Plant Raf-like kinase integrates abscisic acid and hyperosmotic stress signaling upstream of SNF1-related protein kinase2

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Abstract

Plant responses to drought and hyperosmosis is mediated by the phytohormone abscisic acid (ABA), a sesquiterpene compound widely distributed in various embryophyte groups. Exogenous ABA as well as hyperosmosis activates the sucrose nonfermenting 1 (SNF1)-related protein kinase2 (SnRK2), which plays a central role in cellular responses against drought and dehydration, although the details of the activation mechanism are not understood. Analysis of a mutant of the moss Physcomitrella patens with reduced ABA sensitivity and reduced hyperosmosis tolerance revealed that a protein kinase designated "ARK" (for "ABA and abiotic stress-responsive Raf-like kinase") plays an essential role in the activation of SnRK2. ARK encoded by a single gene in P. patens belongs to the family of group B3 Raf-like MAP kinase kinase genes (B3-MAPKKks) mediating ethylene, disease resistance, and salt and sugar responses in angiosperms. Our findings indicate that ARK, as a novel regulatory component integrating ABA and hyperosmosis signals, represents the ancestral B3-MAPKKks, which multiplied, diversified, and came to have specific functions in angiosperms.

Significance

Plants can sense loss of water caused by drought and stimulate internal mechanisms for protecting cells from damage with the aid of the stress hormone abscisic acid (ABA). Analysis of a mutant of the basal land plant, the moss Physcomitrella patens, revealed that an impairment of a protooncogene Raf-like protein kinase, designated “ARK” (for “ABA and abiotic stress-responsive Raf-like kinase”), causes a loss of both ABA sensitivity and osmotic stress tolerance. We show evidence that ARK has a role in integrating ABA and osmonic signals upstream of the sucrose nonfermenting 1-related protein kinase2, known to be a central regulator of stress signaling in plants.


The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511238112/-/DCSupplemental.

This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KT581394), and the microarray data have been deposited in the Gene Expression Omnibus database (accession no. GSE68914).

Published online November 4, 2015.

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SnRK2s is suppressed by group A protein phosphatase 2C (PP2Cs) in the absence of ABA, and binding of ABA to the PYR/PYL/RCAR inhibits PP2Cs, thus allowing activation of SnRK2 (14-16). Phosphorylation in the activation loop of SnRK2 is thought to be completed by autophosphorylation (11), although the details of the mechanism remain to be clarified.

Entire genome sequencing of *P. patens* (17) has revealed that four PYR/PYL/RCARs, two group A PP2Cs, and four SnRK2s are encoded in the moss genome, indicating that the core members needed for the ABA response are conserved from bryophytes to angiosperms (18). All SnRK2s encoded in the *P. patens* genome were assigned to the subclass III category, suggesting that this subclass represents the ancestral members of SnRK2. The *P. patens* *PpOST1*-1 gene, encoding SnRK2, was shown to complement the ABA-insensitive stomatal response of *ost1* in transgenic *Arabidopsis*, and disruption of *PpOST1*-1 in *P. patens* resulted in an ABA-insensitive stomatal response in the sporophyte (19). That study suggested possible conservation of SnRK2 function in land plants. A recent study also indicated a role of group A PP2Cs as negative regulators of ABA signaling in bryophytes. A transgenic line lacking both group A PP2C genes (*PpAB1A* and *PpAB1B*) exhibited constitutive desiccation tolerance with the accumulation of a number of stress-associated transcripts regardless of ABA treatment (20). In this knockout line, however, SnRK2 was activated by exogenous ABA in a manner similar to that in the WT line (20), indicating group A PP2Cs have a minor role in the regulation of SnRK2 and the presence of a previously unidentified mechanism for its activation.

To understand better the molecular mechanisms underlying the ABA response in bryophytes, we mutagenized protonema cells of *P. patens* by UV irradiation and obtained mutant lines that could grow better than the WT line on medium containing the ABA response in bryophytes, we mutagenized protonema cells of *P. patens* by UV irradiation and obtained mutant lines that could grow better than the WT line on medium containing ABA. One of the mutants, designated “AR7,” showed an ABA-insensitive growth response and reduced freezing-stress tolerance (21). AR7 also showed reduced responses to dehydration and cold (22), indicating that the molecule integrating ABA and stress-signaling processes is impaired in AR7. This report characterizes AR7 further and identifies the gene altered in AR7. Comparative genomic sequence analysis between AR7 and the WT line revealed that AR7 has a mutation in the gene encoding a group B3 Raf-like MAP kinase kinase kinase (B3-MAPKKK), subsequently designated “ARK” (for “ABA and abiotic stress responsive Raf-like kinase”). Further analyses indicated that ARK plays a key role in SnRK2-mediated ABA and hypersalinity responses in the moss. On the basis of these collective findings, we propose that, in addition to PYR/PYL/RCAR and PP2C, ARK is an essential element of signaling components for the regulation of SnRK2 in basal land plants.

