Short title: Raf-like kinase as a primary activator of SnRK2

Corresponding author:

Daisuke Takezawa

Graduate School of Science and Engineering, Saitama University, Shimo-ohkubo 255,
Sakura-ku, Saitama 338-8570, Japan

Tel: +81 48 858 3409

Email: takezawa@mail.saitama-u.ac.jp

Article title

**Activation of SnRK2 by the Raf-like kinase ARK represents a common
mechanism of ABA response in embryophytes**

Authors

**Mousona Islam^{1,2*}, Takumi Inoue^{1*}, Mayuka Hiraide¹, Nobiza Khatun¹, Akida
Jahan¹, Keiko Kuwata³, Taishi Umezawa⁴, Izumi Yotsui⁵, Yoichi Sakata⁵,
Daisuke Takezawa¹**

¹Graduate School of Science and Engineering, Saitama University, Shimo-ohkubo
255, Sakura-ku, Saitama 338-8570, Japan; ²Plant Tissue Culture Section, Biological
Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR),
Dhaka 1205, Bangladesh. ³Institute of Transformative Bio-Molecules (WPI-ITbM),
Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan; ⁴Graduate
School of Bio-Applications and Systems Engineering, Tokyo University of
Agriculture and Technology, 2-24-16 Nakacho, Koganei City, Tokyo 184-8588,
Japan; ⁵Department of Bioscience, Tokyo University of Agriculture, 1-1-1
Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan.

*These authors contributed equally to this work.

Author contribution

M.I. and T.I. conducted most of the experiments and organized the entire manuscript
with M.H. and D.T. N.K. contributed to cold acclimation experiments. K.K. analyzed
phosphopeptides. A.J. and I.Y. contributed to transient gene expression assays. T.U.

and Y.S. contributed to experimental design in the transgenic study.

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One sentence summary:

Physiological characterization of various moss mutants revealed a common mechanism for phytohormone responses under water deficit in all land plants.

ABSTRACT

The Raf-like protein kinase ARK previously identified in the moss *Physcomitrella patens* acts as an upstream regulator of subgroup III SnRK2, the key regulator of abscisic acid (ABA) and abiotic stress responses. However, the mechanisms underlying activation of ARK by ABA and abiotic stress for the regulation of SnRK2 including the role of ABA receptor-associated group A PP2C (PP2C-A) are not understood. We identified Ser1029 as the phosphorylation site in the activation loop of ARK, which provided a possible mechanism for regulation of its activity. Analysis of transgenic *ark* lines expressing ARK-GFP with Ser1029-to-Ala mutation indicated that this replacement causes reductions in ABA-induced gene expression, stress tolerance and SnRK2 activity. Immunoblot analysis using an anti-phosphopeptide antibody indicated that ABA treatments rapidly stimulate Ser1029 phosphorylation in wild type. The phosphorylation profile of Ser1029 in ABA-hypersensitive *ppabil* lacking PP2C-A was similar to that in wild type, whereas little Ser1029 phosphorylation was observed in ABA-insensitive *ark* missense lines. Furthermore, newly isolated *ppabil ark* lines showed ABA-insensitive phenotypes similar to those of *ark* lines. These results indicate that ARK is a primary activator of SnRK2, preceding negative regulation by PP2C-A in bryophytes, which provides a prototypal mechanism for ABA and abiotic stress-responses in embryophytes.

Key words:

abiotic stress, abscisic acid, cold, *Physcomitrella patens*, protein kinase

INTRODUCTION

The phytohormone abscisic acid (ABA) regulates a variety of developmental as well as physiological processes in plants. In vegetative tissues, elevated endogenous ABA in response to water deficit in shoots contributes to stomatal closure and tolerance to environmental abiotic stresses such as drought and cold (Rock *et al.*, 2010). Studies on *Arabidopsis* have revealed that ABA elicits these cellular responses by binding to the intracellular ABA receptor PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCAR) (Park *et al.*, 2009; Ma *et al.*, 2009). The ABA-PYR/PYL/RCAR complex binds to group A protein phosphatase 2C (PP2C-A) to inhibit its phosphatase activity, which leads to activation of subclass III SNF1-related protein kinase2 (SnRK2) (Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Nishimura *et al.*, 2010; Rodrigues *et al.*, 2013). Of the three SnRK2 subgroups, subclass III SnRK2 is recognized as the key regulator of ABA signaling since it phosphorylates various cellular substrates including bZIP transcription factors that activate a number of ABA-induced genes (Marcotte *et al.*, 1989; Uno *et al.*, 2000; Fujita *et al.*, 2005). Activity of subclass III SnRK2 is stimulated by ABA-induced phosphorylation at specific Ser residues in the “activation loop” located between subdomains VII and VIII of the kinase domain (Boudsocq *et al.*, 2004; Konrev *et al.*, 2006; Boudsocq *et al.*, 2007). This phosphorylation event is operated by either autophosphorylation by SnRK2 itself or by other protein kinases, though detailed mechanisms have not been clarified.

The roles of ABA in stress responses have been demonstrated not only in angiosperms but also in basal embryophytes, mosses and liverworts. Protonemata of the moss *Physcomitrella patens* acquire tolerance to desiccation, hyperosmosis and freezing upon exogenous ABA treatment (Minami *et al.*, 2003; Khandelwal *et al.*, 2010; Koster *et al.*, 2010). ABA induces expression of genes for late embryogenesis abundant (LEA)-like proteins (Cuming and Lane, 1979) with boiling-soluble characteristics mediated by conserved ABA-responsive promoter elements in the moss protonemata (Knight *et al.* 1995). The *P. patens* genome has four genes for putative PYR/PYL/RCAR, two genes for PP2C-A and four genes for subclass III SnRK2 but no SnRK2 of other subclasses (Rensing *et al.*, 2008; Sakata *et al.*, 2014). Disruption of genes for PP2C-A (*PpABI1A* and *PpABI1B*) in the *ppabil* line resulted in an ABA-hypersensitive response and constitutive desiccation tolerance (Komatsu *et al.*, 2013). Furthermore, disruption of all four subgroup III SnRK2 genes (*PpSnRK2A* to

PpSnRK2D) resulted in a loss of ABA sensitivity (Shinozawa *et al.*, 2019). These findings suggest that core signaling mechanisms for ABA signaling are common in embryophytes including bryophytes and angiosperms.

While the role of PP2C-A in negative regulation of subgroup III SnRK2 has been well documented, the molecules responsible for positive regulation of SnRK2 have not been clarified. By analysis of a *P. patens* mutant designated AR7 that shows reduced SnRK2 activity, we previously reported that ABA- and abiotic stress-responsive Raf-like kinase (ARK) plays a crucial role in integration of ABA and abiotic stress response (Minami *et al.*, 2006; Saruhashi *et al.*, 2015). ARK with 1148 amino acids consists of the C-terminal protein kinase domain with similarity to group B3 Raf-like protein kinase (B3-Raf) and a large non-kinase region with unknown function toward the N-terminus. AR7 was not only insensitive to ABA but also less responsive to hyperosmosis and cold, indicating that ARK might play a role in the integration of ABA and these abiotic signals. AR7 has a missense mutation in Ser532 changed to Phe in the non-kinase region of ARK, suggesting that the region toward the N-terminus to the kinase domain might play a role in the regulation of ARK activity. Null mutations of *ARK* causing loss of ABA-induced desiccation tolerance have also been reported (Yasumura *et al.*, 2015; Stevenson *et al.*, 2016). The kinase domain of ARK fused to glutathione-S-transferase phosphorylated and activated subclass III SnRK2 (*PpSnRK2B* and *PpSnRK2D*) of *P. patens* *in vitro*, indicating that ARK acts as a positive regulator of SnRK2 in the ABA signaling process in bryophytes (Saruhashi *et al.*, 2015; Shinozawa *et al.*, 2019).

