2.3* single mutants, showed strong ABA-insensitive phenotypes in seed germination and root growth inhibition. Changes in seed dormancy and ABA-induced Pro accumulation consistent with ABA insensitivity were also observed. The *snrk2.2 snrk2.3* double mutant had a greatly reduced level of a 42-kD kinase activity capable of phosphorylating peptides from ABF (for ABA Response Element Binding Factor) transcription factors. ABA-induced expression of several genes whose promoters contain an ABA response element (ABRE) was reduced in *snrk2.2 snrk2.3*, suggesting that the mechanism of SnRK2.2 and SnRK2.3 action in ABA signaling involves the activation of ABRE-driven gene expression through the phosphorylation of ABFs. Together, these results demonstrate that SnRK2.2 and SnRK2.3 are redundant but key protein kinases that mediate a major part of ABA signaling in *Arabidopsis*.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a key factor in regulating developmental and physiological processes in plants, including seed dormancy and germination and seedling growth, as well as in controlling many abiotic stress responses (Leon-Kloosterziel et al., 1996; Schroeder et al., 2001; Bray, 2002; Finkelstein and Gibson, 2002; Zhu, 2002; Assmann, 2003; Chow and McCourt, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Forward genetic screens for ABA response mutants in seed germination have identified several mutants showing insensitivity to ABA, such as the dominant *aba insensitive1 (abi1)* and *abi2* and recessive *abi3*, *abi4*, and *abi5* mutants of *Arabidopsis thaliana* (Koorneef et al., 1984; Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). Although *abi1* and *abi2* impair many ABA responses, including the inhibition of seed germination and seedling growth and the promotion of stomatal closure, *abi3*, *abi4*, and *abi5* only show ABA insensitivity in seed germination and early seedling development (Koorneef et al., 1984; Finkelstein and Somerville, 1990; Ooms et al., 1993; Parcy et al., 1994). ABI3, ABI4, and ABI5 are transcription factors that

are expressed mainly in seeds with only low levels of expression in vegetative tissues (Giraudat et al., 1992; Finkelstein et al., 1998, 2002; Finkelstein and Lynch, 2000). ABI1 and ABI2 were found to be protein phosphatases (Meyer et al., 1994; Leung et al., 1994, 1997) that negatively regulate ABA responses (Sheen, 1998), suggesting that there exists a protein kinase(s) as a positive regulator of ABA signaling. With the exception of a protein kinase that positively regulates stomatal responses to ABA (Mustilli et al., 2002; Yoshida et al., 2002), ABA-activated protein kinases as positive regulators of ABA responses have not been reported.

The importance of phosphorylation has also been indicated by analyzing the activation of ABA response element (ABRE) binding factors (ABFs; also referred to as AREBs), which are basic leucine zipper-type (bZIP) transcription factors involved in ABA signaling. ABFs, including ABF1, ABF2 (AREB1), ABF3, ABF4 (AREB2), and ABI5, bind to the ABRE, which is a conserved *cis* element in the promoters of many ABA-induced genes (Guiltinan et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 2006), and activate transcription (Choi et al., 2000; Uno et al., 2000). These ABA-responsive genes encode, for example, putative protective proteins, enzymes required for osmolyte synthesis or transcription factors that in turn regulate still other changes in gene expression (Bray, 2002; Zhu, 2002). Consistent with the ABA-insensitive phenotype of *abi5*, overexpression of ABF3, ABF4, and ABI5 caused ABA hypersensitivity in germination and seedling growth (Lopez-Molina et al., 2001; Kang et al., 2002). It has also been demonstrated that the ABF proteins must themselves be activated in an ABA-dependent manner to increase the expression of target genes (Uno et al., 2000). Several

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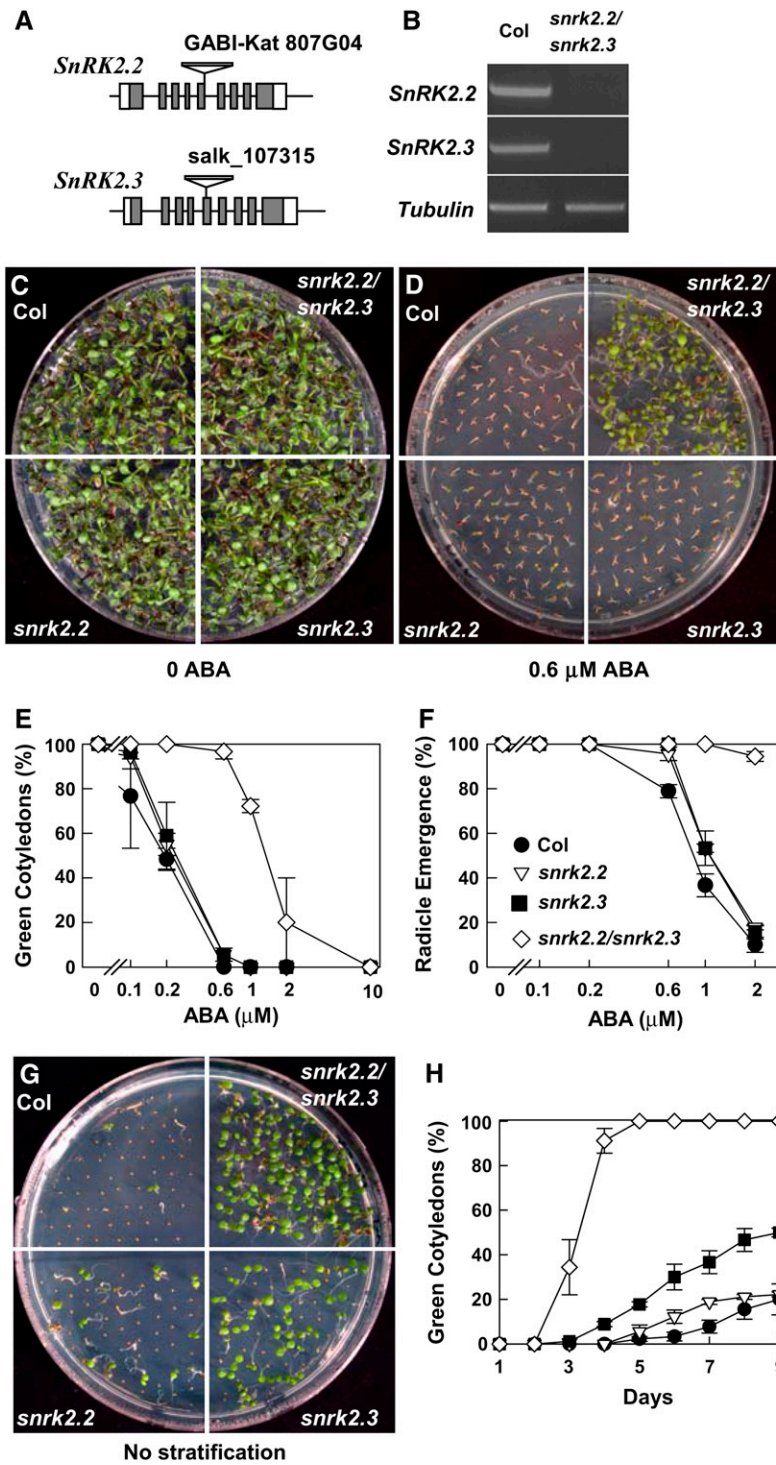