Results

Analysis of Transcriptome and Protein Kinase Activity in AR7. The AR7 line has been characterized as a mutant with reduced ABA sensitivity in various aspects such as growth inhibition, morphological changes leading to brood cell formation, accumulation of soluble sugars and LEA-like boiling-soluble proteins, and tolerance of freezing and dehydration stress (21, 22). Microarray analysis of transcript profiles revealed that the expression of a large portion of the ABA-responsive genes was affected in AR7. Expression of 518 of 579 ABA up-regulated genes in the WT line was reduced significantly in AR7 (Fig. 1A, Left). These genes included 27 LEA-like genes encoding highly hydrophilic polypeptides, which are considered to be associated with dehydration stress tolerance (Table S1). RNA gel-blot analysis of four representative LEA-like transcripts, 17B9 (*Pp1s294_52V6.1*), 6A5 (*Pp1s267_21V6.1*), LEAII (Pp1s118_232V6.1), and 19C6 (*Pp1s52_261V6.1*), confirmed impaired ABA-induced gene expression in AR7 (Fig. 1B). Furthermore, levels of 150 of 165 ABA down-regulated transcripts were higher in AR7 than in the WT line (Fig. 1A, Right). These results indicated that the gene impaired in AR7 encodes an important positive regulator of ABA-responsive gene expression in *P. patens*.

In angiosperms, most of the physiological processes required for the ABA response, including gene expression for various stress-associated proteins, are controlled by subclass III SnRK2s (10). ABA-induced activation of the protein kinase corresponding to the expected size of SnRK2 (39 kDa) has been demonstrated recently in *P. patens* protonema by in-gel kinase assays using histone IIIA as a substrate (20). We found that protonema of *P. patens* treated with 10 μM ABA for the indicated times (C) or were subjected to various treatments (D). ABA, 10 μM ABA for 30 min; Cold, 0 °C for 1 h; Desic, desiccation in a laminar flow for 30 min; Man, 0.5 M mannitol for 30 min.

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Fig. 1. ABA-induced gene expression and SnRK2 activity in the WT line and AR7. (A) Results of comparative microarray analysis showing ABA up-regulated and down-regulated transcripts. (B) RNA gel-blot analysis of WT and AR7 protonema. Total RNA isolated from protonema treated with 10 μM ABA for the indicated times was probed with radiolabeled DNA fragments of the representative ABA-inducible transcripts, 17B9 (*Pp1s294_52V6.1*), 6A5 (*Pp1s267_21V6.1*), LEAII (*Pp1s118_232V6.1*), and 19C6 (*Pp1s52_261V6.1*) (43). (C) and (D) In-gel kinase assays of WT and AR7 protonema for detection of SnRK2 activity. *P. patens* protonema were treated with 10 μM ABA for the indicated times (C) or were subjected to various treatments (D). ABA, 10 μM ABA for 30 min; Cold, 0 °C for 1 h; Desic, desiccation in a laminar flow for 30 min; Man, 0.5 M mannitol for 30 min.
Fig. 2. Characterization of Pp1s462-10V6.1 of P. patens. (A) Structures of the Pp1s462-10V6.1 gene mutated in AR7 and the encoded protein ARK deduced from the sequence of the isolated cDNA (GenBank KT581394). The C-to-T transition in the fifth exon caused an amino acid change from Ser to Phe at position S32 N-terminal to the protein kinase domain (KD). PD indicates a putative PAS domain. (B) Growth of protonemata with or without ABA of WT, AR7, the AR7 knock-in line with the ARK sequence changed to that of WT (AR7/ARKWT), and the WT knock-in line with the ARK sequence changed to that of AR7 type (WT/ARKS532F). (C) Transient assays for detection of ABA-induced gene expression. The protonemata of the lines used in B were bombarded with the Em-
GUS and Ubi-LUC constructs and incubated with or without 10 μM ABA for 1 d, followed by the GUS and LUC assays. Levels of gene expression are represented by GUS/LUC ratio. Error bars indicate the SE (n = 3). ***P < 0.01 compared with ABA-treated WT (t-test). (D) ABA-induced freezing tolerance of the lines used in B. Protonemata were treated for 1 d with 10 μM ABA and either were kept at 4 °C (UF) or were subjected to equilibrium freezing to −3 °C or −4 °C. Survival of the protonemata was estimated by measuring electrolyte leakage from the injured cells. Error bars indicate the SE (n = 3). ***P < 0.001 and **P < 0.01 compared with the ABA-negative (−) tissue of the same line (t-test). (E) Osmotic stress tolerance of the protonema. Pieces of protonemata were grown on BCDAT medium containing 0.4 M mannitol. (F) Cold-acclimation capacities of the protonema. The cells were incubated on BCDAT medium at 0 °C for the indicated number of days and then were subjected to freezing at −3 °C to determine electrolyte leakage. Error bars indicate the SE (n = 3). *P < 0.05 and **P < 0.01 compared with the nonacclimated tissue of the same line (t-test). (G) Accumulation of stress-associated transcripts. RNA extracted from protonemata after treatment with ABA, cold, or hyperosmotic 0.5 M mannitol was electrophoresed, blotted onto a nylon membrane, and reacted with radiolabeled probes of the indicated transcripts. Ethidium bromide-stained rRNA is shown for verification of equal loading.
AR7 showed little activity of the ABA-induced kinase compared with WT protonemata (Fig. 1C). We further compared the kinase activity in the WT line and AR7 in response to other abiotic stresses and found that induction of the kinase activity by cold, desiccation, and hyperosmosis generated by 0.5 M mannitol treatment was also defective in AR7 (Fig. 1D). From these results, we speculated that the gene mutated in AR7 is involved in ABA-induced activation of SnRK2, which controls levels of the majority of transcripts during the response to exogenous ABA.