Although our studies have indicated that ARK is one of key regulators of abiotic stress signaling, little is known about how ARK activity is regulated by ABA. Studies on various eukaryotic Raf-related protein kinases highlighted phosphorylation-mediated activation of the kinases and the role of their N-terminal domains in the regulation of phosphorylation (Köhler and Brummera, 2016). By phosphopeptide mapping of *P. patens* ARK, we showed that ARK is phosphorylated in the activation loop of the kinase domain, providing a possible mechanism for regulation of ARK (Saruhashi *et al.*, 2015). In the present study, we therefore focused on phosphorylation and activation of ARK during ABA and stress response using *P. patens* lines for which site-specific mutations have been introduced. The results of our analysis indicated that ARK phosphorylation in the activation loop is critical for

SnRK2 regulation during ABA response. We also analyzed changes in ARK phosphorylation during ABA and abiotic stress treatments using an anti-phosphopeptide antibody in various mutants as well as in WT *P. patens*.

RESULTS

Analysis of ARK-GFP lines with mutations in putative phosphorylation sites

Our previous study showed by phosphopeptide mapping that Ser1029 and Ser1030 in the activation loop of the ARK kinase domain are putative phosphorylation sites (Saruhashi *et al.*, 2015). To determine which serine residues are critical for activation of ARK, ARK-GFP constructs with or without mutations in these phosphorylation sites (ARK^{S1029A}-GFP ARK^{S1030A}-GFP ARK^{S1029A/S1030A}-GFP) (Fig. 1A) were generated and introduced into AR7 with S532F mutation in ARK. Growth tests on a medium containing 10 μ M ABA indicated that protonemal growth of AR7 and AR7 expressing non-mutated ARK-GFP and ARK^{S1030A}-GFP was severely inhibited by ABA. In contrast, growth of AR7 expressing ARK^{S1029A}-GFP and ARK^{S1029A/S1030A}-GFP was less sensitive to ABA. We also generated AR7 expressing ARK^{S532F}-GFP for comparison. As expected, there was little inhibition of growth of AR7 expressing ARK^{S532F}-GFP by ABA (Fig. 1B, Fig. S1).

Using these lines, we analyzed ABA-induced gene expression by transient assays using the beta-glucuronidase (*GUS*) reporter gene fused to the ABA-inducible *Em* promoter (*proEm*) (Knight *et al.*, 1995; Sakata *et al.*, 2010). The results of assays indicated that AR7 lines expressing ARK^{S1029A}-GFP and ARK^{S1029A/S1030A}-GFP showed only a partial ABA response compared with the lines with ARK-GFP and ARK^{S1030A}-GFP (Fig. 1C). We also analyzed ABA-induced freezing tolerance in these lines. We previously showed that ABA treatment of protonemata increases freezing tolerance of the cells in WT but not in AR7 (Minami *et al.*, 2006; Bhyan *et al.*, 2011). Tests for freezing tolerance indicated that AR7 lines with ARK-GFP and ARK^{S1030A}-GFP showed similar levels of freezing tolerance induced by ABA treatment, whereas the lines with ARK^{S1029A}-GFP and ARK^{S1029A/S1030A}-GFP showed only a slight enhancement in the tolerance induced by ABA treatment (Fig. 1D). These results indicated that the mutation in Ser1029 to Ala causes reductions in ABA response for gene expression and stress tolerance.

Since ARK is postulated to be an activator of SnRK2, we carried out in-gel kinase assays of ARK-GFP mutant lines using a gel containing histone IIIS, by which we previously showed that AR7 has little SnRK2 kinase activity (Saruhashi *et al.*, 2015). The results of assays indicated that the lines with ARK^{S532F}-GFP and ARK^{S1029A}-GFP showed little SnRK2 activity, while the ARK^{S1030A}-GFP line showed ABA-induced SnRK2 activity similar to that of the ARK-GFP line (Fig. 1E). These results indicated that Ser1029 of ARK might be a critical phosphorylation site for activation of SnRK2 in *P. patens*.

To determine the importance of Ser1029 phosphorylation, anti-phosphopeptide antibody specific to Ser1029 phosphorylation (anti-P-S1029) was raised and used for immunoblot analysis (Fig. 2A). We detected immuno-reacting signals around the expected molecular mass of ARK-GFP (157 kDa) that were enhanced by 15-min treatment with 10 μ M ABA in the non-mutated ARK-GFP line. Similar enhancement of signals by ABA was also detected in the ARK^{S1030A}-GFP lines. In contrast, the signals were very faint in ARK^{S532F}-GFP and ARK^{S1029A}-GFP lines (Fig. 2A). Phosphopeptide analysis of ARK-GFP proteins immunoprecipitated from ABA-treated protonemata using anti-GFP antibody microbeads indicated that Ser phosphorylation in the peptide of amino acids 1029-1043 occurred in the ARK-GFP and ARK^{S1030A}-GFP lines but not in the ARK^{S1029A}-GFP and ARK^{S532F}-GFP lines (Table S2). The anti-P-S1029 antibody was also used for detection of phosphorylation of native ARK (Fig. 2B). The signal detected at around 130 kilodaltons by the anti-P-S1029 antibody was increased after 15 min of ABA treatment, while the levels of ARK polypeptide detected by an antibody against ARK C-terminal peptide (anti-ARKc) were unchanged. The level of Ser1029 phosphorylation was highest at 30 min and gradually decreased with time, but the enhanced phosphorylation was still observed even after 3 h (Fig. S2). We also found that osmotic treatment with 0.5 M mannitol stimulates the Ser1029 phosphorylation, though to a lesser extent than that with ABA treatment (Fig. S3).

Analysis of AR144 (*ark*^{D995N}) with mutation in the kinase catalytic core

The fact that AR7 with the S523F mutation in the non-kinase region but with an intact kinase domain showed little Ser1029 phosphorylation and ABA response raised the

question about whether Ser1029 phosphorylation requires catalytic activity of ARK itself. To determine the role of the kinase domain of ARK, we searched for new *ark* alleles by screening of more than 6×10^6 mutagenized protonema cells, and we isolated the ABA-insensitive AR144 line, in which Asp995 in the kinase domain of ARK has been changed to Asn (Fig. 3A). This Asp residue corresponds to a highly conserved Asp in the “HRD motif” in the catalytic loop of various eukaryotic protein kinases (Fig. 3A). This Asp residue is essential for the kinase activity since it is assigned to be responsible for correct orientation of the target hydroxyl group of Ser in the substrate peptide (Kannan and Neuwald 2005; Kornev *et al.*, 2006).

When grown on a medium containing 10 μ M ABA, protonemata of AR144 showed ABA-insensitive growth similar to that of AR7 (Fig. 3B). In freezing tolerance tests, freezing-induced leakage of electrolytes in WT protonemata was remarkably reduced by pretreatment with ABA, whereas the leakage was not mitigated by ABA in AR144 protonemata as in the case of AR7 (Fig. 3C). Transient reporter assays indicated that there was no ABA-increased gene expression in AR144 (Fig. 3D) in contrast to AR7 with an intact kinase domain showing a slight increase. We analyzed phosphorylation of ARK in AR144 by using the anti-P-Ser1029 antibody to examine whether the ARK kinase activity is necessary for its phosphorylation and is correlated with activation of SnRK2. The results of analyses indicated that while both Ser1029 phosphorylation and SnRK2 activity were enhanced by ABA in WT, neither Ser1029 phosphorylation nor SnRK2 activity was detected in AR144 as well as in AR7 (Fig. 3E). These results indicated that the catalytic activity of ARK is necessary for Ser1029 phosphorylation and cellular ABA response.

Effect of cold on ARK phosphorylation

We previously reported that the freezing tolerance of *P. patens* protonema cells is enhanced by exposure to low temperatures (Minami *et al.*, 2005). This cold acclimation process accompanied accumulation of LEA-like proteins that mitigate freezing damage (Minami *et al.*, 2005; Sasaki *et al.*, 2014). Comparison of freezing-induced electrolyte leakage in WT and AR7 indicated that cold treatment (0°C) enhanced freezing tolerance in WT but only slightly in AR7 (Fig. 4A), correlated with accumulation of the LEA-like 17B9 protein in boiling-soluble

fractions (Fig. 4B). Immunoblot analysis of WT and AR7 protonemata with anti-P-S1029 and anti-ARKc antibodies revealed that phosphorylation of Ser1029 was enhanced in WT with increased abundance of ARK polypeptide during cold acclimation (Fig. 4B). The Ser1029 phosphorylation was correlated with enhancement of SnRK2 activity. In AR7, however, little enhancement of both Ser1029 phosphorylation and SnRK2 activity was observed during cold acclimation, although cold-enhanced accumulation of ARK polypeptide was observed. These results suggest that Ser1029 phosphorylation of ARK plays a role in the cold acclimation process in the moss cells.