Figure 1. Seed Germination and Dormancy Assays.

(A) Diagrams of *SnRK2.2* and *SnRK2.3* showing positions of the T-DNA insertions.

(B) RT-PCR analysis with *SnRK2.2*, *SnRK2.3*, and *Tubulin* primers using total RNA extracted from seedlings of the wild type (Columbia [Col-0]) and *snrk2.2 snrk2.3* as the template.

(C) and (D) Photographs of Col-0, *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* seedlings on control (Murashige and Skoog [MS]) medium with 3% sucrose (C) or 0.6 μ M ABA medium (D) at 9 d after the end of stratification.

lines of evidence have shown that phosphorylation is involved in the activation of ABFs. ABI5 was phosphorylated after ABA treatment (Lopez-Molina et al., 2001). Likewise, the protein kinase inhibitor staurosporine suppressed the activation of ABF2 by ABA (Uno et al., 2000). Glutathione S-transferase (GST)-fused ABF2 or ABF4 proteins could be phosphorylated in vitro by extracts from ABA-treated plants (Uno et al., 2000). Also, expression of a phosphorylation-mimicking, and presumably constitutively active, form of ABF2 caused reduced germination and seedling growth and increased the expression of ABA-responsive genes in plants under unstressed conditions (Furihata et al., 2006). These results are consistent with the existence of protein kinases that act as positive regulators of ABA signaling.

In the regulation of stomatal aperture, SnRK2.6 (for SNF1-related protein kinase2.6/OST1) has been identified as a positive regulator of ABA signaling (Mustilli et al., 2002; Yoshida et al., 2002). SnRK2.6 is a member of the SnRK family of protein kinases. The *Arabidopsis* genome contains 38 SnRKs, of which 10 (SnRK2.1 to SnRK2.10) are SnRK2s (Hrabak et al., 2003). There are also 10 SnRK2 class kinases (SAPK1 to SAPK10) in rice (*Oryza sativa*). Although related to yeast SNF1, SnRK2s, as well as SnRK3s, appear to be plant-specific classes of kinases. Many of these kinases have been assigned different names in past reports, but the SnRK2.1 to SnRK2.10 designations are now the accepted nomenclature (Hrabak et al., 2003). Several experiments have shown that the kinase activity of SnRK2s can be activated by ABA and that SnRK2s phosphorylate ABFs in several tissues. In *Vicia faba*, the SnRK2-type protein kinase AAPK is activated by ABA in guard cells and regulates stomatal closure (Li et al., 2000). Of the *Arabidopsis* SnRK2s, SnRK2.2, SnRK2.3, SnRK2.6, SnRK2.7, and SnRK2.8 could be activated by ABA when expressed in *Arabidopsis* protoplasts (Boudsocq et al., 2004). Consistent with this finding, green fluorescent protein-fused SnRK2.2, SnRK2.3, SnRK2.6, SnRK2.7, and SnRK2.8 expressed in T87 cells were also activated by ABA and phosphorylated GST-fused fragments of ABF2 and ABF4 (Furihata et al., 2006; Yoshida et al., 2006). In rice, SAPK8, SAPK9, and SAPK10, which are homologous with SnRK2.2, SnRK2.3, and SnRK2.6, were activated by ABA in a protoplast system (Kobayashi et al., 2004). These SAPKs phosphorylated TRAB1, which is a rice ortholog of the *Arabidopsis* ABFs (Kobayashi et al., 2005). In wheat (*Triticum aestivum*), PKABA1, which is induced by ABA at the transcript level, phosphorylates Ta ABF (Anderberg and Walker-Simmons, 1992; Johnson et al., 2002).

The *snrk2.6* mutation affected leaf water loss through the regulation of stomatal closure (Mustilli et al., 2002; Yoshida et al., 2002). However, seed dormancy and germination were not

affected in *snrk2.6* (Mustilli et al., 2002; Yoshida et al., 2002). It is possible that another SnRK2 protein kinase may function in seed germination and other ABA-regulated traits besides stomatal closure. Because forward genetic screens have failed to identify this kinase, there might be several redundant protein kinases that mediate ABA signaling in these processes, and a reverse genetics approach may be necessary to identify them.

SnRK2.2 and SnRK2.3 are two protein kinases most closely related to SnRK2.6 (Hrabak et al., 2003). In this study, we isolated *snrk2.2* and *snrk2.3* single mutants and a *snrk2.2 snrk2.3* double mutant. The double mutant was insensitive to ABA in seed germination and seedling growth. These results suggest that SnRK2.2 and SnRK2.3 are the key protein kinases mediating ABA signaling during seed germination and seedling growth. We also present data indicating that the effect of these two protein kinases is mediated at least in part by phosphorylating ABFs and regulating ABA-responsive genes.

RESULTS

snrk2.2 snrk2.3 Is Insensitive to ABA in Seed Germination

To analyze the function of SnRK2.2 and SnRK2.3, we obtained T-DNA insertion lines (Figure 1A) from the GABI and Salk T-DNA collections and isolated *snrk2.2* and *snrk2.3* homozygous mutants by PCR screening. The single mutants were crossed, and further PCR screening was done to obtain a *snrk2.2 snrk2.3* double mutant. RT-PCR analysis confirmed that expression of both *SnRK2.2* and *SnRK2.3* was abolished in the double mutant (Figure 1B).