Identification of the Gene Mutated in AR7. To identify the mutation locus in the genome of AR7, comparative genomic sequence analysis of AR7 and the WT line was carried out using the Illumina HiSeq 2000 sequencer. More than 286 million and 101 million reads of 100-bp nucleotide fragments of the WT line and AR7, covering roughly 58 and 21 times the genome size, respectively, were analyzed against the reference genome sequence of P. patens subsp. patens V1.6. The analysis revealed a total of 2,068 mutations in the AR7 genome, causing 47 nonsynonymous substitutions of amino acids in the encoded proteins (Dataset S1). To examine whether mutations of any of these 47 candidate genes caused ABA-insensitive phenotypes in AR7, the cDNA clones for these genes were isolated from the WT line and transiently expressed in the AR7 protonemata by particle bombardment. The candidate cDNA was co-bombarded with the ABA-induced Em reporter fused to the β-glucuronidase reporter gene (Em-GUS) (23, 24) to determine whether any of the clones restore ABA-induced GUS expression in AR7, which otherwise shows very little GUS expression. Introduction of one cDNA clone corresponding to the gene model Pp1s462-10V6.1 was found to restore the ABA insensitivity of AR7. The Pp1s462-10V6.1 gene consisted of 17 exons, and analysis of the longest cDNA revealed that it encodes a 1,148-amino acid protein having a C-terminal protein kinase domain similar to plant Raf-like MAPKKks belonging to B3-MAPKKks (Fig. 2A). The stretch of amino acids from positions 86–185 in the N-terminal nonkinase domain contained a putative PAS domain possibly involved in molecular interactions (25), which typically is conserved in group B2 MAPKKks (B2-MAPKKks) categorized in angiosperms (26). The Pp1s462-10V6.1 gene was designated as “ARK.” Sequence analysis revealed that Ser-532 (TCC) located in a position N-terminal to the kinase domain was changed to Phe-532 (TTC) by a single C-to-T transition in the fifth exon of ARK in AR7 (Fig. 2A).

To determine why the Ser-to-Phe mutation at position 532 found in AR7 caused ABA insensitivity, we analyzed the accumulation of ARK proteins in the WT line and AR7 by immunoblot analysis using an antibody raised against the C-terminal amino acids of ARK. We detected immunoreactive bands of ~120 kDa consistent with the length of 1,148 amino acids of ARK. However, the accumulation of ARK proteins appeared to be similar in the WT line and AR7 and did not change significantly during ABA treatment (Fig. S1). To confirm that the Ser-to-Phe mutation at position 532 caused the ABA-insensitive phenotype, a gene knock-in experiment was conducted. A genomic fragment of ARK from the WT line was fused with the neomycin phosphotransferase gene cassette so the marker would be inserted into a position downstream of the polyadenylation site. The resultant construct was introduced into AR7 by polyethylene glycol-mediated protoplast transformation to obtain transgenic plants resistant to the antibiotic G418. After confirmation of gene targeting to the ARK locus (Fig. S2), the generated knock-in line (ARK/ARKWT) was used for various physiological analyses of ABA responses. Exogenous ABA inhibited protonemal growth of AR7/ARKWT and facilitated subsequent formation of brood cells similar to that in the WT line (Fig. 2B). Transient expression analysis showed ABA-induced Em-GUS expression in the AR7/ARKWT protonemata similar to that in the WT line (Fig. 2C). Furthermore, impaired ABA-induced freezing tolerance of AR7 was recovered in AR7/ARKWT to a level similar to that in the WT line (Fig. 2D) concomitant with accumulation of LEA-like boiling-soluble proteins (Fig. S3).

