Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress

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Abscisic acid (ABA) is an important phytohormone regulating seed dormancy, germination, seedling growth, and plant transpiration. We report here an Arabidopsis triple mutant that is disrupted in 3 SNF1-related protein kinase subfamily 2 (SnRK2s) and nearly completely insensitive to ABA. These SnRK2s, SnRK2.2, SnRK2.3, and SnRK2.6 (also known as OST1), are activated by ABA and can phosphorylate the ABA-responsive element binding factor family of b-ZIP transcription factors, which are important for the activation of ABA-responsive genes. Although stomatal regulation of *snrk2.6* and seed germination and seedling growth of the *snrk2.2/2.3* double mutant are insensitive to ABA, ABA responses are still present in these mutants, and the growth and reproduction of these mutants are not very different from those of the WT. In contrast, the *snrk2.2/2.3/2.6* triple mutant grows poorly and produces few seeds. The triple mutant plants lose water extremely fast when ambient humidity is not high. Even on 50 μM ABA, the triple mutant can germinate and grow, whereas the most insensitive known mutants cannot develop on 10 μM ABA. In-gel kinase assays showed that all ABA-activated protein kinase activities are eliminated in the triple mutant. Also, the expression of ABA-induced genes examined is completely blocked in the triple mutant. These results demonstrate that the protein kinases SnRK2.2, SnRK2.3, and SnRK2.6 have redundant functions, and suggest that ABA signaling is critical for plant growth and reproduction.

abscisic acid | seed germination | SnRK2 | stomatal regulation

The phytohormone abscisic acid (ABA) is a key factor in regulating various developmental and physiological processes in plants, including seed maturation, dormancy, seedling development, and stomatal behavior (1–7). Forward genetics screens identified several ABA-deficient mutants such as *aba1*, *aba2*, and *aba3*, and several ABA-insensitive mutants such as *abi1*, *abi2*, *abi3*, *abi4*, and *abi5* that have helped to illuminate the physiological functions of ABA (8–16). The phenotypes of the ABA-deficient mutants, which included loss of dormancy, reduced size and wilting, supported the importance of ABA in developmental and physiological responses (8). However, no recessive mutant in ABA response pathways exhibits similarly strong defects. Although *abi1-1* and *abi2-1* mutants are insensitive to ABA and have wilty phenotypes, they are dominant mutants; the effect of the mutations on the pathway is not straightforward. In fact, ABI1 and ABI2 function as negative regulators, because *abi1* or *abi2* null mutants are more sensitive to ABA (9, 17–20). To understand ABA signaling, it is important to identify recessive mutants with strong ABA-insensitive phenotypes, which would define key positive regulators in the pathway.

Because ABI1 and ABI2 are protein phosphatases, there must be kinases that act as positive regulators in ABA signaling. Phosphorylation is involved in ABA-induced activation of transcription factors, the ABA-responsive element (ABRE)-binding factors (ABFs; also referred to as AREBs). ABFs, which include ABF1, ABF2 (AREB1), ABF3, ABF4 (AREB2), and ABI5, are b-ZIP transcription factors that bind to ABRE, a conserved *cis*-element in the promoters of many ABA-induced genes, and activate their transcription (21–24). Because these ABA-responsive genes encode proteins important for stress tolerance such as protective proteins and enzymes for osmolyte synthesis (3, 25), ABFs are among the most important transcription factors for ABA function. Activation of ABF2 by ABA was suppressed by the protein kinase inhibitor staurosporine (23). ABA treatment induced phosphorylation of ABI5 (26). When a phosphorylation-mimicking form of ABF2 was expressed, germination and seedling growth were inhibited, and ABA-induced genes were expressed even under unstressed conditions (27). In-gel kinase assays showed that extracts from ABA-treated but not control plants had kinase activities that could phosphorylate ABF2, ABF4, and ABI5 (23, 28). The ABA-activated kinases were identified as SNF1-related protein kinase (SnRK) 2.2, SnRK2.3, and SnRK2.6 (27–29).

SnRK2s are a family of plant-specific protein kinases containing 10 members (SnRK2.1–2.10) in *Arabidopsis*, and 10 members (SAPK1–10) in *Oryza sativa* (30). The involvement of SnRK2 in the ABA pathway was first reported for wheat PKABA1, which is induced by ABA at the transcript level (31), and which phosphorylates TaABF (32). In *Vicia faba*, the SnRK2 protein kinase AAPK is activated by ABA in guard cells and regulates stomatal closure (33). SnRK2.6 (also known as OST1), the *Arabidopsis* ortholog of AAPK, is also required for stomatal regulation, because the *ost1* (open stomata 1) mutant plants are defective in stomatal closure (34). SnRK2.2, SnRK2.3, and SnRK2.6 were found to be strongly activated by ABA in *Arabidopsis* protoplasts (35). GFP-fused SnRK2.2, SnRK2.3, and SnRK2.6 expressed in T87 cells were also activated by ABA and phosphorylated GST-fused ABF2 and ABF4 fragments (27, 36). SAPK8, SAPK9, and SAPK10, which are rice orthologs of SnRK2.2, SnRK2.3, and SnRK2.6, were activated by ABA in a protoplast system (37), and phosphorylated TRAB1, which is a rice ortholog of the *Arabidopsis* ABFs (38). Cooverexpression of PKABA1 and ABI5 induced phosphorylation of ABI5 in vivo and inhibited seed germination (39).