Roles of PP2C-A in regulation of ARK phosphorylation and SnRK2 activity

PP2C-A associated with the PYR/PYL/RCAR receptor is thought to be a critical regulator of SnRK2 in Arabidopsis (Park *et al.*, 2009; Umezawa *et al.*, 2009). Komatsu *et al.* (2013) reported that the *P. patens ppabil* line lacking both of the two genes for PP2C-A showed desiccation tolerance without ABA treatment. Protonemata of *ppabil* frequently form spherical “brood cells” (Arif *et al.*, 2019) under normal growth conditions, while these cells are formed by ABA treatment for several days in WT protonemata (Fig. 5A). Interestingly, the *ppabil* protonemata are still responsive to ABA and can form more brood cells upon ABA treatment (Fig. 5A). In-gel kinase assays of *ppabil* indicated that ABA-dependent SnRK2 activity in *ppabil* was greater than that in WT, though the activity remained low without exogenous ABA (Komatsu *et al.*, 2013), indicating that PP2C-A is not the only regulator of SnRK2. Since ARK has been identified as a positive regulator of SnRK2, we suspected that PP2C-A might affect SnRK2 through regulation of ARK. Comparison of ABA-induced changes in Ser1029 phosphorylation of ARK and SnRK2 activity in the same plant extract indicated that phosphorylation of ARK was stimulated by ABA in *ppabil* in a manner similar to that of WT, in contrast to a remarkable enhancement of SnRK2 activity in *ppabil* (Fig. 5B). These results indicated that PP2C-A is not required for ABA-induced activation of ARK phosphorylation.

To determine the roles of ARK under the *ppabil* background, we mutagenized protonemata of *ppabil* by ultraviolet light and isolated lines that grow well on a medium containing 1 μ M ABA, which severely inhibit growth of *ppabil*. Of several

isolated mutant lines, we identified ppuv4 (*ark*^{S1069S}), ppuv6 (*ark*^{S1069L}) and ppuv9 (*ark*^{S998L}) lines (Fig. 6A, Fig. S4A) that all had missense mutations in the kinase domain. These mutant lines showed ABA-insensitive growth similar to that of AR7 (*ark*^{S532F}), forming no brood cells upon ABA treatment (Fig. 6B, Fig. S4B). A comparison of ABA-induced accumulation of boiling-soluble proteins and the LEA-like 17B9 protein therein in WT, *ppabi1*, AR7 and ppuv4 indicated that AR7 and ppuv4 accumulated little of these proteins with or without ABA treatment (Fig. 6C). Furthermore, in-gel kinase assays for SnRK2 activity (Fig. 6D), freezing tolerance tests (Fig. 6E) and gene expression studies (Fig. S5) indicated that ppuv4 is insensitive to ABA. Transient reporter assays using *proEm-GUS* for determination of exogenous ABA- and ARK-dependent gene expression indicated that, while *ppabi1* shows *GUS* expression even without exogenous ABA due to its hypersensitivity, ppuv4 shows little expression with or without exogenous ABA, which can be only restored by introduction of *ARK* cDNA (Fig. 6F). These results indicated that mutations in ARK cause ABA insensitivity even under the *ppabi1* background.

DISCUSSION

Activation of Raf-like kinase ARK by Ser1029 phosphorylation is a key event in ABA response

We demonstrated in this study that ABA and abiotic stress responses in *P. patens* require activation of ARK, which belongs to the family of B3-Raf kinases and has been suggested to be an upstream activator of subclass III SnRK2. Previous studies indicated that nearly 90% and 80% of ABA-regulated gene expression are under the control of ARK and subgroup III SnRK2, respectively (Saruhashi *et al.*, 2015; Shinozawa *et al.*, 2019). Consistently, approximately 88% of ABA-increased phosphorylation was found to be ARK-dependent (Amagai *et al.*, 2018), where drastic reduction in the levels of ABA-activated phosphorylation of both SnRK2 and AREB transcription factors in AR7 was observed. Our results showing correlations of Ser1029 phosphorylation with ABA-induced gene expression, stress tolerance and SnRK2 activity indicate that activation of ARK by phosphorylation is a crucial event during ABA response in *P. patens*. The role of ARK in SnRK2 activation may not be restricted to bryophytes; we previously showed that ectopic expression of three

Arabidopsis B3-Raf kinases (At1G18160, At1G73660 and At4G24480) can restore ABA sensitivity of AR7 (Saruhashi *et al.*, 2015). It has recently been reported that these B3-Raf kinases are necessary for activation of SnRK2 kinases during ABA and osmotic responses in Arabidopsis (Takahashi *et al.*, 2020; Lin *et al.*, 2020). The serine residue corresponding to Ser1029 of ARK is conserved in Arabidopsis B3-Raf kinases (Fig. S6). Considering the functional conservation of subclass III SnRK2 in *P. patens* and Arabidopsis (Chater *et al.*, 2011; Shinozawa *et al.*, 2019), the results of this study suggest that sequential activation of B3-Raf kinase and subclass III SnRK2 might be an evolutionarily conserved mechanism for ABA and stress responses in embryophytes. The presence of Arabidopsis B3-Raf kinases with specific functions such as CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) for ethylene signaling (Kieber *et al.*, 1998), ENHANCED DISEASE RESISTANCE1 (EDR1) for non-host resistance (Frye and Innes, 1998; Hiruma *et al.*, 2011) and SUGAR INSENSITIVE8 (SIS8) for sugar sensing (Huang *et al.*, 2014) suggests functional diversification of B3-Raf kinases in angiosperms.

Ser1029 phosphorylation of ARK requires a functional kinase domain

The present study revealed the importance of both the kinase domain and the N-terminal region of ARK for its activation (Fig. 3E). AR144 lacking the critical Asp residue in the catalytic loop of the kinase domain showed no ABA response and Ser1029 phosphorylation, suggesting that the phosphorylation requires a functional kinase domain. This indicates that Ser1029 is not phosphorylated by other protein kinases but is phosphorylated by ARK itself (i.e., autophosphorylation). Consistently, bacterially expressed recombinant GST-ARK proteins readily undergo autophosphorylation in the activation loop (Table S3). What controls this autophosphorylation upon ABA and cold treatment *in vivo* is yet to be clarified. In animal Raf kinases, the N-terminal domains play a crucial role in regulation of the catalytic activity (Matallanas *et al.*, 2011). For human c-Raf, the “autoinhibitory block” consisting of the Ras-binding domain and the C1 domain directly interacts with the C-terminal kinase domain and inhibits its catalytic activity, and upon stimulation, binding of Ras-GTP releases the kinase domain from this autoinhibition. The partially activated c-Raf undergoes autophosphorylation in the activation loop, which fully activates the kinase activity. For *P. patens* ARK, the N-terminal

non-kinase region might also play a role in regulation of the kinase domain, since AR7 with S532F mutation showed little Ser1029 phosphorylation and catalytic activity toward SnRK2 (Fig. 2A and Fig. 3E). Given that the Ser1029 phosphorylation is done by autophosphorylation, activity of ARK must be controlled by the action of an unidentified negative regulatory (NR) factor that inhibits the catalytic activity of the kinase domain, possibly through interaction with a specific domain in the N-terminal region, and by ABA-dependent removal of the NR factors.