When we analyzed ABA responses in *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3*, *snrk2.2 snrk2.3* was found to be ABA-insensitive in seed germination and early seedling growth. This was true in assays measuring the emergence of green cotyledons (Figures 1C to 1E) as well as when germination was scored by radicle emergence (Figure 1F). In all of these assays, the *snrk2.2* and *snrk2.3* single mutants showed either no significant difference from the wild type or a much weaker ABA-insensitive phenotype relative to the double mutant. This finding suggests significant functional redundancy between SnRK2.2 and SnRK2.3.

ABA also controls seed dormancy, and we observed decreased seed dormancy in *snrk2.2 snrk2.3*. For the dormancy experiments, plants of each genotype tested were grown in different sections of the same pot and seeds were harvested at the same time to minimize the effect of seed maturation and storage conditions. *snrk2.2 snrk2.3* seeds showed green cotyledons at 5 d after sowing, whereas almost none of the wild-type seeds had germinated at this time (Figures 1G and 1H). In this

Figure 1. (continued).

(E) Quantification of the percentage of seedlings with green cotyledons after 6 d on the indicated concentrations of ABA (means \pm SE; $n = 3$). Each measurement consisted of at least 30 seeds. For symbols, see (F).

(F) Quantification of radicle emergence of each genotype at 3 d after the end of stratification (means \pm SE; $n = 3$). Medium used was MS medium without sucrose.

(G) Germination of nonstratified seeds at 7 d after sowing on MS medium with 3% sucrose.

(H) Germination time course (measured by appearance of green cotyledons) for nonstratified seeds of each genotype (means \pm SE; $n = 3$). For symbols, see (F).

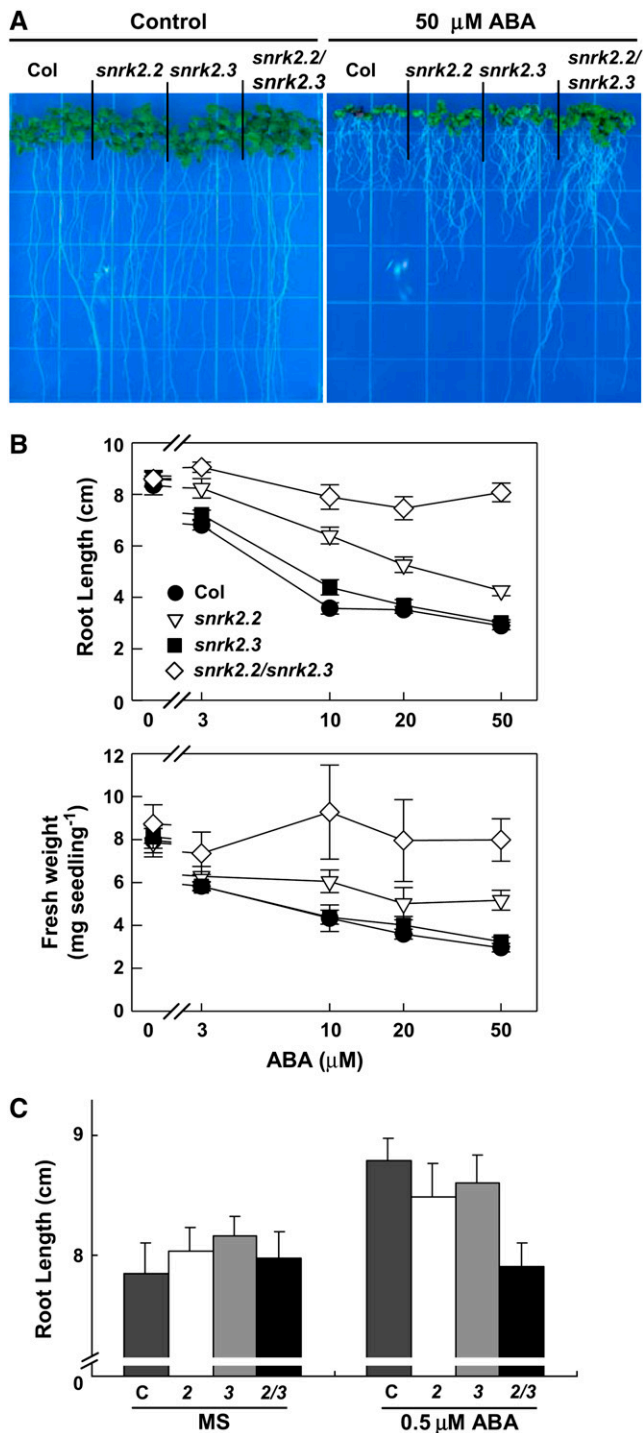


Figure 2. ABA Inhibition of Seedling Growth.

(A) Photographs of seedlings at 14 d after transfer to control medium (MS medium with 3% sucrose) or medium containing 50 μM ABA. Seedlings were 4 d old at the time of transfer and had equal root lengths at that time.

(B) Quantification of root length and seedling fresh weight for seedlings treated as described for (A). For fresh weight determination, seven seedlings were weighed at one time and the result was divided by seven. Data are means \pm SE ($n = 28$ for root length and $n = 4$ for fresh weight).

case, the single mutants, especially *snrk2.3*, also germinated more than the wild type but less than *snrk2.2 snrk2.3*.

snrk2.2 snrk2.3 Is Also Insensitive to ABA Regulation in Seedling Growth

After germination is complete, ABA still can regulate seedling growth. We analyzed this aspect of ABA sensitivity in our mutants and *snrk2.6* by transferring 4-d-old seedlings germinated on agar plates to the same medium with or without ABA. The length of the primary root and seedling fresh weight were then measured 14 d later. Based on both of these measures, *snrk2.2 snrk2.3* grew more than the wild type on medium containing 3 to 50 μM ABA (Figures 2A and 2B). *snrk2.2*, but not *snrk2.3*, also grew more than the wild type but less than *snrk2.2 snrk2.3* under these conditions. Low concentrations of ABA ($<1 \mu\text{M}$) are known to stimulate root growth (Ephritikhine et al., 1999). In our experiments, there was a slight stimulation of root elongation in the wild type in response to 0.5 μM ABA (Figure 2C). This stimulation by 0.5 μM ABA was not observed in the *snrk2.2 snrk2.3* double mutant. Thus, in these traits as well, *snrk2.2 snrk2.3* is insensitive to regulation by ABA, and there is redundancy between the functions of SnRK2.2 and SnRK2.3.