Loss-of-function mutations in *SnRK2.6* enhanced leaf water loss because of failure of ABA-induced stomatal closure (29, 34). Because of functional redundancy between SnRK2.2 and SnRK2.3, *snrk2.2* and *snrk2.3* single mutants did not have clear ABA response phenotypes, but *snrk2.2/2.3* double mutant was insensitive to ABA in terms of seed dormancy, seed germination, and seedling growth (28). Thus, these kinases are important for ABA signaling, and the results suggested functional segregation between SnRK2.6 and SnRK2.2/2.3, i.e., SnRK2.6 functions in guard cells, whereas SnRK2.2/2.3 specialize in seed germination and seedling growth regulation (28). However, the phenotypes of these kinase mutants are milder than those of ABA-deficient mutants. The growth of *snrk2.6* and *snrk2.2/2.3* is similar to that

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of the WT, whereas several ABA-deficient mutants are smaller than the WT, and their stems frequently wilt even under standard greenhouse conditions (8). Also, ABA-induced gene expression is reduced, but not blocked in *snrk2.2/2.3* (28) and *snrk2.6* (29). Thus, it is possible that there are remaining activities from redundant protein kinases in these mutants. Because *SnRK2.6* is expressed not only in guard cells, but also in several other tissues (39), and the expression of *SnRK2.2* and *SnRK2.3* was detected in all tissues examined (28), these functionally segregated kinases may still work redundantly in some tissues. To test this hypothesis, we constructed and analyzed a *snrk2.2/2.3/2.6* triple mutant. Our results show that the SnRK2 kinases are partially redundant, but together, are essential for ABA responses, and suggest that protein phosphorylation mediated by these kinases is required for all aspects of ABA signaling.

Results

The *snrk2.2/2.3/2.6* Triple Mutant Plants Are Severely Impaired in Growth and Reproduction. The triple mutant *snrk2.2/2.3/2.6* was constructed by crossing the double mutant *snrk2.2/2.3* (28) with *snrk2.6*. Although several homozygous *snrk2.2/2.3/2.6* plants were identified from the F2 generation by PCR-based genotyping, the triple mutant plants did not produce any seeds. To try to obtain seeds from the *snrk2.2/2.3/2.6* triple mutant, we grew a plant homozygous for *snrk2.2* and *snrk2.3*, but heterozygous for *snrk2.6* (here referred to as *snrk2.2/3/6+*). Among the progenies of *snrk2.2/3/6+*, more *snrk2.2/2.3/2.6* triple mutant plants were identified. Although the triple mutants could grow, bolt, and flower under our greenhouse conditions (40–60% relative humidity), they were very small and failed to produce seeds (Fig. 1 *A–C*). Their stems and flowers frequently wilted (Fig. 1 *D* and *E*). Even when a flower did not wilt, no pollen was released from anthers, resulting in no fertilization and consequently no elongation of pistils. Because *snrk2.6* is severely impaired in stomatal regulation and loses water rapidly (29, 34), we considered the possibility that the greenhouse humidity may be too low for the *snrk2.2/2.3/2.6* triple mutant. We then covered the plants with transparent plastic wrap to maintain near saturation humidity. High humidity (RH $\approx$ 99%) helped the *snrk2.2/2.3/2.6* triple mutant to grow larger and to occasionally produce seeds, although more than half of the flowers still failed to produce seeds. Although the efficiency of rescue (by high humidity) varied, the best rescued line had $\approx$60% of the plant height and 60% of the length of the largest leaf compared with those of the WT (Col-0) that grew in the same pot under plastic wrap.