Roles of ARK in cold responses

Many plant species can acquire tolerance to freezing upon exposure to low, non-freezing temperatures. In Arabidopsis, cold provokes expression of a number of transcripts such as those for COR/DHN with characteristics similar to LEA proteins. Expression of these transcripts is driven by CBF transcription factors, genes for which are also up-regulated by cold (Thomashow, 2010; Shi *et al.*, 2018). Several distinct mechanisms for regulation of *CBF* genes have been proposed, although the molecules for cold sensing and the early signal transduction process have not been identified. It has been proposed in Arabidopsis that subclass III SnRK2 OST1 activated by cold positively regulates *CBF* gene expression by phosphorylation of the transcriptional regulators ICE1 and BTF3 (Ding *et al.*, 2015; 2018). Bryophytes also undergo cold acclimation accompanied by enhancement of SnRK2 activity (Saruhashi *et al.*, 2015). The fact that cold-enhanced Ser1029 phosphorylation of ARK and activation of SnRK2 were not observed in AR7 (Fig. 4B) therefore indicates the role of ARK in activation of SnRK2 for the cold response in *P. patens*. We also showed an increased accumulation of ARK polypeptide during the cold treatment (Fig. 4b), which might provide a novel mechanism for the control of ARK by cold. Interestingly, ARK transcript levels are apparently not affected by cold (Fig. 4B). It is also likely that this process does not require functional ARK, since accumulation of ARK polypeptide by cold was also observed in AR7 accumulating reduced amounts of the 17B9 protein and transcripts (Fig. 4B; Fig. S7).

PP2C-A functions downstream to ARK

PP2C-A, which consists of nine members in Arabidopsis, is recognized as a primary regulator of SnRK2 (Umezawa *et al.*, 2009; Vlad *et al.*, 2009). The role of PP2C-A in negative regulation of ABA signaling is likely to be conserved in both mosses and liverworts consisting of the basal lineage of embryophytes, because overexpression of PP2C-A genes of these plants caused abolishment of ABA-responsive gene expression and loss of abiotic stress tolerance (Komatsu *et al.*, 2009; Tougane *et al.*, 2010). Furthermore, the fact that the *ppabil* plants still responded to ABA and could activate SnRK2 (Komatsu *et al.*, 2013) indicates the presence of PP2C-A-independent ABA-response mechanisms in bryophytes. Consistently, results of phosphoproteome analysis of *P. patens* indicated that only a small portion (23 out of 143) of ABA-stimulated phosphoproteins detected in WT were hyperphosphorylated in *ppabil*, while 126 of those were dependent on ARK (Amagai *et al.*, 2018).

The present study on *ppuv4* (*ppabil ark*) demonstrated that, with or without PP2C-A, *P. patens* cannot respond to ABA without functional ARK (Fig. 6A-F). Abolishment of SnRK2 activity in *ppuv4* as in AR7 indicates that SnRK2 cannot undergo regulation by PP2C-A unless it has been pre-activated by ARK (Fig. 6D). Furthermore, our results suggested that PP2C-A may not be a primary regulator of ARK phosphorylation (Fig. 5B). Figure 7 and Figure S8 illustrate working models for the control of SnRK2 in bryophytes, focusing on the role of ARK for positive regulation and PP2C-A for subsequent negative regulation. These models are supported not only by abolishment of ABA responses in *ppuv4* but also by the results of our experiment with *MpPYL1* (Fig. S9). *MpPYL1* is a major ABA receptor in vegetative tissues of *M. polymorpha* and has recently been shown to have ABA-independent inhibitory activity towards PP2C-A (Jahan *et al.*, 2019; Sun *et al.*, 2019). Our experiment indicated that transient overexpression of *MpPYL1* enhances the activity of the *GUS* reporter gene fused to the ABA-inducible *Em* promoter without ABA treatment, but such enhancement was not observed in AR7 with or without ABA (Fig. S9), suggesting that inhibition of PP2C-A by PYR/PYL/RCAR causes activation of the promoter only in the presence of functional ARK. Our previous analysis indicated spatial separation of ARK and PP2C-A in bryophytes; The localization of the GFP-fusion protein of ARK was mainly in the cytoplasm and partly in the endoplasmic reticulum, while that of PP2C-A was only in the nucleus (Komatsu *et al.*, 2009; Tougane *et al.*, 2010; Saruhashi *et al.*, 2015). The working models we

proposed for bryophytes in this study represent the basic ABA response machinery prototypal to that in angiosperms possessing diverse PP2C-A molecules. Evolutionary diversification of PP2C-A molecules in angiosperms resulted in localization of some PP2C-A molecules in the nucleus for regulation of transcription factors, while others are in the cytoplasm for regulation of angiosperms-specific substrates such as plasma membrane-localized ion channels. Takahashi *et al.* (2020) hypothesized from the results of *in vitro* and *Xenopus* oocyte reconstitution assays that Arabidopsis B3-Raf kinases play a role in reactivation of SnRK2 that had been dephosphorylated by PP2C-A for regulation of the AKS1 transcription factor and the S-type ion channel SLAC1. We do not rule out the possibility of a role of *P. patens* ARK in reactivation of SnRK2 because *P. patens* SnRK2 is localized in both the cytoplasm and nucleus (Saruhashi *et al.*, 2015), but verification of its possible role requires further investigation.

MATERIALS AND METHODS

Chemicals and plant materials

Chemicals were purchased from Wako Jun-yaku (Osaka, Japan) unless otherwise stated. Absciscic acid was from Sigma (A4906, St. Louis, MO, USA). Culture and ABA, osmotic and cold treatments of protonemata of *P. patens* wild type (WT) and mutant lines were carried out as described previously (Minami *et al.*, 2003; 2005).

Transient reporter assays

P. patens protonemata cultured on cellophane-overlaid BCDAT agar medium for 5 days were used for particle bombardment (Marella *et al.*, 2006). The protonema cells were bombarded with DNAs of the *proEm-GUS* reporter construct (Marcotte *et al.*, 1989) and the *proUbi-LUC* reference construct (Bruce *et al.*, 1989) with or without *ARK* effector constructs by using the PDS-1000He particle delivery system (Bio-Rad, Hercules, CA). The bombarded cells were incubated in a medium with or without ABA and were used for GUS and LUC assays (Bruce *et al.*, 1989; Jefferson *et al.*, 1989; Marella *et al.*, 2006; Komatsu *et al.*, 2009).

Generation of transgenic *P. patens*

P. patens protonemata were subjected to PEG-mediated transformation according to

the protocol described by Nishiyama *et al.* (2000). Constructs of ARK-green fluorescent protein (ARK-GFP) without mutation and that with mutations in Ser532 to Phe (ARK^{S532F}-GFP), Ser1029 to Ala (ARK^{S1029A}-GFP), Ser1030 to Ala (ARK^{S1030A}-GFP) and Ser1029/Ser1030 to Ala/Ala (ARK^{S1029A/S1030A}-GFP) driven by the rice actin promoter were made using the pFL-Not vector (Saruhashi *et al.*, 1995).

Mutant screening

Partially homogenized protonemata spread on a cellophane-overlaid agar medium were cultured for two days prior to irradiation of ultraviolet light under conditions described by Minami *et al.* (2006). After incubation in the dark at 25°C for one day, the protonemata were cultured under continuous light for 1 or 2 days at 25°C. They were then transferred with cellophane onto a medium containing 10 µM ABA for WT and onto a medium containing 1 µM ABA for *ppabi1*.

Protein gel electrophoresis and immunoblot analysis

For protein extraction, protonemata were homogenized in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 25 mM NaF, 2 mM dithiothreitol, 1 mM o-vanadate, and 50 mM β-glycerophosphate and a 1/100 volume of proteinase inhibitor mixture (P9599; Sigma, MO) on ice. After centrifugation at 14,000 × g for 10 min at 4 °C, the supernatants were used as protein samples for electrophoresis. The proteins (30 µg) were electrophoresed on 8% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane for immunoblot analysis. After blocking with 1% bovine serum albumin (for the anti-phosphopeptide antibody) or 3% skim milk (for other antibodies) in TBS-T (25 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.05%(w/v) Tween-20), the proteins were reacted with the following primary antibodies: anti-GFP (MBL, Nagoya, Japan), anti-ARK C-terminal 15 amino acids (LGGTPKSGLSRDL) (anti-ARKc) (Saruhashi *et al.*, 2015) and anti-Ser1029-phosphorylated peptide (FLTSKpSSAGTPEWMAPE) (anti-P-Ser1029). The anti-P-Ser1029 antibody raised in rabbits was affinity-purified using the same phosphopeptide and then cross-absorbed against the dephosphorylated GST-ARK protein to remove antibody that might recognize non-phosphorylated ARK. The membrane that had been reacted with a primary antibody was reacted with a 1:10000 dilution of horseradish peroxidase-conjugated secondary antibody (MBL, Nagoya, Japan). The membrane was then immersed in chemiluminescence reagent

(Chemi-Lumi One, Nacalai Tesque, Kyoto, Japan) and exposed to X-ray film for signal detection.