Hyperosmotic- and cold-stress responses also were examined in the AR7/ARKWT line. Although growth of AR7 protonemata was susceptible to 0.4 M mannitol contained in the medium, AR7/ARKWT protonemata grew on the medium in a manner similar to that of the WT line (Fig. 2E). Furthermore, AR7 showed reduced cold acclimation capacity (i.e., cold-induced freezing tolerance) compared with that of the WT line (22), but AR7/ARKWT acclimatized in a manner similar to that of the WT.

To confirm the phenotypic reversion by the change in a single amino acid of ARK, a similar gene knock-in experiment was carried out to introduce an AR7-type mutation (Ser-532 to Phe) into the ARK locus of the WT line. The resultant WT/ARKS532F line was found to mimic AR7 phenotypes in protonemata growth. In addition, that line was insensitive to ABA, cold, and hyperosmotic treatment as observed in AR7 (Fig. 2A–F). When expression profiles of representative transcripts of WT, AR7, AR7/ARKWT, and WT/ARKS532F were compared in terms of responsiveness to ABA, hyperosmosis, and cold, the results were consistent with the growth and stress-tolerance phenotypes in these lines (Fig. 2G). These results indicated that ARK plays a crucial role in cellular responses to ABA and abiotic stresses in P. patens and that the single Ser-to-Phe change at the amino acid position 532 of ARK was sufficient to alter the responses.

ARK Kinase Activity Toward SnRK2. To examine changes in ABA-induced protein kinase activity in the ARK knock-in lines, in-gel kinase assays were carried out using histone IIIS as a substrate. Protonemata of AR7/ARKWT had ABA-induced kinase activity similar to that of the WT line, whereas protonemata of the WT/ARKS532F line showed very little kinase activity, comparable to that of AR7 (Fig. 3A). From these results, we speculated that ARK is either directly or indirectly involved in the activation of SnRK2s. We found that PpSnRK2B (PpIs240_91V6.1), one of the SnRK2s of P. patens, is activated by ABA, as shown by in-gel kinase assays of transgenic P. patens expressing PpSnRK2B fused to GFP (PpSnRK2B-GFP). PpSnRK2B-GFP was also activated by hyperosmosis (Fig. S4). Cellular localization of ARK-GFP and of PpSnRK2B-GFP was determined by confocal microscopy. ARK-GFP was localized mainly in the cytosol, and PpSnRK2B-GFP was localized in the cytosol and nucleus in the protonema cells, suggesting possible colocalization of PpSnRK2B with ARK in the cytosol (Fig. 3B). Similar experiments using the onion epidermal cells also indicated possible colocalization of PpSnRK2B and ARK in the cytosol (Fig. S5).

To determine the role of ARK in the possible regulation of PpSnRK2B, phosphorylation assays were carried out using recombinant proteins. The protein kinase domain of ARK fused to GST (GST-ARKK12) and PpSnRK2B fused to maltose-binding protein (MBP-PpSnRK2B) were expressed in E. coli, and affinity-purified recombinant proteins were used for in vitro phosphorylation assays. Results of assays indicated that MBP-PpSnRK2B showed some autophosphorylation activity, but GST-ARKK12 phosphorylated MBP-PpSnRK2B more strongly (Fig. 3C). To examine the effect of this phosphorylation of MBP-PpSnRK2B on its kinase activity, in-gel kinase assays of nonphosphorylated, autophosphorylated, and ARK-phosphorylated MBP-PpSnRK2B were carried out using histone IIIS as a substrate. Results of assays indicated that the activity of MBP-PpSnRK2B was increased by phosphorylation catalyzed by GST-ARKK12 but was not affected by autophosphorylation (Fig. 3D). Phosphopeptide mapping revealed that GST-ARKK12 phosphorylated specific serine residues (Ser-165 and Ser-169) of the MBP-PpSnRK2B protein (Fig. 3E and Fig. S6). The phosphorylation site corresponding to Ser-165, which lies between subdomains VII

Saruhashi et al. PNAS Published online November 4, 2015 | E6391

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and VIII in the activation loop, has been identified as the critical Ser residue for the activation of Arabidopsis SnRK2s (27).