The *snrk2.2/2.3/2.6* Triple Mutant Loses Water Extremely Fast. The phenotype described above suggested that the *snrk2.2/2.3/2.6* triple mutant is severely impaired in the control of transpirational water loss. To analyze the effect of *snrk2.2/2.3/2.6* triple mutations on transpiration, leaf water loss of the mutants was measured. Consistent with previous reports (28, 29, 34), *snrk2.6* plants exhibited an increased leaf water loss relative to the WT, whereas water loss from *snrk2.2/2.3* was only slightly faster than from the WT (Fig. 2). Water was lost much more quickly from leaves of the *snrk2.2/2.3/2.6* triple mutant than from the leaves of *snrk2.6* (Fig. 2). Under our laboratory conditions (RH $\approx$ 35–50%), within 30 min after detachment from green house-grown plants without the plastic cover, leaves of the *snrk2.2/2.3/2.6* triple mutant were shrunken (Fig. 2*A*). Similar sized leaves detached from the WT, *snrk2.2/2.3*, or *snrk2.6* did not lose water nearly as quickly as leaves of the *snrk2.2/2.3/2.6* triple mutant (Fig. 2*B*). Same aged leaves of the WT, *snrk2.2/2.3*, or *snrk2.6*, which were larger than those of the triple mutant, also kept >60% of initial fresh weight 30 min after detachment. Also, when the plastic wrap used to maintain high humidity was removed, stems of the triple mutant invariably wilted and did not recover, whereas stems of the WT, *snrk2.2/2.3*, and *snrk2.6* did not wilt. Even when some stems of *snrk2.6* occasionally withered, they recovered within 3 h later (Fig. 2*C*). These results suggest that *snrk2.2/2.3/2.6* is severely impaired in stomatal regulation and is, thus, very sensitive to low to moderate humidity conditions. Therefore, we conclude that SnRK2.2/2.3 and SnRK2.6 function redundantly in controlling water loss.

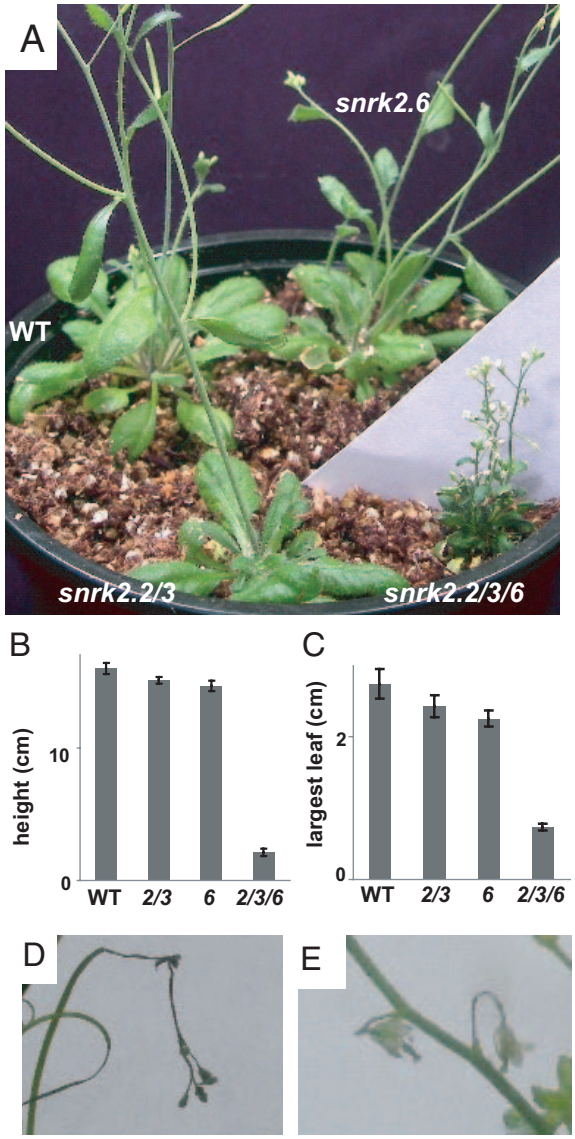

Fig. 1. Size and morphology of the *snrk2.2/2.3/2.6* triple mutant. (*A*) WT, *snrk2.2/2.3*, *snrk2.6*, and *snrk2.2/2.3/2.6* plants growing in soil under greenhouse conditions. A sheet of white paper was used to increase contrast in the photograph. (*B* and *C*) Plant height and length of the largest leaf of the WT, *snrk2.2/2.3* (2/3), *snrk2.6* (6), and *snrk2.2/2.3/2.6* (2/3/6) growing in soil (means $\pm$ SE; $n = 9$). (*D* and *E*) Examples of a wilted stem (*D*) and wilted flowers (*E*) of *snrk2.2/2.3/2.6*.