Analysis of phosphopeptides

Protein extracts were prepared from protonemata of AR7 expressing ARK-GFP constructs using the buffer used for immunoblot analysis. Immunoprecipitation was carried out using the μ MACS GFP Tagged Protein Isolation Kit (Miltenyi Biotech). Protein samples were electrophoresed using 8% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue, and stained bands corresponding to ARK-GFP near 150 kilodaltons were excised. After proteolytic enzyme digestion, the phosphopeptides were analyzed by nano-flow reverse-phase liquid chromatography followed by tandem MS using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific).

Extraction of total RNA, reverse transcription (RT)-PCR and RT-PCR analysis

RNA extraction, first-strand cDNA synthesis and PCR reaction were carried out as described previously (Saruhashi *et al.*, 2015). The RNA was reverse-transcribed with oligo-dT and used for semi-quantitative PCR and SYBR Green-based quantitative PCR. Sequences of oligonucleotide primers are listed in Table S1.

In-gel kinase assay for SnRK2 activity

An in-gel kinase assay was carried out according to the protocol described by Suzuki and Shinshi (1995). Protein extracts used for immunoblot analysis were subjected to electrophoresis using a 10% SDS-polyacrylamide gel containing 0.5 mg/mL histone HHS (Sigma-Aldrich) as a substrate. The gel was successively washed for 30 min twice in buffer I (50 mM Tris-Cl, pH 8.0 and 20% isopropanol) and buffer II (50 mM Tris-Cl, pH 8.0 and 5 mM 2-mercaptoethanol), denaturation buffer (buffer II containing 6 M guanidine hydrochloride), and renaturation buffer (buffer II containing 0.04% Tween 40) at room temperature. The gel was then incubated in renaturation buffer at 4°C for 16 h and again washed once in renaturation buffer at room temperature for 30 min. After incubation in buffer III (40 mM HEPES-KOH, pH 7.5, 15 mM MgCl₂, 0.1 mM ethylene glycol tetraacetic acid and 2 mM dithiothreitol) for 30 min at room temperature, the gel was reacted with 50 μ M [γ -³²P] ATP (1.5 TBq/mmol) in buffer III for 1 h at 25°C. After removal of the reaction solution, the gel

was washed for 30 min for at least six times with washing solution (5% trichloroacetic acid and 1% sodium pyrophosphate) before drying and exposure to X-ray film.

Tests for freezing tolerance

Protonema tissues placed in a glass test tube containing 0.5 mL of distilled water were set in a programmable cooling bath. The tissues were seeded with ice at -1°C , kept at -1°C for 50 min, and then cooled at a rate of $-2.4^{\circ}\text{C h}^{-1}$ to desired temperatures. Electrolyte leakage from the damaged tissues was determined after thawing by measurement of conductivity in the water and represented as the percentage against conductivity of total ions released by subsequent boiling.

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LITERATURE CITED

- Amagai A, Honda Y, Ishikawa S, Hara Y, Takezawa D, Sakata Y, Shinozaki K, Umezawa T. 2018. Phosphoproteomic profiling reveals ABA-responsive phosphosignaling pathways in *Physcomitrella patens*. *Plant Journal* 94: 699-708.
- Arif MA, Hiss M, Tomek M, Busch H, Meyberg R, Tintelnot S, Reski R, Rensing SA, Frank W. 2019. ABA-Induced vegetative diaspore formation in *Physcomitrella patens*. *Frontiers in Plant Science* 10: 315.
- Bhyan SB, Minami A, Kaneko Y, Suzuki S, Arakawa K, Sakata Y, Takezawa D. 2011. Cold acclimation in the moss *Physcomitrella patens* involves abscisic acid-dependent signaling. *Journal of Plant Physiology* 169: 137-145.
- Boudsocq M, Barbier-Brygoo H, Laurie`re C. 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 279: 41758–41766.

- Boudsocq M, Droillard MJ, Barbier-Brygoo H, Laurie`re C. 2007. Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Molecular Biology* 63: 491–503.
- Bruce WB, Christensen AH, Klein T, Fromm M, Quail PH. 1989. Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proceedings of the National Academy of Sciences of the United States of America* 86: 9692–9696.
- Chater C, Gray JE. 2011. Stomatal closure: the old guard takes up the SLAC. *Current Biology* 25: R271-R273.
- Cuming AC, Lane BG. 1979. Protein synthesis in imbibing wheat embryos. *European Journal of Biochemistry* 99: 217-224.
- Ding Y, Li H, Zhang X, Xie Q, Gong Z, Yang S. 2015. OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in Arabidopsis. *Developmental Cell* 32: 278-289.
- Ding Y, Jia Y, Shi Y, Zhang X, Song C, Gong Z, Yang S. 2018. OST1-mediated BTF3L phosphorylation positively regulates CBFs during plant cold responses. *EMBO Journal* 37: e98228.
- Frye CA, Innes RW. 1998. An Arabidopsis mutant with enhanced resistance to powdery mildew. *Plant Cell* 10: 947–956.
- Fujita Y, Fujita M, Satoh R, Maruyama, K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K. 2005. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. *Plant Cell* 17: 3470-3488.
- Hiruma K, Nishiuchi T, Kato T, Bednarek P, Okuno T, Schulze-Lefert P, Takano Y. 2011. Arabidopsis ENHANCED DISEASE RESISTANCE 1 is required for pathogen-induced expression of plant defensins in nonhost resistance, and acts through interference of MYC2-mediated repressor function. *Plant Journal* 67: 980-992.
- Huang Y, Li CY, Qi Y, Park S, Gibson SI. 2014. SIS8, a putative mitogen-activated protein kinase kinase kinase, regulates sugar-resistant seedling development in Arabidopsis. *Plant Journal* 77:577–588.
- Jahan A, Komatsu K, Wakida-Sekiya M, Hiraide M, Tanaka K, Ohtake R, Umezawa T, Toriyama T, Shinozawa A, Yotsui I, Sakata Y, Takezawa D. 2019. Archetypal

roles of the PYR/PYL/RCAR-like receptor in drought and sugar responses in liverworts. *Plant Physiology* 179: 317-328.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* 6: 3901-3907.

Kannan N, Neuwald AF. 2005. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *Journal of Molecular Biology* 351: 956-972.

Khandelwal A1, Cho SH, Marella H, Sakata Y, Perroud PF, Pan A, Quatrano RS. 2010. Role of ABA and ABI3 in desiccation tolerance. *Science* 327: 546.

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. *Cell* 72: 427-441.

Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, Coates D, Quatrano RS, Bahadur S, Stockley PG, Cumming AC. 1995. Molecular responses to abscisic acid and stress are conserved between moss and cereals. *Plant Cell* 7: 499-506.

Köhler M, Brummer T. 2016. B-Raf activation loop phosphorylation revisited. *Cell Cycle* 15: 1171-1173.

Komatsu K, Nishikawa Y, Ohtsuka T, Taji T, Quatrano RS, Tanaka S, Sakata Y. 2009. Functional analyses of the ABI1-related protein phosphatase type 2C reveal evolutionarily conserved regulation of abscisic acid signaling between *Arabidopsis* and the moss *Physcomitrella patens*. *Plant Molecular Biology* 70:327-340.

Komatsu K, Suzuki N, Kuwamura M, Nishikawa Y, Nakatani M, Ohtawa H, Takezawa D, Seki M, Tanaka M, Taji T et al. 2013. Group A PP2Cs evolved in land plants as key regulators of intrinsic desiccation tolerance. *Nature Communications* 4: 2219.

Kornev AP, Haste NM, Taylor SS, Ten Eyck LF. 2006. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proceedings of the National Academy of Sciences of the United States of America*. 103:17783-17788.