As was the case for seed germination, no difference between *snrk2.6* and the wild type was observed in the sensitivity of seedling growth to ABA (see Supplemental Figure 1 online).

snrk2.2 snrk2.3 Is Little Affected in Leaf Water Loss, in Contrast with *snrk2.6*

Next, leaf water loss of the mutants was examined. Previous studies have shown that the defining phenotype of *snrk2.6* is impaired stomatal closure leading to greater leaf water loss (Mustilli et al., 2002; Yoshida et al., 2002). *snrk2.2* and *snrk2.3* had only slight increases in leaf water loss (Figure 3). The effect on leaf water loss in *snrk2.2 snrk2.3* was greater, approximately the sum of the *snrk2.2* and *snrk2.3* single mutant losses; however, it was still much smaller than the effect in *snrk2.6* (Figure 3). Thus, these data suggested that SnRK2.2 and SnRK2.3 have only minor roles in stomatal control and further distinguished their function from that of SnRK2.6.

SnRK2.2 and SnRK2.3 Are Expressed in Various Tissues

SnRK2.6, which plays a role in stomatal regulation, is expressed specifically in guard cells (Mustilli et al., 2002; Yoshida et al., 2002). Because *snrk2.2 snrk2.3* showed impairment in various aspects of ABA responses, the expression patterns of *SnRK2.2* and *SnRK2.3* were analyzed.

We examined the expression of *SnRK2.2* and *SnRK2.3* using RT-PCR and promoter: β -glucuronidase (GUS) fusions. RT-PCR analysis showed the expression of *SnRK2.2* in all tissues examined (Figure 4A). *SnRK2.3* was expressed at a lower level but still

(C) Quantification of root length for seedlings at 14 d after transfer to control medium or medium containing 0.5 μM ABA. Data are means \pm SE ($n = 21$). Col-0, *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* are indicated by C, 2, 3, and 2/3, respectively.

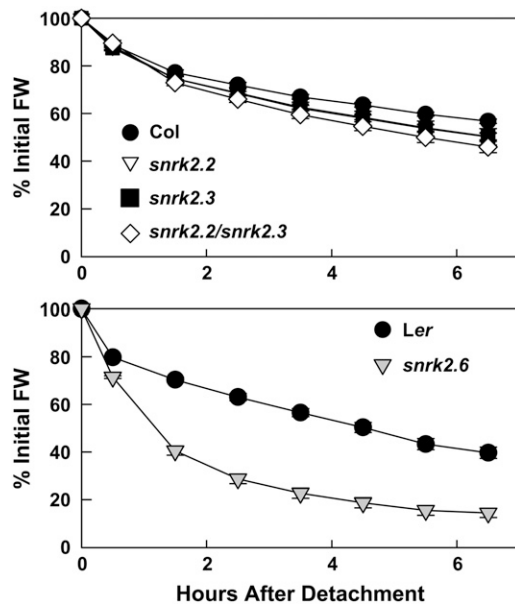


Figure 3. Leaf Water Loss Assay.

Water loss was measured using detached leaves of Col-0, *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* (top panel) or Landsberg *erecta* (Ler) and *snrk2.6/ost1* (bottom panel). Data are means \pm SE ($n = 4$ to 8). FW, fresh weight.

could be detected in all tissues by increasing the number of PCR cycles (Figure 4A). For a more detailed analysis of the expression patterns, promoter fragments covering 2 kb upstream of the translational start site were cloned for *SnRK2.2*, *SnRK2.3*, and *SnRK2.6*, fused to GUS, and transformed into Col-0 wild-type plants. GUS staining of the transgenic plants revealed *SnRK2.2* and *SnRK2.3* expression throughout cotyledon and leaf tissues, with similar or lower levels of expression in stems, roots, flowers, and siliques (Figures 4B and 4C; see Supplemental Figure 2 online). *SnRK2.3* also showed particularly strong expression in root tips (Figure 4B; see Supplemental Figure 2 online). Examination of leaf tissues at higher magnification confirmed that the *SnRK2.2* and *SnRK2.3* promoter:GUS lines had staining in all cell types in the epidermis (Figure 4D). Consistent with previous reports (Mustilli et al., 2002), staining was detected only in guard cells of *SnRK2.6* promoter:GUS lines. This was true in leaf tissue (Figures 4B to 4D) as well as in stems and sepals (see Supplemental Figure 2 online). Thus, the widespread expression in various tissues is consistent with a role of *SnRK2.2* and *SnRK2.3* in regulating various aspects of ABA response, whereas *SnRK2.6* functions specifically in guard cell regulation.

***snrk2.2 snrk2.3* Is Impaired in ABA-Inducible Phosphorylation of ABFs in Vitro**

The activation status of SnRK2s in vivo can be detected by in-gel kinase assay using *Arabidopsis* crude extracts (Yoshida et al., 2002; Boudsocq et al., 2004). In-gel kinase assays using histone as a substrate showed that an \sim 44-kD ABA-induced kinase activity, but not a 42-kD activity, was missing in *snrk2.6* (Yoshida

et al., 2006). The phosphorylatable fragment (Gly-73 to Gln-119) of ABF2 can also be used as a substrate of SnRK2.2 and SnRK2.3 (Furihata et al., 2006). We used the *snrk2* mutants to determine whether the SnRK2.2 and SnRK2.3 phosphorylation activity could be activated by ABA in vivo and whether ABFs were possible substrates. Protein extracts were prepared from Col-0, *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* seedlings grown under control conditions or after exposure to 100 μ M ABA for 30 min. Recombinant GST-fused fragments of ABF2 (Gly-73 to Gln-119), ABI5 (Arg-132 to Gln-190), and ABF1 (Gly-83 to Glu-131) were used for in-gel kinase activity assays. Similar kinase activity was detected for each of these substrates (Figures 5A to 5C). Two bands of ABA-induced phosphorylation activity were detected (Figures 5A to 5C). The stronger band of kinase activity was at 42 kD, and this activity was reduced in all three of the mutants. *snrk2.2* had a greater reduction in the 42-kD activity than did *snrk2.3*, and the reduction was additive in the double mutant. These results suggest that the 42-kD activity is derived