Seed Germination and Early Seedling Growth of the *snrk2.2/2.3/2.6* Triple Mutant Is Almost Completely Insensitive to ABA. Because the *snrk2.2/2.3/2.6* triple mutant rarely produces seeds, we used progeny of *snrk2.2/3/6+* for further experiments. To analyze the ABA response of the triple mutant, we planted *snrk2.2/3/6+* progeny on plates containing Murashige-Skoog medium (MS) plus 10 μM ABA, on which the growth of WT, *snrk2.2/2.3*, and *snrk2.6* is arrested and cannot develop green cotyledons. Of 155

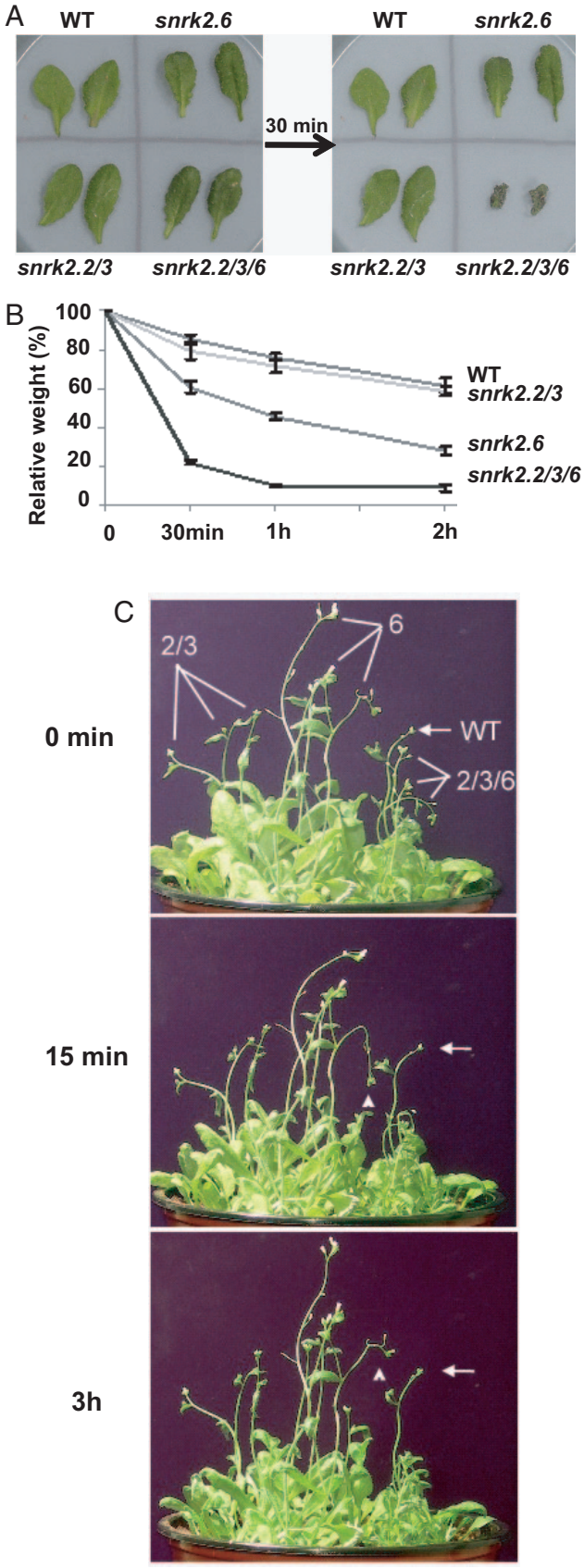

Fig. 2. Rapid water loss from the *snrk2.2/2.3/2.6* triple mutant. (*A* and *B*) Water loss was measured by using detached leaves of WT, *snrk2.2/2.3*, *snrk2.6*, and *snrk2.2/2.3/2.6*. (*A*) Photographs were taken just after detachment (*Left*) and 30 min after detachment (*Right*). (*B*) Data are normalized relative to weight just after detachment (means ± SE; $n = 5$). (*C*) Wilting of stems after removal of the plastic wrap that maintained high humidity. Photographs of the same view of the WT, *snrk2.2/2.3* (2/3), *snrk2.6* (6), and *snrk2.2/2.3/2.6* (2/3/6) were taken just after (*Top*), 15 min after (*Middle*), and 3 h after (*Bottom*) the plastic wrap was removed. Two other stems of the WT are behind the stems of *snrk2.6*. Arrows indicate the same stem of the WT. Note that stems of *snrk2.2/2.3/2.6* that can be seen below the arrow in *Top* are wilted and cannot been seen in the *Middle* and *Bottom*. In comparison, the stem of *snrk2.6* that is wilted in the *Middle* (arrowhead) has recovered in the *Bottom*.