Koster KL, Balsamo RA, Espinoza C, Oliver MJ. 2010. Desiccation sensitivity and tolerance in the moss *Physcomitrella patens*: assessing limits and damage. *Plant Growth Regulation* 62: 293-302.

- Lin, Z., Li, Y., Zhang, Z. Liu X, Hsu C-C, Du Y, Sang T, Zhu C, Wang Y, Satheesh V
et al. 2020. A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling
in higher plants. Nature Communications 11, 613.
- Ma Y, Szostkiewicz, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009.
Regulators of PP2C phosphatase activity function as abscisic acid sensors.
Science 324:1064-1068.
- Marcotte WR Jr, Russel SH, Quatrano RS. 1989. Absciscic acid-responsive sequences
from the Em gene of wheat. Plant Cell 1: 969-976.
- Marella HH, Sakata Y, Quatrano RS. 2006. Characterization and functional analysis of
ABSCISIC ACID INSENSITIVE3-like genes from *Physcomitrella patens*. Plant
Journal 46: 1032-1044.
- Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A,
Kolch W. 2011. Raf family kinases: old dogs have learned new tricks. Genes and
Cancer 2: 232–260.
- Minami A, Nagao M, Arakawa K, Fujikawa S, Takezawa D. 2003. Absciscic
acid-induced freezing tolerance in the moss *Physcomitrella patens* is
accompanied by increased expression of stress-related genes. Journal of Plant
Physiology 160: 475-483.
- Minami A, Nagao M, Ikegami K, Koshiba T, Arakawa K, Fujikawa S, Takezawa D.
2005. Cold acclimation in bryophytes: low-temperature-induced freezing
tolerance in *Physcomitrella patens* is associated with increases in expression
levels of stress-related genes but not with increase in level of endogenous abscisic
acid. Planta 220: 414-423.
- Minami A, Togawa S, Nagao M, Takezawa D. 2006. Altered freezing tolerance in the
Physcomitrella patens mutant with reduced sensitivity to abscisic acid.
Cryobiology and Cryotechnology 52: 135-139.
- Nishimura N, Sarkeshik A, Nito K, Park SY, Wang A, Carvalho PC, Lee S, Caddell
DF, Cutler SR, Chory J et al. 2010. PYR/PYL/RCAR family members are major
in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. Plant
Journal 61: 290-299.
- Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M. 2000. Tagged
mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle
mutagenesis. DNA Research 7: 9-17.

629 Niwa Y. 2003. A synthetic green fluorescent protein gene for plant biotechnology.
630 Plant Biotechnology 20: 1-11.

631 Nolen B, Taylor S, Ghosh G. 2004. Regulation of protein kinases: controlling activity
632 through activation segment conformation. Molecular Cell 15: 661-675.

633 Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J,
634 Rodrigues A, Tsz-fung FC et al. 2009. Absciscic acid inhibits type 2C protein
635 phosphatases via the PYR/PYL family of START proteins. Science
636 324:1068-1071.

637 Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T,
638 Perroud PF, Lindquist EA, Kamisugi Y et al. 2008. The *Physcomitrella* genome
639 reveals evolutionary insights into the conquest of land by plants. Science 319:
640 64-69.

641 Rock CD, Sakata Y, Quatrano RS. 2010. Stress Signaling I: The Role of Absciscic Acid
642 (ABA). Abiotic Stress Adaptation in Plants. Springer, 33-73.

643 Rodrigues A, Adamo M, Crozet P, Margalha L, Confraria A, Martinho C, Elias A,
644 Rabissi A, Lumberras V, González-Guzmán et al. 2013. ABI1 and PP2CA
645 phosphatases are negative regulators of Snf1-related protein kinase1 signaling in
646 Arabidopsis. Plant Cell 25: 3871-3884.

647 Sakata Y, Komatsu K, Takezawa D. 2014. ABA as a universal plant hormone.
648 Progress in Botany 75: 58-96.

649 Sakata Y, Nakamura I, Taji T, Tanaka S, Quatrano RS. 2010. Regulation of the
650 ABA-responsive Em promoter by ABI3 in the moss *Physcomitrella patens*: role
651 of the ABA response element and the RY element. Plant Signaling and Behavior
652 5: 1061-1066.

653 Saruhashi M, Ghosh TK, Arai K, Ishizaki Y, Hagiwara K, Komatsu K, Shiwa Y,
654 Izumikawa K, Yoshikawa H, Umezawa T et al. 2015. Plant Raf-like kinase
655 integrates absciscic acid and hyperosmotic stress signaling upstream of
656 SNF1-related protein kinase2. Proceedings of the National Academy of Sciences
657 of the United States of America 112: E6388-6396.

658 Sasaki K, Christov NK, Tsuda S, Imai R. 2013. Identification of a novel LEA protein
659 involved in freezing tolerance in wheat. Plant and Cell Physiology 55:136-147.

660 Shi Y, Ding Y, Yang S. 2018. Molecular regulation of CBF signaling in cold
661 acclimation. Trends in Plant Science 23: 623-637.

- Shinozawa A, Otake R, Takezawa D, Umezawa T, Komatsu K, Tanaka T, Amagai A, Ishikawa S, Hara Y, Kamisugi Y et al. 2019. SnRK2 protein kinases represent an ancient system in plants for adaptation to a terrestrial environment. *Communications Biology* 2: 30.
- Stevenson SR, Kamisugi Y, Trinh CH, Schmutz J, Jenkins JW, Grimwood J, Muchero W, Tuskan GA, Rensing SA, Lang D et al. 2016. Genetic analysis of *Physcomitrella patens* identifies ABSCISIC ACID NON-RESPONSIVE, a regulator of aba responses unique to basal land plants and required for desiccation tolerance. *Plant Cell* 28:1310-1327.
- Sun Y, Harpazi B, Wijerathna-Yapa A, Merilo E, de Vries J, Michaeli D, Gal M, Cuming AC, Kollist H, Mosquna A. 2019. A ligand-independent origin of abscisic acid perception. *Proceedings of the National Academy of Sciences of the United States of America* 116: 24892-24899.
- Suzuki K, Shinshi H. 1995. Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. *Plant Cell* 7, 639-647.
- Takahashi Y, Zhang J, Hsu P, Ceciliato PHO, Zhang L, Dubeaux G, Munemasa S, Ge C, Zhao Y, Hauser F, Schroeder JI. 2020. MAP3Kinase-dependent SnRK2-kinase activation is required for abscisic acid signal transduction and rapid osmotic stress response. *Nature Communications* 11, 12.
- Thomashow MF. 2010. Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiology* 154: 571–577.
- Tougane K, Komatsu K, Bhyan SB, Sakata Y, Ishizaki K, Yamato KT, Kohchi T, Takezawa D. 2010. Evolutionarily conserved regulatory mechanisms of abscisic acid signaling in land plants: characterization of ABSCISIC ACID INSENSITIVE1-like type 2C protein phosphatase in the liverwort *Marchantia polymorpha*. *Plant Physiology* 152: 1529-1543.
- Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K. 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 106: 17588-17593.
- Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic

acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences of the United States of America 97: 11632-11637.

Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Laurière C, Merlot S. 2009. Plant cell protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. Plant Cell 21:3170-3184.

Yasumura Y, Pierik R, Kelly S, Sakuta M, Voesenek LA, Harberd NP. 2015. An ancestral role for CONSTITUTIVE TRIPLE RESPONSE1 proteins in both ethylene and abscisic acid signaling. Plant Physiology 169:283-298.

SUPPORTING MATERIAL

Table S1: Oligonucleotide primers used for gene expression studies.

Table S2: Analysis of phosphopeptides immunoprecipitated from AR7 expressing ARK-GFP with or without mutations in Ser1029 and Ser1030.

Table S3: Autophosphorylation in the peptide SSAGTPEWMAPEVLR in the activation loop of recombinant GST-ARK.

Figure S1: Growth responses to ABA of transgenic AR7 lines expressing various ARK-GFP constructs.

Figure S2: Detection of Ser1029 phosphorylation of native ARK.

Figure S3: Effects of hyperosmotic mannitol on ARK Ser1029 phosphorylation.

Figure S4: Growth responses to ABA of three ppuv mutants isolated by ultraviolet light mutagenesis of *ppabil*.