To explore possible regulation of ARK, we also examined the phosphorylation status of ARK. Proteins extracted from transgenic *P. patens* expressing ARK-GFP (Fig. 3B) were subjected to purification by magnetic beads with the anti-GFP antibody attached, and the purified proteins were used for phosphopeptide mapping. We identified phosphorylation of a peptide corresponding to amino acids 1029–1043 of ARK, with phosphorylation on either Ser-1029 or Ser-1030 (Fig. 4). These residues were located within the activation loop in the kinase domain, comparable with phosphorylation sites of other eukaryotic MAPKKKs (Fig. 4B). To determine the role of phosphorylation of ARK, mutation constructs were generated by replacing both Ser-1029 and Ser-1030, or either, with Ala, and the constructs were used for transient assays in AR7 cells with *Em-GUS* to determine their capacity to restore ABA responsiveness (Fig. 4C). We found that ARK with Ala substitution of Ser-1029 (S1029A) does not restore ABA-induced *Em-GUS* expression in AR7. When both Ser-1029 and Ser-1030 were replaced with Ala (S1029A/S1030A), the expression level was as low as in the Ser-1029 mutation, whereas replacing only Ser-1030 with Ala (S1029A/S1030A) restored *Em-GUS* expression to a level similar to that in nonmutated ARK. These results indicate that phosphorylation of ARK at Ser-1029 in the activation loop is essential for its activation.

### Functional Complementation of the Ark Mutation by B3-MAPKKKs from Other Land Plant Sources

To determine the diversity of ARK-related genes in land plants, phylogenetic analysis of group B1–B4 Raf-like MAPKKKs from *P. patens, A. thaliana,* and Selaginella moellendorffii (Fig. 5A) was conducted. Three and six members of B3-MAPKKKs are encoded in the genomes of *S. moellendorffii* and *A. thaliana,* respectively, whereas *ARK* is the only B3-MAPKKK in *P. patens.* Furthermore, the *S. moellendorffii* and *A. thaliana* genomes encode two and six B2-MAPKKKs, respectively, but they were not found in the *P. patens* genome. The genes for group B1 MAPKKK (B1-MAPKKK) appeared to be the closest relatives of *ARK* in *A. thaliana* and *S. moellendorffii* (Fig. 5B), and their ion-current chromatograms are shown. The B1-MAPKKKs from *P. patens* and *A. thaliana* form a clade with the B3-MAPKKKs from *S. moellendorffii*.

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### Figure 3

**A** AK as a possible regulator of SnRK2. (Upper) In-gel kinase assays of protonemata of WT, AR7, and ARK knock-in lines for the detection of ABA-activated protein kinases. Proteins were electrophoresed on an SDS-polyacrylamide gel containing histone IIIS. After denaturation and renaturation procedures, the proteins were reacted with γ[^32P]ATP. (Lower) Coomassie Brilliant Blue (CBB) staining of ribulose 1,5-bisphosphate carboxylase/oxygenase. (B) The AK-GFP construct fused to the CaMV35 promoter was introduced into protonema cells of *P. patens.* Localization of GFP and *PpSnRK2B-GFP* is shown for comparison. (C) Kinase activity of GST-ARK([K]) on MBP-PpSnRK2B. Recombinant proteins of MBP-PpSnRK2B (1 μg) (lanes 1 and 3) and GST-ARK([K]) (0.05 μg) (lanes 2 and 3) were reacted with γ[^32P]ATP and electrophoresed on an SDS-polyacrylamide gel, followed by Coomassie Brilliant Blue staining and autoradiography ([^32P]). Closed and open arrowheads indicate the positions of MBP-PpSnRK2B and GST-ARK([K]), respectively. (D) In-gel kinase assays of the recombinant MBP-PpSnRK2B (1 μg) with or without autophosphorylation (Auto-P) or phosphorylation by GST-ARK([K]) (0.05 μg). Positions of molecular-weight markers are shown in kilodaltons. Results of staining with Coomassie Brilliant Blue and immunoblot analysis using the anti-ARK antibody (Anti-ARK) are shown for comparison. Closed and open arrowheads indicate the positions of MBP-PpSnRK2B and GST-ARK([K]), respectively. (E) Phosphorylation sites in the activation loop of MBP-PpSnRK2B identified by phosphopeptide mapping. The amino acid sequence and probable phosphorylation sites (asterisks) of the identified phosphopeptide (m/z = 924.46) and its ion-current chromatograms are shown. The MBP-PpSnRK2B protein was either phosphorylated with GST-ARK([K]) or autophosphorylated (Auto-P) for 15 min. (Details are shown in Fig. S6.)
ABA before protein extraction for the GUS and LUC assays. Levels of gene fused to the rice actin promoter and introduced into the AR7 cells with or without mutations in the predicted phosphorylation sites. The cDNA was (46), MLK3 (47), MEKK1 (48), and cRAF (49) are indicated by bold letters.