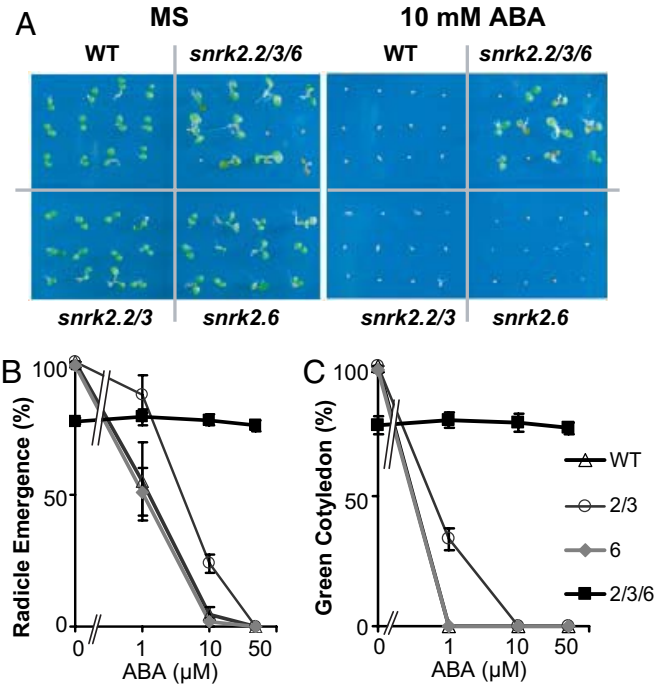

Fig. 3. Seed germination phenotypes of the different genotypes. (*A*) Photographs of the WT, *snrk2.2/2.3*, *snrk2.6*, and *snrk2.2/2.3/2.6* seedlings growing on the control medium (MS with 3% sucrose) or 10 μM ABA medium 5 days after the end of stratification. (*B* and *C*) Quantification (mean ± SE; $n = 3$) of radicle emergence of each genotype 4 days after the end of stratification on MS, without sucrose and with different concentrations of ABA (*B*), and the percentage of seedlings with green cotyledons 5 days after the end of stratification on MS with 3% sucrose and with different concentrations of ABA (*C*). Note that data for Col-0 and *snrk2.6* mostly overlap.

seeds from selfed *snrk2.2/3/6+*, 39 produced green cotyledons. We transferred these seedlings and 36 other seedlings that were arrested and did not develop green cotyledons to MS medium without ABA to determine their genotypes. All seedlings with green cotyledons on the ABA-supplemented MS medium were of the *snrk2.2/2.3/2.6* triple mutant genotype, whereas all seedlings without green cotyledons on the ABA-supplemented MS medium had at least 1 *SnRK2.6* WT allele. The result indicated that the germination and early seedling growth of the *snrk2.2/2.3/2.6* triple mutant is insensitive to 10 μM ABA. This phenotype was confirmed when sufficient *snrk2.2/2.3/2.6* seeds were later collected and tested (Fig. 3). Approximately 20% of the *snrk2.2/2.3/2.6* triple mutant seeds did not germinate even on MS medium without ABA, probably because these seeds did not mature properly. The same percentage of the triple mutant seeds did not germinate on ABA-supplemented MS medium. Of the rest that did germinate, virtually all produced visible, green cotyledons even on MS medium with 50 μM ABA (Fig. 3). The *snrk2.2/2.3/2.6* mutant appears to have lost sensitivity to ABA. The triple mutant is clearly the most ABA-insensitive mutant thus far, because *snrk2.2/2.3* (Fig. 3) and *abi1-1*, the most