Figure S5: ABA-induced gene expression of *LEA*-like transcripts in wild type (WT), *ppabil*, AR7 and ppuv4.

Figure S6: Amino acid sequence alignment in the activation loops of ARK and Arabidopsis B3-Raf kinases.

Figure S7: Expression of *ARK* and *LEA*-like *17B9* transcripts during cold acclimation in WT and AR7.

Figure S8: Details of the working model shown in Figure 7.

Figure S9: Effect of MpPYL1 on ABA-inducible promoter in WT and AR7.

FIGURE LEGENDS

Figure 1: Analysis of *Physcomitrella patens* lines expressing ARK-GFP constructs. (A) Schematic representation of ARK-GFP showing positions of Ser532 (S532) in the non-kinase domain and putative phosphorylation sites, Ser1029 (S1029) and Ser1030 (S1030), located in the activation loop of the kinase domain. (B) Growth responses to ABA of wild type (WT), AR7, and AR7 lines expressing ARK-GFP without mutation (ARK) and with S532F, S1029A, S1030A and S1029A/S1030A mutations. Protonemata of these lines were cultured with or without 10 μ M ABA for one week. (C) ABA-induced gene expression in transgenic lines. Protonemata were bombarded with plasmid constructs of the *Em* promoter fused with the *beta-glucuronidase* (*proEm-GUS*) gene and the rice *ubiquitin* promoter fused with the *luciferase* gene (*proUbi-LUC*) and were cultured with or without 10 μ M ABA for one day before GUS and LUC assays. Error bars indicate standard error of the mean. $*p < 0.05$ in the t-test ($n = 3$) compared with the ABA-treated ARK line. (D) Freezing tolerance of ARK-GFP lines. Protonemata were cultured with or without 10 μ M ABA for one day and subjected to freezing at 10°C. After thawing, electrolyte leakage was determined to estimate the damage caused by freezing. Error bars indicate standard error of the mean. $*p < 0.05$ in the t-test ($n = 3$) compared with the ABA-treated ARK line. (E) In-gel kinase assays for detection of SnRK2 activity. Proteins extracted from protonemata were electrophoresed using SDS-polyacrylamide gel polymerized with histone IIIS as a substrate. After denaturation and renaturation processes, the proteins were reacted with 32 P-ATP and radioactive signals were detected by exposure to X-ray film. Staining of the large subunit of ribulose biphosphate carboxylase (rbcL) is

shown as a control.

Figure 2: ABA-stimulated phosphorylation of Ser1029 detected by anti-phosphopeptide antibody. (A) Proteins from protonemata of transgenic *Physcomitrella patens* AR7 lines expressing ARK-GFP (ARK) and ARK-GFP with S532F, S1029A, and S1030A mutations with or without treatment with 10 μ M ABA for 15 min were subjected to immunoblot analysis using an anti-phospho-Ser1029 (P-S1029) antibody and an anti-GFP antibody. Since levels of ARK-GFP accumulation vary among these lines, the amount of proteins loaded per lane was adjusted using the anti-GFP antibody. Note that ARK^{S1030A}-GFP tends to give enhanced signals for an unknown reason. (B) Detection of native ARK in WT *P. patens*. Protonemata were treated with 10 μ M ABA for the indicated time periods, and extracted proteins were reacted with the anti-P-S1029 antibody and an antibody that recognizes the C-terminal 15-amino-acid peptide of ARK (anti-ARKc).

Figure 3: ABA response of AR144 of *Physcomitrella patens* with a mutation in the catalytic core of the kinase domain of ARK. (A) Comparison of amino acid sequences in the catalytic core of protein kinase A (PKA), cyclin-dependent protein kinase2 (CDK2), p38, casein kinase2 (CK2), and epidermal growth factor receptor (EGFR) (adapted from Nolen *et al.*, 2004) with ARK. The conserved Asp (D) has been changed to Asn (N) in AR144. (B) Growth responses to ABA. Protonemata of WT, AR7 and AR144 were grown on a medium with 10 μ M ABA for one week. (C) Comparison of freezing tolerance. Protonemata incubated with or without 10 μ M ABA for one day were frozen to -4°C. After thawing, electrolyte leakage was measured to estimate the extent of freezing injury. Error bars indicate standard error of the mean. (D) Transient gene expression assays of WT, AR7 and AR144. Protonemata were bombarded with plasmid constructs of *proEm-GUS* and *proUbi-LUC* and were cultured with or without 10 μ M ABA for one day for GUS and LUC assays. Error bars indicate standard error of the mean. ** $p < 0.01$ in the t-test ($n = 3$) compared with control WT without ABA treatment. (E) Effect of ABA on Ser1029 phosphorylation in WT, AR7 and AR144. Protonemata were treated with 10 μ M ABA for different times and used for immunoblot analysis with anti-phospho-Ser1029 (P-S1029) and anti-ARKc antibodies. Staining of the large subunit of ribulose biphosphate carboxylase (rbcL) is shown as a control. Results of an in-gel kinase

assay for detection of SnRK2 are also shown.

Figure 4: Cold responses mediated by ARK in *Physcomitrella patens*. (A) Changes in freezing tolerance during cold acclimation in wild type (WT) and AR7 (*ark*^{S532F}). Protonemata that had been cold-acclimated for the indicated days were frozen to -3°C and injury rate was estimated by measurement of electrolyte leakage after thawing. Error bars indicate standard error of the mean. **p* < 0.05 in the t-test (n = 3) compared with non-acclimated protonemata of each line. (B) Immunoblot analysis with anti-phospho-Ser1029 (P-S1029) and anti-ARKc antibodies. Coomassie Brilliant Blue staining of the large subunit of ribulose biphosphate carboxylase (rbcL), results of immunoblot analysis for cold-induced 17B9 protein, and SnRK2 activity analyzed by an in-gel kinase assay are also shown.

Figure 5: ABA response of the *ppabil* line of *Physcomitrella patens*. (A) Growth responses to ABA in protonemata of WT and *ppabil*. The protonemata were grown on a medium with or without 10 µM ABA for one week. (B) Effect of ABA treatment on Ser-1029 phosphorylation in WT and *ppabil*. Protonemata were treated with 10 µM ABA for different times and used for immunoblot analysis with anti-ARKc and anti-phospho-Ser1029 (P-S1029) antibodies. Coomassie Brilliant Blue staining of the large subunit of ribulose biphosphate carboxylase (rbcL) and SnRK2 activity analyzed by an in-gel kinase are also shown.

Figure 6: ABA response of *ppabil ark* lines of *Physcomitrella patens*. (A) Positions of mutations found in the ABA-insensitive *ppuv4* line isolated by ultraviolet mutagenesis of *ppabil*. (B) Effects of ABA on growth of wild type (WT), *ppabil*, AR7 and *ppuv4*. (C) ABA-induced accumulation of boiling-soluble LEA-like proteins in WT, *ppabil*, AR7 and *ppuv4*. Total soluble proteins of these lines were boiled for 1 min and centrifuged, and the boiling-soluble fraction (BSF) in the supernatant was analyzed by SDS-PAGE. The proteins were either stained with Coomassie Brilliant Blue or used for immunoblot analysis using an antibody against the LEA-like 17B9 protein. Staining of the large subunit of ribulose biphosphate carboxylase (rbcL) of the total soluble proteins is shown as a control. (D) SnRK2 activity in WT and the mutant lines analyzed by in-gel kinase assays using histone H1S as a substrate. (E) Freezing tolerance tests of WT and mutant lines. Protonemata were treated with or

without 10 μ M ABA for one day and frozen to -4°C . Electrolyte leakage (%) was measured after thawing to determine freezing injury. Error bars indicate standard error of the mean. $*p < 0.05$ in the t-test ($n = 3$). (F) Gene expression with or without 10 μ M ABA treatment in WT and the mutant lines. Transient gene expression analysis was carried out using the construct of ARK fused to the rice actin promoter (*proAct*) or that without ARK (Vec). Error bars indicate standard error of the mean. $*p < 0.05$, $**p < 0.01$ in the t-test ($n = 4$) compared with the values of WT (Vec, -ABA).