MAPKKKs. Predicted phosphorylation sites for ARK (S1029 and S1030), TAK1 (T183 and Y185), and cRAF (S532) are indicated in bold.

We further tested whether B3-MAPKKK genes from *A. thaliana* and *S. moellendorfii* can complement ABA sensitivity of AR7 by transient assays. Fig. 5C shows that *A. thaliana* cDNA clones for At1g73660 and At4g24480 and the *S. moellendorfii* cDNA clone for Sm269874 restored ABA-induced Em-GUS expression in AR7.

Results of experiments using other B3-MAPKKK clones indicated that At1g18160 also restored the GUS expression in AR7, but At5g11850, At5g03730, and At1g08720 had little effect (Fig. S7). These results indicated that specific genes of vascular plants encoding B3-MAPKKK can functionally complement the impairment of ARK in the moss.

**Discussion**

Although subclass III SnRK2 is recognized as a key regulator of ABA signaling, the mode of kinase activity regulation has not been fully elucidated. A generally accepted mechanism of SnRK2 activation is that ABA suppresses PP2C activity via binding to PYR/PYL/RCAR receptors, thus facilitating enhancement of autophosphorylation of SnRK2 (11). In *Arabidopsis*, activity of SnRK2.2, SnRK2.3, and SnRK2.6 was increased in the triple mutant of group A PP2C (*abi1hab1pp2ca*), indicating that inhibition of the PP2C by the ABA-receptor complex is the key mechanism for activation of SnRK2 by autophosphorylation (28).

The present study of ARK, a group B3 Raf-like kinase, provides evidence of positive regulation of ABA signaling in addition to negative regulation by group A PP2Cs in bryophytes. Plant Raf-like kinases have been shown to function in various types of biotic and abiotic signaling, but they are not thought to function in a typical MAP kinase pathway involving MAP kinase kinases and MAP kinases, and there is little biochemical evidence regarding their target substrates (29). This result is consistent with our observation of little ABA-induced kinase activity when myelin basic protein, a substrate commonly used for detection of MAP kinases, was used for in-gel kinase assays. Our study showing that a specific Raf-like kinase functions for activation of SnRK2 provides new insights into a signaling pathway for a possible connection between these kinases through unidentified mechanisms operating in plant ABA response.

Our study also indicated the possible mechanism for hyperosmotic-induced activation of plant SnRK2s mediated by an upstream protein kinase. SnRK2s representing all three subclasses in *Arabidopsis* and rice are activated by hyperosmosis (8, 10), but the mechanism of activation has not been elucidated. Results of a previous report indicated that hyperosmotic-induced SnRK2 activation might involve unidentified upstream protein kinases that phosphorylate SnRK2 (27). The reduced hyperosmosis-induced kinase activity in AR7 indicated that ARK might be involved in the activation of SnRK2 (Fig. 1C) toward osmotic stress tolerance (Fig. 2C). Hyperosmotic response by both ABA-dependent and independent mechanisms in the moss has been demonstrated recently using an ABA-deficient mutant of *P. patens* (30). Given that both mechanisms are mediated by ARK, this kinase is possibly a regulatory target of ABA and hyperosmotic signals upstream of SnRK2.