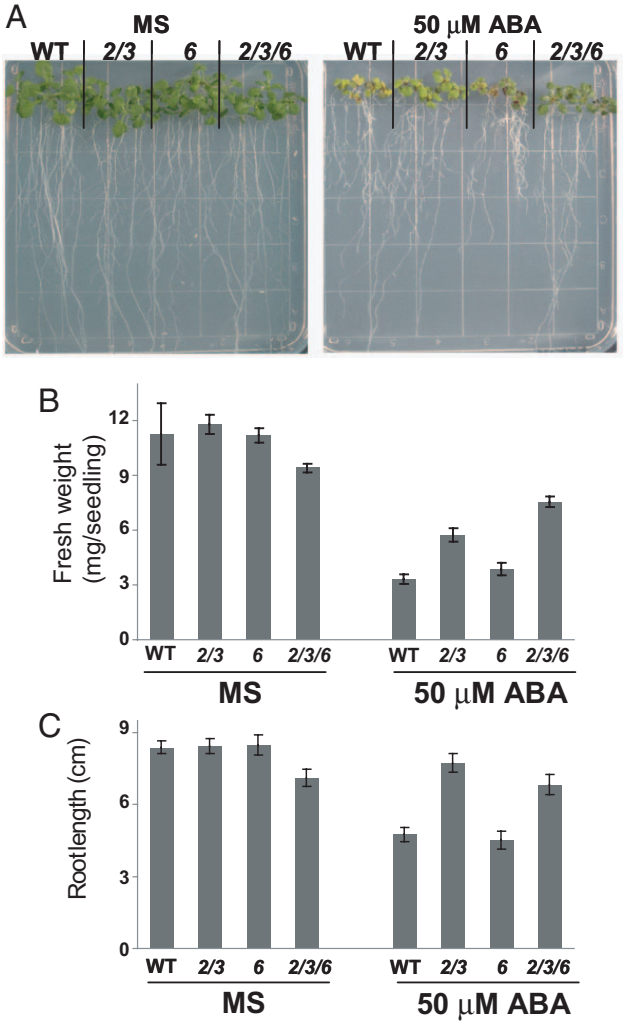

Fig. 4. ABA-mediated inhibition of seedling growth. (*A*) Photographs of the WT, *snrk2.2/2.3* (2/3), *snrk2.6* (6), and *snrk2.2/2.3/2.6* (2/3/6) seedlings taken 10 days after transfer to MS with 3% sucrose or MS with 3% sucrose and 50 μM ABA. Seedlings had almost equal root lengths and were 7-days old at the time of transfer. (*B* and *C*) Seedling fresh weight (*B*) and primary root length (*C*) for seedlings treated as in *A*. For fresh weight determination, 5 seedlings were weighed together and then averaged. Values are means ± SE ($n = 3$ for fresh weight, and $n = 15$ for root length). The WT, *snrk2.2/2.3*, *snrk2.6*, and *snrk2.2/2.3/2.6* are indicated by WT, 2/3, 6, and 2/3/6, respectively.

ABA-insensitive mutants that have been reported, cannot develop green cotyledons on 10 μM ABA. Our results suggest that SnRK2.2/2.3 and 2.6 have redundant roles in ABA signaling during seed germination and early seedling growth.

Shoot and Root Growth of the *snrk2.2/2.3/2.6* Triple Mutant Is Insensitive to ABA. In addition to inhibiting seed germination, high concentrations of ABA suppress seedling growth. ABA inhibition of seedling growth was investigated by transferring 7-day-old seedlings of mutants to the MS medium supplemented with 50 μM ABA. Fresh weight and root length were measured 10 days after transfer as indicators of shoot growth and root growth, respectively. As expected, the *snrk2.2/2.3/2.6* triple mutant was more resistant than *snrk2.2/2.3* to ABA-mediated inhibition of shoot growth; the fresh weight of *snrk2.2/2.3/2.6* was the highest among the different genotypes on 50 μM ABA, although it was lower than the rest in the absence of ABA (Fig. 4 *A* and *B*). However, in the presence of

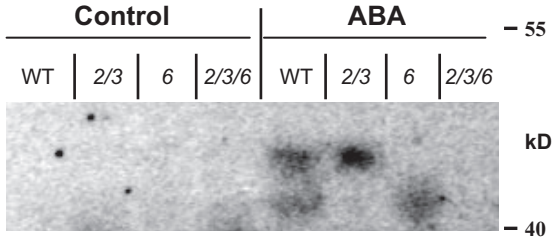

Fig. 5. ABA-activated protein kinase activities in the different genotypes. In-gel kinase assay with proteins extracted from the WT, *snrk2.2/2.3* (2/3), *snrk2.6* (6), and *snrk2.2/2.3/2.6* (2/3/6) seedlings grown under control conditions or 30 min after treatment with 100 μM ABA. GST-fused ABF2 fragment (amino acids Gly-73 to Gln-119) was used as the phosphorylation substrate. The positions of molecular mass markers are shown on the right.

50 μM ABA, the *snrk2.2/2.3/2.6* triple mutant roots were slightly shorter than those of *snrk2.2/2.3*, albeit still longer than those of the WT and *snrk2.6* (Fig. 4 *A* and *C*). These results indicate that SnRK2.2/2.3 and SnRK2.6 function redundantly in ABA-mediated inhibition of shoot growth. Also, the shorter roots and lower fresh weight of the *snrk2.2/2.3/2.6* seedlings in the absence of ABA suggest that ABA signaling is required for optimal growth of Arabidopsis.

ABA-Activated Protein Kinase Activities Are Eliminated in the *snrk2.2/2.3/2.6* Triple Mutant. The in vivo activation status of SnRK2s can be monitored by an in-gel kinase assay using *Arabidopsis* crude extracts (29, 35). In-gel kinase assays using an ABF2 fragment (Gly-73 to Gln-119) as substrate showed that ABA activated protein kinases migrate at ≈42 and ≈44 kDa. The lower band was missing in the *snrk2.2/2.3* double mutant (28). In-gel kinase assays using histone as a substrate (36) suggest that the upper band corresponds to the activity of SnRK2.6. We analyzed the ABA-activated kinase activities in the *snrk2.2/2.3/2.6* triple mutant. Protein extracts were prepared from WT, *snrk2.2/2.3*, *snrk2.6*, and *snrk2.2/2.3/2.6* seedlings grown under control conditions or after exposure to 100 μM ABA for 30 min. Consistent with previous reports, the lower and upper ABA-induced bands disappeared in *snrk2.2/2.3* and *snrk2.6*, respectively. In the *snrk2.2/2.3/2.6* triple mutant, neither the lower nor the upper band was detected (Fig. 5). Together with previous studies (27, 28, 35, 36), these data demonstrate that the ABA-activated protein kinase activities are derived solely from SnRK2.2/2.3 and SnRK2.6, and suggest that ABA signaling through the SnRK2 kinases is completely blocked in the triple mutant.