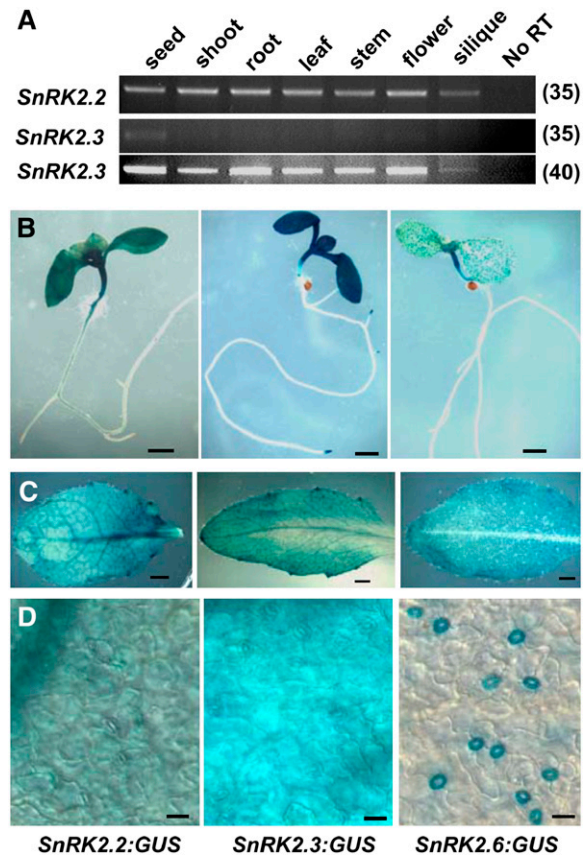


Figure 4. Expression of *SnRK2.2* and *SnRK2.3*.

(A) RT-PCR analysis of tissue distribution of *SnRK2.2* and *SnRK2.3* expression. Numbers at right indicate the number of PCR cycles performed.

(B) to (D) GUS staining of plants with promoter:GUS expression driven by 2-kb promoter fragments of *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* (arranged from left to right in each row of photographs). Bars in (B), (C), and (D) = 1 mm, 1 mm, and 20 μ m, respectively.

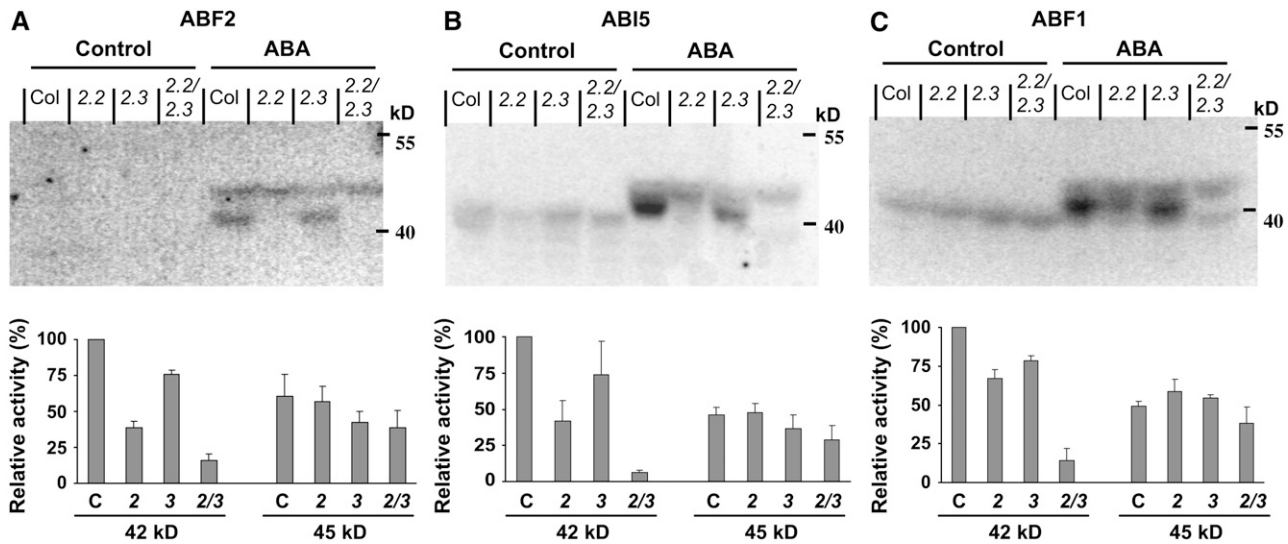


Figure 5. In-Gel Kinase Assay.

In-gel kinase assay with proteins extracted from the wild type (Col-0), *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* seedlings under control conditions or 30 min after 100 μ M ABA treatment. The ABF2 fragment (amino acids Gly-73 to Gln-119 [A]), ABI5 fragment (Arg-132 to Gln-190 [B]), and ABF1 fragment (Gly-83 to Glu-131 [C]) were used as substrates. The graphs at bottom indicate relative radioactivity (mean \pm SE; $n = 4$ for ABF2, $n = 3$ for ABI5 and ABF1) of the 42- and 45-kD bands after ABA treatment normalized relative to the 42-kD activity of the wild type. In the graphs, Col-0, *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* are indicated by C, 2, 3, and 2/3, respectively.

from SnRK2.2 and SnRK2.3. In contrast with the 42-kD kinase activity, the 45-kD activity was weaker and was relatively unaffected in *snrk2.2* or *snrk2.3*. It was, however, somewhat weaker in *snrk2.2 snrk2.3*. SnRK2.2 and SnRK2.3 may indirectly affect the 45-kD activity, which likely corresponds to SnRK2.6 (Yoshida et al., 2006).

In all lanes, regardless of ABA treatment, strong bands of \sim 69 kD were detected and weak bands of \sim 39 kD were sometimes detected besides the 42- and 45-kD ABA-inducible activities (data not shown). Together with previous studies (Boudsocq et al., 2004; Furihata et al., 2006; Yoshida et al., 2006), these data demonstrate that SnRK2.2 and SnRK2.3 have ABA-inducible kinase activity and together are likely to play a major role in phosphorylating and activating ABF2, ABI5, ABF1, and possibly other ABFs.