Phosphopeptide mapping and mutational analysis of transient assays indicated that ARK itself might be activated by phosphorylation in the activation loop (Fig. 4.4 and C). Activation by phosphorylation in the activation loop is well defined in mammalian and yeast MAPKKKs (Fig. 4B). Phosphorylation and activation events in these kinases typically are modulated through their nonkinase domain by dimerization, binding of other regulatory proteins, or phosphorylation by upstream MAPKKK kinases (31). The loss of ARK function with the Ser-to-Phe substitution at position 532 in the nonkinase domain (Fig. 2A–G) suggests that the region around Ser-532 functions as a regulatory domain, and the Ser-to-Phe alteration might have resulted in a drastic change in microenvironment of the region because of the introduction of a bulky hydrophobic residue, leading to the abolishment of the kinase activity by an unidentified mechanism. The role of the N-terminal nonkinase domain in the regulation of kinase activity is well defined in Ste11p MAPKK in budding yeast (32), wherein an intramolecular autoinhibitor sequence in the N-terminal domain interacts with the C-terminal kinase domain to inhibit its catalytic activity. The inhibition is enhanced by the binding of Ste50p to the N-terminal domain and is ameliorated...
upon signal perception by phosphorylation at the specific Ser and Thr residues by an upstream kinase Ste20p. The role of the N-terminal domain in stimulating ARK phosphorylation and how ABA facilitates this process have not been clarified.

ARK is the sole B3-MAPKKK in *P. patens*, but angiosperms have multiple B3-MAPKKKs with various functions. *CONSTITUTIVE TRIPLE RESPONSE1* (CTR1, At5g03730) is a known negative regulator of ethylene signaling (33). *ENHANCED DISEASE RESISTANCE1* (EDR1, At1g08720) and At1g73660 have been demonstrated to be involved in salicylic acid-induced defense responses (34) and in salt tolerance (35), respectively. Evidence of signal crosstalk between these apparently unrelated stimuli has been provided by analysis of *Arabidopsis* mutants. For instance, the *ctr1* mutant allele has been isolated as a mutant that enhances ABA insensitivity of *abi1-1* (36). The *ctr1* allele also has been identified as a mutant defective in sugar response (37, 38). From studies using various other mutants, signal crosstalk among ABA, sugar, and ethylene in *Arabidopsis* has been proposed (39), although details of the mechanism have not been clarified. *At1g73660*, identified as the *at6* locus that improves salt-stress tolerance (35), also has been identified as the gene responsible for *sugar insensitive*8 (sis8) showing sugar-resistant seedling growth (40, 41). The loss-of-function mutant of *At1g73660*, however, exhibited a WT response to ABA, the gibberellic acid biosynthesis inhibitor paclobutrazol, and the ethylene precursor aminocyclopropane carboxylic acid in germination experiments (35, 40), indicating that *At1g73660* has a distinct function specific to salt and sugar. We hypothesize that the prototype B3-MAPKKK (ARK), mainly engaged in ABA and hyperosmotic responses in ancestral land plants, had diversified in vascular plants by acquiring more specialized functions toward sugar, salt, ethylene, and pathogenic signals. It should be noted that restoration of ABA-induced gene expression in AR7 by some of *Arabidopsis* B3-MAPKKKs (Fig. 5C) does not necessarily reflect physiological events during the ABA response in angiosperms. Whether any of the B3-MAPKKKs in angiosperms are involved in the positive regulation of SnRK2 would be clarified by detailed analysis of the mutants of B3-MAPKKKs with respect to ABA response and SnRK2 activation.

**Experimental Procedures**

**Plant Materials, Growth Conditions, and Acclimation Treatment.** Protonema tissues of *P. patens* (Hedw.) Bruch & Schimp. were grown on cellophane-overlaid 0.8% agar plates of BCDAT medium supplemented with 0.5% glucose (42). The protonemata were cultured in a controlled environment growth chamber at 25 °C under continuous illumination (35 μmol photons m⁻² s⁻¹). ABA, cold, and hyperosmotic treatments of the protonemata were carried out as described previously (22, 30).

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**Fig. 5.** Phylogenetic and functional analyses of group B3 Raf-like MAPKKKs in land plants. (A) Phylogenetic analysis of plant Raf-like MAPKKKs belonging to groups B1–B4 in *P. patens*, *S. moellendorffii*, and *A. thaliana*. (B and C) Transient assays of *P. patens* protonemata using various MAPKKK DNA clones. The cDNA clones were fused to the rice actin promoter and were cotransintegrated with Em-GUS and Ubi-LUC into protonemata. The protonemata were incubated with or without ABA for 1 d and were used for GUS and LUC assays. Error bars indicate the SE (n = 3). ***P < 0.001 and **P < 0.01 compared with the vector control (t-test). (B) Results for ARK and two group B1 MAPKKKs, *Pp1s63_65V6.1* and *Pp1s75_1V6.1*. (C) Results for ARK, two Arabidopsis genes, *At1g73660* and *At4g24480*, and one Selaginella gene, *Sm269874*.
Tests for Freezing Tolerance and Hypersalinity-Stress Tolerance. The freezing tolerance of *P. patens* protonemata was determined as described previously (30). In brief, protonema tissues placed in a glass test tube were seeded with ice at –1 °C, kept at –1 °C for 60 min, and then cooled at a rate of –2 °C h−1 to desired temperatures. Electrolyte leakage from the damaged tissues was measured after thawing by using a conductivity meter and was represented as the percentage of total ions released after subsequent boiling (1). For osmotic stress treatment, the tissues were placed on a medium containing 0.4 M mannitol and were grown for 7 d under continuous light.