ABA-Induction of Gene Expression Is Blocked in the *snrk2.2/2.3/2.6* Triple Mutant. To examine the effect of the *snrk2.2/2.3/2.6* triple mutations on expression of ABA-induced genes, we performed Northern blot analysis on the ABA-inducible genes *RD29A*, *COR15A*, *RAB18*, *RD22*, and *NCED3*. RNA was extracted from plants before and 3 h after they were sprayed with 100 μM ABA. In the *snrk2.2/2.3/2.6* triple mutant, the induction of these genes by ABA was eliminated, whereas the induction was reduced, but not eliminated, in the *snrk2.2/2.3* double mutant (Fig. 6). ABA-induction of these genes was little affected in the *snrk2.6* mutant. These results suggest that SnRK2.2, SnRK2.3, and SnRK2.6 function redundantly and protein phosphorylation mediated by these kinases is essential for ABA-induction of gene expression.

Discussion

Our data show that *snrk2.2/2.3/2.6* is a recessive mutant that is extremely insensitive to ABA. To our knowledge, no mutant with such a strong insensitivity to ABA has been reported

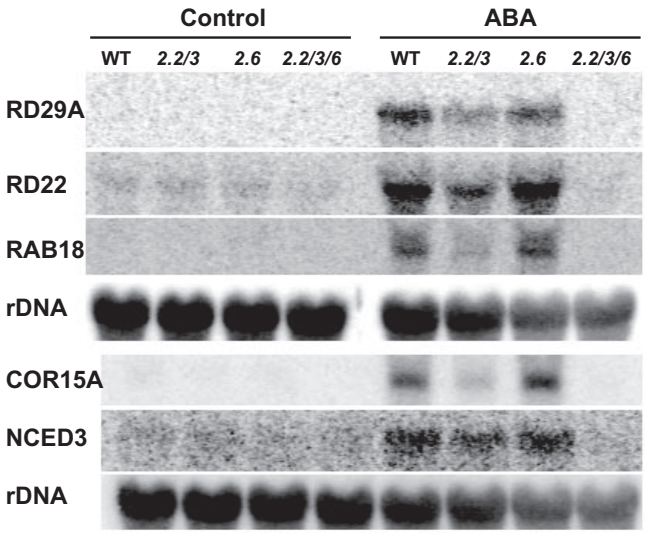

Fig. 6. Expression of ABA-regulated genes in the *snrk2.2/2.3/2.6* triple mutant. Northern blot analysis with RNA extracted from the WT, *snrk2.2/2.3* (2/3), *snrk2.6* (6), and *snrk2.2/2.3/2.6* (2/3/6) seedlings under control conditions or 3 h after treatment with 100 μM ABA. The RNA blots were sequentially probed with the indicated probes.

previously. Our results suggest that the SnRK2s-mediated protein phosphorylation is required for all aspects of ABA function including seed germination, seedling development, and stomatal regulation. Our data show that SnRK2.2, SnRK2.3, and SnRK2.6 function redundantly in these processes. Leaves of the *snrk2.2/2.3/2.6* triple mutant were shrunken within 30 min after detachment (Fig. 2 *A* and *B*). Stems of the *snrk2.2/2.3/2.6* triple mutant also withered quickly under low humidity (Fig. 2*C*). Although water was lost more rapidly from the *snrk2.6* mutant than from the WT (29, 34), water was lost much more rapidly from the triple mutant. The *snrk2.6* mutation has been shown to impair stomatal regulation (29, 34). Our results suggest that stomatal responses to ABA in the triple mutant are completely disrupted. Because water loss was little affected by the *snrk2.2/2.3* double mutations (28), a role of SnRK2.2 and SnRK2.3 in stomatal regulation has been unclear. Our results suggest that SnRK2.2 and SnRK2.3 have important contributions to stomatal regulation.

In the *snrk2.2/2.3/2.6* triple mutant, seed germination and early seedling development and growth were insensitive to ABA (Fig. 3). Although the *snrk2.2/2.3* double mutant was insensitive to ABA (28), the *snrk2.2/2.3/2.6* triple mutant was much more insensitive (Fig. 3). Because the sensitivity of seed germination to ABA was similar between *snrk2.6* mutant and the WT (29), the role of SnRK2.6 in seed germination has been unclear. Thus, our results with the *snrk2.2/2.3/2.6* triple mutant clarify that SnRK2.6 shares an important role with SnRK2.2/2.3 in seed germination, early seedling development, and seedling growth.