SnRK2.2 and SnRK2.3 Are Required for ABA-Induced Changes in Gene Expression and Pro Accumulation

ABA induces the expression of many genes that are important for adaptation to stress. Some well-documented examples include *RESPONSIVE TO DESSICATION29B* (*RD29B*), *RD22*, *RD29A*, *RESPONSIVE TO ABA18* (*RAB18*), *NCED3* (for 9-*cis*-Epoxy-carotenoid Dioxygenase3), and *P5CS1* (for Δ^1 -Pyrroline-5-Carboxylate Synthetase1) (Yamaguchi-Shinozaki and Shinozaki, 2006). Based on their activity in phosphorylating the ABF2 fragment and the other phenotypes observed above, we hypothesized that SnRK2.2 and SnRK2.3 play a role in ABA-induced gene expression changes. To examine the expression of these genes, quantitative RT-PCR was performed. In the wild type, treatment with 100 μ M ABA for 3 h induced all of these genes (Figure 6A). Induction of these genes

was consistently less in all three of the mutants, although the extent to which induction by ABA was blocked varied among the genes examined. As in the other phenotypes examined above, it was also consistently observed that the *snrk2.2 snrk2.3* double mutant had the greatest reduction in ABA-induced gene expression.

Some of these same ABA-regulated genes are also expressed in seeds. We found that expression of *RD29B* and *RAB18* was greatly reduced in imbibed seeds of *snrk2.2 snrk2.3* (Figure 6B). This finding confirmed that SnRK2.2 and SnRK2.3 are also responsible for regulating the expression of genes with an ABRE in their promoter during seed dormancy and germination. Other genes examined in imbibed seeds were expressed at very low levels (*NCED3*, *RD22*, and *RD29A*) and did not differ between any of the genotypes tested (data not shown).

Consistent with their role in ABA induction of the Pro biosynthesis gene *P5CS1*, we found that SnRK2.2 and SnRK2.3 also affected ABA-induced Pro accumulation. In seedlings exposed to 10 μ M ABA for 96 h, Pro accumulation in *snrk2.2*, *snrk2.3*, and *snrk2.2 snrk2.3* was less than that in wild-type Col-0. *snrk2.2* had a greater effect than *snrk2.3*, and the effect was additive in the double mutant (Figure 6C). Pro content in control seedlings was low (0.15 μ mol/g fresh weight) and did not differ between the wild type and the mutants. By contrast, Pro accumulation was not reduced in *snrk2.6* seedlings exposed to 10 μ M ABA (1.74 \pm 0.17 μ mol/g fresh weight for *Ler* versus 2.18 \pm 0.15 μ mol/g fresh weight for *snrk2.6*).

DISCUSSION

ABA regulates many important plant processes, including seed germination, dormancy, seedling growth, and stomatal aperture.

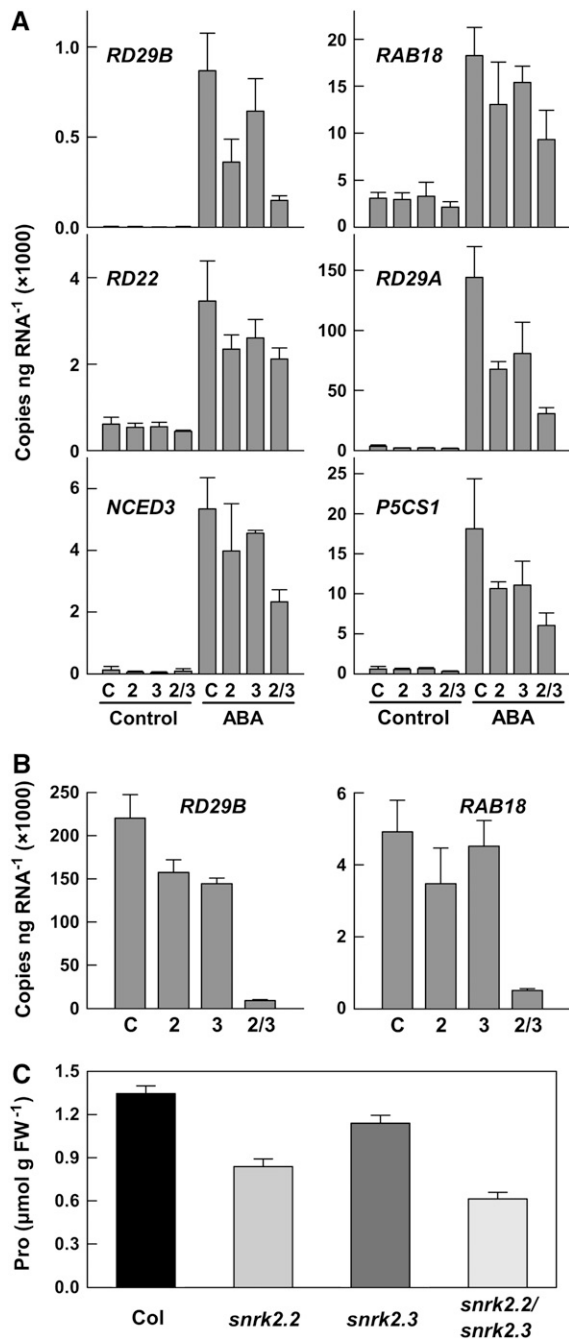


Figure 6. Expression of ABA-Regulated Genes and ABA-Induced Pro Accumulation.

(A) Expression of the ABA-upregulated genes *RD29B*, *RAB18*, *RD22*, *RD29A*, *NCED3*, and *P5CS1* assayed by quantitative RT-PCR in Col-0 (C), *snrk2.2* (2), *snrk2.3*(3), and *snrk2.2 snrk2.3* (2/3) seedlings under control conditions or after 3 h of exposure to 100 μ M ABA. Data are means \pm SE ($n = 3$). In some cases, such as with *RD29B*, expression in control seedlings was too low to be visible on the graphs.

(B) Expression of *RD29B* and *RAB18* in seeds imbibed for 24 h (means \pm SE; $n = 3$).

(C) Pro contents of seedlings after transfer to plates containing 10 μ M ABA for 96 h. Data are means \pm SE ($n = 6$ to 8). FW, fresh weight.

Although many genes have been reported to be involved in ABA signaling, some key components are still missing, and ABA signaling appears to involve a highly branched network. SnRK2.6 has been identified as a positive regulator of ABA signaling but functions only in guard cell ABA responses (Mustilli et al., 2002; Yoshida et al., 2002), whereas ABI1 and ABI2 function as negative regulators (Sheen, 1998) in seed germination, seedling growth, and stomatal closure (Leung et al., 1997). This raises the question of which protein kinases might positively regulate ABA signaling in seed germination and seedling growth.