Analysis Using GFP-Fusion Constructs. GFP-fusion constructs of *AR* and *PpSnrk2* were generated by fusing the cDNA to CaMV35S::gGFP (65) using an in vitro solution at starter T7 promoter. Fifty-day-old *P. patens* protonemata were bombarded with the GFP-fusion constructs, and cells were observed under a confocal laser-scanning microscope.

Preparation of Recombinant Proteins. For preparation of MBP-PpSnrk, the entire coding sequence of *PpSnrk* cDNA (*Pp1s240_91V6.1*) was amplified from reverse transscripts of *P. patens* protonemata and fused in-frame to the Ndel-digested pMAL-CX vector (New England Biolabs). The MBP-fusion protein was purified using amylase resin according to the manufacturer’s instructions. For GST-ARK(65), the region of the protein kinase domain (amino acids 867–1148) was fused in-frame to the BamHI/Ndel-digested pGEX-5 x 3 vector (GE Healthcare). The GST-fusion protein was purified using glutathione-Sepharose resin. Both recombinant proteins were subjected to ultrafiltration using a Nanosep 30 kDa size-exclusion column ( Pall) for concentration and removal of low-molecular-weight materials.

Immunoblot Analysis. Proteins electrophoresed on 10% (w/v) SDS-polyacrylamide gels were transferred onto PVDF membranes, reacted with primary and secondary antibodies, and detected by either nitroblue tetrazolium/s-bromo-4-chloro-3-indolyl phosphate reagent or chemiluminescent reagents. For preparation of ARK antibodies, antisera were raised against the C-terminal 15 amino acids (LGGTPKSGLSDRDL) of ARK was subjected to affinity purification using the recombinant GST-ARK(65) protein fixed on a PVDF membrane.

Protein Kinase Assays. In vitro phosphorylation reactions were carried out using affinity-purified recombinant proteins at 30 °C for 15 min in 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 50 μM γ-[32P]ATP. The reaction mixture was electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue, and phosphoproteins were detected by autoradiography. In-gel kinase assays were carried out essentially as described previously (20). In brief, 50 μg of total soluble proteins extracted from AB-treated and nontreated protonemata were electrophoresed using SDS-polyacrylamide gel containing histone H11 (HS055; Sigma). After denaturation and renaturation procedures, the gel was incubated with 50 μM γ-[32P]ATP. The gel was washed, dried, and exposed to X-ray film to detect kinase activity. For recombinant proteins, the kinase reaction was carried out at 30 °C for 15 min in 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, and 1 mM DTT with 50 μM nonradioactive ATP, and the reaction mixture was loaded onto the histone H11s-polyacrylamide gel for in-gel kinase assays.

Phosphopeptide Mapping. Protonemata of *P. patens* expressing ARK-GFP were used for protein extraction. The ARK-GFP proteins were purified by using anti-GFP magnetic microbeads (Milltenyi Biotech) according to the manufacturer’s instructions. Determination of phosphorylation sites of proteins was carried out essentially as described previously (12). Phosphorylated proteins digested with Lys-C and trypsin were analyzed by a nanoLC-MS-MS system for peptide identification, quantification, and prediction of phosphorylation sites. For enrichment of phosphopeptides, the digested peptides were subjected to hydroxyl acid-associated metal oxide affinity chromatography.

Acknowledgments. We thank Ralph Quarato for *Em-GUS*, Tuan-hua David Ho for *Ubi-LUC*, Yasuo Niwa for 35S::gGFP (65), Mitsuyasu Hasebe for *PtNB2*, and the NODAI Genome Research Center of Tokyo University of Agri- culture, for expression of the cell-wall enzyme pectin methylesterase. This study was supported in part by Grants-in-Aid for Scientific Research 26291004, 23119504, and 25119705 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to T.U.), 24570058 and 16H02353 (to Y. Sakata), and 23684038 and 15H04363 (to T.U.), by Grant from Basic Science Research Projects 110683 from The Sumitomo Foundation (to Y. Sakata); and by Precuriosity Research for Embryonic Science and Technology (PRESTO) from Japan Science and Technology Agency (T.U.).