In ABA signaling, SnRK2.2, SnRK2.3, and SnRK2.6 are expected to relay directly or indirectly information from ABA receptors to transcription factors controlling ABA-responsive genes. ABA-induced phosphorylation on the ABF2 fragment in the in-gel kinase assay was eliminated in the *snrk2.2/2.3/2.6* triple mutant (Fig. 5). Phosphorylation of ABFs triggers the expression of genes that have the ABRE element in their promoters (21–24). That is consistent with our finding that expression of the ABA-induced genes examined was eliminated in the kinase triple mutant (Fig. 6), because the genes examined, except *RD22*, have ABRE in their promoters. Impairment in *RD22* expression, which is controlled by Myc and Myb transcription factors (40), in the *snrk2.2/2.3/2.6* triple mutant suggests that the SnRK2s-mediated protein phosphorylation is required for the expression and/or activity of the Myb and/or Myc transcription factors.

In-gel kinase assays using extract from protoplasts or T87 cells overexpressing SnRK2.7 or 2.8 revealed that SnRK2.7 and SnRK2.8 can also be activated by ABA (35, 36). This result suggests that SnRK2.7 and SnRK2.8 may work redundantly with SnRK2.2/2.3/2.6 in ABA pathways. However, in an in-gel kinase assay using crude extract from seedlings kinase activity $\approx$39 kDa, which is the expected mobility of SnRK2.7 and SnRK2.8, was not induced by ABA (28, 29). These results along with the near complete failure of the *snrk2.2/2.3/2.6* triple mutant to respond to ABA indicate that the roles of SnRK2.7 and SnRK2.8 in ABA signaling, if any, are marginal.

The virtual elimination of ABA responses in the *snrk2.2/2.3/2.6* triple mutant strongly suggests that ABA signaling pathways converge at the 3 protein kinases, and that protein phosphorylation mediated by SnRK2.2, SnRK2.3, and SnRK2.6 is absolutely essential for ABA signaling. The *snrk2.2/2.3/2.6* triple mutant can serve as a valuable genetic tool for elucidating the roles of ABA and ABA signaling in plant growth, development, and environmental responses. The growth and seed production defects of the triple mutant even under near 100% humidity conditions suggest that ABA has important roles in plant growth and reproduction that have not been fully appreciated previously. Further characterization of this triple mutant may unravel other new roles of ABA and ABA signaling. Future studies using the *snrk2.2/2.3/2.6* triple mutant will also be important for understanding how ABA perception is connected to the various downstream effects of ABA.

Materials and Methods

T-DNA Insertion Lines. The *snrk2.2/2.3* double mutant was described (28). The seeds of a *snrk2.6* T-DNA insertion line (Salk_008068) were obtained from the Arabidopsis Biological Resource Center (41). Homozygous plants were obtained by PCR screening by using the primers: LP, 5′-CATATCTTTAGACGAGGGGCC-3′; and RP, 5′-GTGAGTGGTCCAATGGATTTG-3′. For seedling growth assay, in-gel kinase assay, and Northern blot analysis, we plated *snrk2.2/3/6+* seeds on MS medium containing 10 μM ABA, selected seedlings with green cotyledons as the *snrk2.2/2.3/2.6* triple mutant on the third day, and then transferred these seedlings to MS medium for further growth without ABA.

Physiological Assays. Plants in soil and seedlings in agar plates were routinely grown 16-h light ($\approx$75 μmol m^{-2} sec^{-1}) and 8-h dark cycle at 23 °C. For germination assays, seeds were plated on MS medium containing no sucrose (for scoring radicle emergence) or 3% sucrose (for scoring the production of green cotyledons) and ABA as indicated. In each experiment, at least 20 seeds per genotype were stratified at 4 °C for 3 days, and radicle emergence or the presence of green cotyledons was scored after the indicated time interval.

Measurement of Leaf Water Loss. Leaves of similar size were detached from 4-week-old plants. Because leaves of the triple mutant were small, 3 leaves were weighed at one time for all genotypes.

In-Gel Kinase Assay. The in-gel kinase assay was performed as described (28).

Northern Blot Analysis. Total RNA (20 μg/lane) was size-fractionated by electrophoresis, and blotted onto a nitrocellulose membrane; ^{32}P-labeled probes were generated with the Random Primer Labeling Kit (GE Healthcare). Radioactivity was detected by using a Typhoon 9410 imager (Molecular Dynamics). Gene-specific fragments of $\approx$500 bp for *RD29A*, *RAB18*, *RD22*, and *NCED3* were amplified by PCR with primers as described (28). Primers used to amplify *COR15A* partial cDNA were 5′-AAACATGAAGAGAGAGGATATGGA-3′ and 5′-TGGTGAGAAAGCGAAAGACT-3′; rDNA was produced as described (42).

Note Added in Proof: Park et al. (43) reported recently that the activities of the ABA-activated SnRK2 kinases are substantially reduced in the Arabidopsis ABA receptor quadruple mutant *pyr1pyl1pyl2pyl4*.

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