We have used a reverse genetics approach to study the functions of SnRK2.2 and SnRK2.3. *snrk2.2 snrk2.3* plants are insensitive to ABA in seed germination, indicating that SnRK2.2 and SnRK2.3 are the protein kinases that positively regulate ABA signaling in seed germination. SnRK2.2 and SnRK2.3 also have highly redundant functions in seed dormancy, inhibition of seedling growth by ABA, ABA-induced Pro accumulation, and ABA-induced gene expression. This redundancy likely explains why SnRK2.2 and SnRK2.3 were not identified in the extensive forward genetic screening for ABA-insensitive mutants that has been conducted by a number of laboratories.

snrk2.2 snrk2.3 is only slightly defective in leaf water loss compared with *snrk2.6*. The very distinctive phenotype of *snrk2.2 snrk2.3* compared with *snrk2.6* raises the question of the basis for this specificity. Such differences could be caused by a difference in gene expression or their substrate specificity or activation. As described above, experiments to date have not yet found a difference in substrate specificity among the SnRK2s: SnRK2.2, SnRK2.3, SnRK2.6, and SnRK2.8 were all able to phosphorylate ABF2 (Furihata et al., 2006). Likewise, ABA activation of all of these kinases was observed (Boudsocq et al., 2004; Furihata et al., 2006). This leaves differences in expression as the most likely basis for the specific roles of SnRK2.2 and SnRK2.3 versus SnRK2.6 in different ABA responses. Consistent with this hypothesis, our results show widespread expression of SnRK2.2 and SnRK2.3, particularly in leaf and stem tissues, whereas SnRK2.6 is largely confined to the guard cells. SnRK2.2 and SnRK2.3 do have a small effect on leaf water loss, and this is consistent with their low level of expression in guard cells.

Our experiments showed a >80% reduction in *snrk2.2 snrk2.3* of a 42-kD ABA-activated kinase activity capable of phosphorylating an ABF2, ABI5, or ABF1 fragment in vitro. This finding is consistent with several previous studies that have shown activation of SnRK2 activity, including SnRK2.2, SnRK2.3, and SnRK2.6, by ABA and/or osmotic stress (Boudsocq et al., 2004; Kobayashi et al., 2004; Furihata et al., 2006; Yoshida et al., 2006). However, the activation mechanism of SnRK2s is still unclear. The C-terminal domain of rice SAPK8 can confer ABA responsiveness in protoplasts. Likewise, the C-terminal region of SnRK2.6 is important for ABA response (Yoshida et al., 2006) and has been shown to bind to ABI1 in a yeast two-hybrid assay. This interaction appears to be important in vivo, as SnRK2.6 is not fully activated by ABA in *abi1-1* (Yoshida et al., 2006). The molecular mechanism by which SnRK2s are activated is not known but must involve a posttranslational modification resistant to SDS treatment, because the activation status of endogenous SnRK2s is maintained after SDS-PAGE. Many kinases are themselves activated by autophosphorylation or phosphorylation by upstream kinase(s),

and the hyperosmotic stress activation of rice SAPK1 and SAPK2 is mediated by phosphorylation (Kobayashi et al., 2004). The identification of SnRK2.2 and SnRK2.3 as ABA-activated kinases important in ABA signaling sets the stage for further experiments to identify upstream kinases or other regulatory mechanisms.

Our data suggest that the ABF bZIP family transcription factors are downstream targets of SnRK2.2 and SnRK2.3 in ABA signaling. The substantial loss of ABF phosphorylation activity in *snrk2.2 snrk2.3* also suggests that SnRK2.2 and SnRK2.3 may be the predominant kinases that phosphorylate the transcription factors in response to ABA in seedlings. Because ABI5 and ABF1 could also be substrates of SnRK2.2 and SnRK2.3 (Figures 5B and 5C), other ABFs may also be phosphorylated by SnRK2.2 and SnRK2.3. It is possible that other SnRK2.2 or SnRK2.3 substrates outside of the ABF family may also be involved in ABA signaling.

Most of the ABA-induced genes for which we observed reduced ABA responsiveness in *snrk2.2 snrk2.3* contain ABREs in their promoters. *RD29A*, *RD29B*, and *P5CS1* have all been reported to contain ABREs in their promoters (Yamaguchi-Shinozaki and Shinozaki, 1994; Yoshida et al., 1999). Our own examination of promoter sequences also revealed an ABRE ~75 bp upstream of the transcriptional start site of *NCED3* and ~300 bp upstream of the start site in the *RAB18* promoter. Thus, SnRK2.2 and SnRK2.3 are likely to affect the expression of these genes through phosphorylation of one or more ABFs, thus influencing ABF binding to the ABRE. However, *RD22* has no distinct ABRE in its promoter region and instead has been reported to be regulated by MYC and MYB transcription factors (Abe et al., 1997). *RD22* expression is also reduced in *snrk2.2 snrk2.3*. Thus, there is a question regarding whether this phenotype can be accounted for by an indirect mechanism (i.e., that SnRK2.2 and SnRK2.3 regulate the expression of a gene that in turn regulates *RD22* expression) or whether there is a more direct, as yet unknown, mechanism by which SnRK2s can regulate gene expression independently of the ABRE.

METHODS

T-DNA Insertion Lines

The seeds of *Arabidopsis thaliana snrk2.2* and *snrk2.3* T-DNA insertion lines (GABI-Kat 807G04 and Salk_107315) were obtained from the Max Planck Institute for Plant Breeding Research (Rosso et al., 2003) and the ABRC (Alonso et al., 2003), respectively. Homozygous plants were obtained by PCR screening using primers designed by the I-Sect website (<http://signal.salk.edu/cgi-bin/tDNAexpress>; primer sequences are given Supplemental Table 1 online). The *snrk2.6* mutant used here is the original *ost1* mutant (Mustilli et al., 2002) and is in the *Ler* background. For clarity in the comparison with *snrk2.2* and *snrk2.3*, we refer to *ost1* as *snrk2.6* throughout this report.

Production of Transgenic Plants and GUS Staining

Fragments covering 2 kb upstream of the translational start sites of *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* were amplified by PCR using genomic DNA as templates. For *SnRK2.2* and *SnRK2.3*, this region contains endogenous *HindIII* or *BamHI* sites. Therefore, two different fragments

covering the 2-kb region were amplified, with each fragment having different portions of *HindIII* and *BamHI* sites on each end such that mixing, denaturing, and reannealing the fragments generated heterodimers with *HindIII/BamHI* cohesive ends. These fragments were cloned into *HindIII/BamHI*-digested pBI101. For the 2-kb fragment upstream of *SnRK2.6*, the PCR product was cloned into pGEM-T easy vector, digested with *PstI*, and blunted. After digestion with *BamHI*, the fragment was subcloned into pBI101 vector at blunted *HindIII* and *BamHI* sites. Primers used are given in Supplemental Table 1 online. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Col-0 plants were transformed by floral dip infiltration, and kanamycin-resistant transgenic seedlings of T2 plants and other tissues of T1 plants were used for GUS staining. For GUS staining, tissues were incubated in 5 mM $K_4Fe(CN)_6$, $K_3Fe(CN)_6$, 0.3% Triton X-100, 0.1 M phosphate buffer (a mixture of KH_2PO_4 and K_2HPO_4 , pH 7.0), and 3 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (cyclohexylammonium salt; Gold Bio Tech) at 37°C for 16 h and incubated in 70% ethanol at 37°C for 16 h.

Kinase Assays

The ABF2, ABI5, and ABF1 fragments used for in vitro kinase assays were obtained by reverse transcription and amplification of the portion of the ABF2, ABI5, or ABF1 cDNA corresponding to amino acids Gly-73 to Gln-119 of ABF2, Arg-132 to Gln-190 of ABI5, and Gly-83 to Glu-131 of ABF1. The template for cloning was total RNA extracted from 12-d-old seedlings or genomic DNA of Col-0. The PCR product was digested with *BamHI* and *XhoI* (ABF2 and ABF1) or *EcoRI* (ABI5) and subcloned into pGEX4T1 vector (Amersham) for the production of GST fusion protein using *Escherichia coli* Rosetta cells (Novagen). Fusion proteins were purified using glutathione-agarose beads (Sigma-Aldrich) and dialyzed with 10 mM Tris-HCl, pH 8.8, for 16 h.

In-gel kinase assays were performed according to the protocol of Furihata et al. (2006) with some modifications. Plant materials used consisted of 10-d-old seedlings grown on control medium (MS agar with 1% sucrose) or seedlings sprayed with ABA and harvested 3 h later. Proteins were extracted in 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na_3VO_4 , 50 mM β -glycerophosphate, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 \times protease inhibitor cocktail (Sigma-Aldrich), and 50 mM HEPES-KOH, pH 7.5. Proteins (40 μ g/lane) were separated on a SDS-PAGE gel containing 0.5 mg/mL GST-tagged substrate peptide. The gel was washed for 3 \times 30 min with 0.5 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4 , 0.5 mg/mL BSA, 0.1% Triton X-100, and 25 mM Tris-HCl, pH 7.5, and proteins were renatured with 1 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4 , and 25 mM Tris-HCl, pH 7.5, for 2 \times 30 min and 16 h at 4°C. After 30 min of incubation in reaction solution (2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4 , and 25 mM Tris-HCl, pH 7.5) at room temperature, the gel was incubated in 12 mL of reaction solution supplemented with 50 μ Ci of [γ - ^{32}P]ATP and 250 nM cold ATP for 90 min at room temperature. The gel was washed with 5% TCA and 1% sodium pyrophosphate more than five times for 30 min each, incubated with 10% glycerol, and dried. Radioactivity was quantified using a Typhoon 9410 imager (Molecular Dynamics).

Physiological Assays

Soil-grown plants and seedlings on agar plates were routinely grown under continuous light ($\sim 75 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 23°C. For germination assays, seeds were plated on MS medium containing sucrose and ABA as indicated in the text and figure legends. Seeds were then stratified at 4°C for 4 d, and radicle emergence or the presence of green cotyledons was scored after the indicated time intervals.

For Pro analysis, seeds were plated onto half-strength MS medium with 6 mM MES, pH 5.7, without the addition of sucrose or other sugars,

according to the protocols of Verslues et al. (2006), and stratified for 4 d at 4°C. ABA treatment for the analysis of Pro accumulation was performed by transferring 6-d-old seedlings to half-strength MS plates without sugar but with 10 μ M ABA added to the medium. Pro was assayed using a ninhydrin-based colorimetric assay (Bates et al., 1973).

For leaf water loss measurements, fully expanded leaves were removed from 4- to 5-week-old plants and incubated under the same conditions used for seedling growth, and each sample (consisting of three to four individual leaves) was weighed at the indicated times.

RT-PCR and Quantitative PCR Analysis of Gene Expression

Total RNA was purified from seedlings, leaves, stems, and inflorescences using Trizol (Invitrogen) according to the manufacturer's instructions or from imbibed seeds and green siliques according to Penfield et al. (2005). In either case, the extracted total RNA was dissolved in 100 μ L of RNase-free water and purified using the RNAeasy kit (Qiagen) according to the manufacturer's instructions, including DNase treatment. Reverse transcription reactions were performed using 0.05 μ g of total RNA and SuperScriptII reverse transcriptase (Invitrogen). PCR was then performed for 20 cycles and one-twentieth of the initial PCR sample was used to set up a second PCR sample, which was amplified for an additional 15 or 20 cycles. The total number of PCR cycles used for both reactions is indicated in the figures.

Real-time quantitative PCR analysis was performed with a 7700 sequence detection system (Applied Biosystems) using primers and TaqMan probes designed using Primer Express software (Applied Biosystems). BHQ/FAM-labeled TaqMan probes were obtained from Biosearch Technologies. Total RNA was extracted from control or 100 μ M ABA-treated seedlings using the RNAeasy kit (Qiagen) including DNase treatment. Reverse transcription and PCR (10 μ L) were performed with 0.1 μ g of total RNA using the One-Step RT-PCR kit (Qiagen). To quantify the copy number of each RNA, the C_t (threshold cycle) value was compared with a standard curve generated using PCR products for each gene that had been purified and quantified by UV light absorbance (Zhu et al., 2005). Three biological and three technical replicates were performed for each experiment. Primers used for both RT-PCR and quantitative RT-PCR are given in Supplementary Table 1 online.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *SnRK2.2*, At3g50500; *SnRK2.3*, At5g66880; *SnRK2.6*, At4g33950.

Supplemental Data

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

Supplemental Figure 1. ABA Sensitivity of Germination and Seedling Growth of *snrk2.6*.

Supplemental Figure 2. GUS Staining of Flowers, Stems, and Siliques of *SnRK2.2*, *SnRK2.3*, and *SnRK2.6* Promoter:GUS Transgenic Lines.

Supplemental Table 1. Oligonucleotides Used in This Study.

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