Figure 7: Working models showing ARK-mediated ABA responses in *Physcomitrella patens*. ABA stimulates ARK autophosphorylation and inhibits PP2C-A, causing activation of SnRK2 regulating downstream factors, in which phosphorylation of SnRK2 by ARK proceeds negative regulation by PP2C-A. ABA-stimulated activation of both ARK and SnRK2 occurs without PP2C-A, suggesting the presence an unidentified negative regulator (NR). Details of these models are shown in Fig. S8.

A **B** **C** **D** **E**

Figure 1: Analysis of *Physcomitrella patens* lines expressing ARK-GFP constructs. **(A)** Schematic representation of ARK-GFP showing positions of Ser532 (S532) in the non-kinase domain and putative phosphorylation sites, Ser1029 (S1029) and Ser1030 (S1030), located in the activation loop of the kinase domain. **(B)** Growth responses to ABA of wild type (WT), AR7, and AR7 lines expressing ARK-GFP without mutation (ARK) and with S532F, S1029A, S1030A and S1029A/S1030A mutations. Protonemata of these lines were cultured with or without 10 μ M ABA for one week. **(C)** ABA-induced gene expression in transgenic lines. Protonemata were bombarded with plasmid constructs of the *Em* promoter fused with the *beta-glucuronidase* (*proEm-GUS*) gene and the rice *ubiquitin* promoter fused with the *luciferase* gene (*proUbi-LUC*) and were cultured with or without 10 μ M ABA for one day before GUS and LUC assays. Error bars indicate standard error of the mean. * $p < 0.05$ in the t-test ($n = 3$) compared with the ABA-treated ARK line. **(D)** Freezing tolerance of ARK-GFP lines. Protonemata were cultured with or without 10 μ M ABA for one day and subjected to freezing at 10°C. After thawing, electrolyte leakage was determined to estimate the damage caused by freezing. Error bars indicate standard error of the mean. * $p < 0.05$ in the t-test ($n = 3$) compared with the ABA-treated ARK line. **(E)** In-gel kinase assays for detection of SnRK2 activity. Proteins extracted from protonemata were electrophoresed using SDS-polyacrylamide gel polymerized with histone IIIS as a substrate. After denaturation and renaturation processes, the proteins were reacted with 32 P-ATP and radioactive signals were detected by exposure to X-ray film. Staining of the large subunit of ribulose biphosphate carboxylase (rbcL) is shown as a control.

Figure 2: ABA-stimulated phosphorylation of Ser1029 detected by anti-phosphopeptide antibody. **(A)** Proteins from protonemata of transgenic *Physcomitrella patens* AR7 lines expressing ARK-GFP (ARK) and ARK-GFP with S532F, S1029A, and S1030A mutations with or without treatment with 10 μ M ABA for 15 min were subjected to immunoblot analysis using an anti-phospho-Ser1029 (P-S1029) antibody and an anti-GFP antibody. Since levels of ARK-GFP accumulation vary among these lines, the amount of proteins loaded per lane was adjusted using the anti-GFP antibody. Note that ARK^{S1030A}-GFP tends to give enhanced signals for an unknown reason. **(B)** Detection of native ARK in WT *P. patens*. Protonemata were treated with 10 μ M ABA for the indicated time periods, and extracted proteins were reacted with the anti-P-S1029 antibody and an antibody that recognizes the C-terminal 15-amino-acid peptide of ARK (anti-ARKc).

A

PKA	162	LIYRDLKPENLLI	174
CDK2	123	VLHRDLKPQNLLI	135
p38	149	IIHRDLKPGNLAV	161
CK2	152	IMHRDVKPHNVM	164
EGFR	809	LVHRDLAARNVLV	821
IRK	1128	FVHRDLAARNCMV	1140
ARK	991	IVHRDLKSPNLLV	1003

↓
N (AR144)

B **C** **D** **E**

Figure 3: ABA response of AR144 of *Physcomitrella patens* with a mutation in the catalytic core of the kinase domain of ARK. **(A)** Comparison of amino acid sequences in the catalytic core of protein kinase A (PKA), cyclin-dependent protein kinase2 (CDK2), p38, casein kinase2 (CK2), and epidermal growth factor receptor (EGFR) (adapted from Nolen *et al.*, 2004) with ARK. The conserved Asp (D) has been changed to Asn (N) in AR144. **(B)** Growth responses to ABA. Protonemata of WT, AR7 and AR144 were grown on a medium with 10 μ M ABA for one week. **(C)** Comparison of freezing tolerance. Protonemata incubated with or without 10 μ M ABA for one day were frozen to -4°C. After thawing, electrolyte leakage was measured to estimate the extent of freezing injury. Error bars indicate standard error of the mean. **(D)** Transient gene expression assays of WT, AR7 and AR144. Protonemata were bombarded with plasmid constructs of *proEm-GUS* and *proUbi-LUC* and were cultured with or without 10 μ M ABA for one day for GUS and LUC assays. Error bars indicate standard error of the mean. ** $p < 0.01$ in the t-test ($n = 3$) compared with control WT without ABA treatment. **(E)** Effect of ABA on Ser1029 phosphorylation in WT, AR7 and AR144. Protonemata were treated with 10 μ M ABA for different times and used for immunoblot analysis with anti-phospho-Ser1029 (P-S1029) and anti-ARKc antibodies. Staining of the large subunit of ribulose biphosphate carboxylase (rbcL) is shown as a control. Results of an in-gel kinase assay for detection of SnRK2 are also shown.

A **B**

Figure 4: Cold responses mediated by ARK in *Physcomitrella patens*. **(A)** Changes in freezing tolerance during cold acclimation in wild type (WT) and AR7 (*ark^{S532F}*). Protonemata that had been cold-acclimated for the indicated days were frozen to -3°C and injury rate was estimated by measurement of electrolyte leakage after thawing. Error bars indicate standard error of the mean. * $p < 0.05$ in the t-test ($n = 3$) compared with non-acclimated protonemata of each line. **(B)** Immunoblot analysis with anti-phospho-Ser1029 (P-S1029) and anti-ARKc antibodies. Coomassie Brilliant Blue staining of the large subunit of ribulose biphosphate carboxylase (rbcL), results of immunoblot analysis for cold-induced 17B9 protein, and SnRK2 activity analyzed by an in-gel kinase assay are also shown.

Figure 5: ABA response of the *ppabi1* line of *Physcomitrella patens*. **(A)** Growth responses to ABA in protonemata of WT and *ppabi1*. The protonemata were grown on a medium with or without 10 μ M ABA for one week. **(B)** Effect of ABA treatment on Ser-1029 phosphorylation in WT and *ppabi1*. Protonemata were treated with 10 μ M ABA for different times and used for immunoblot analysis with anti-ARKc and anti-phospho-Ser1029 (P-S1029) antibodies. Coomassie Brilliant Blue staining of the large subunit of ribulose biphosphate carboxylase (rbcl) and SnRK2 activity analyzed by an in-gel kinase are also shown.

Figure 6

Figure 6: ABA response of *ppabi1 ark* lines of *Physcomitrella patens*. **(A)** Positions of mutations found in the ABA-insensitive *ppuv4* line isolated by ultraviolet mutagenesis of *ppabi1*. **(B)** Effects of ABA on growth of wild type (WT), *ppabi1*, AR7 and *ppuv4*. **(C)** ABA-induced accumulation of boiling-soluble LEA-like proteins in WT, *ppabi1*, AR7 and *ppuv4*. Total soluble proteins of these lines were boiled for 1 min and centrifuged, and the boiling-soluble fraction (BSF) in the supernatant was analyzed by SDS-PAGE. The proteins were either stained with Coomassie Brilliant Blue or used for immunoblot analysis using an antibody against the LEA-like 17B9 protein. Staining of the large subunit of ribulose biphosphate carboxylase (rbcl) of the total soluble proteins is shown as a control. **(D)** SnRK2 activity in WT and the mutant lines analyzed by in-gel kinase assays using histone H1S as a substrate. **(E)** Freezing tolerance tests of WT and mutant lines. Protonemata were treated with or without 10 μ M ABA for one day and frozen to -4°C. Electrolyte leakage (%) was measured after thawing to determine freezing injury. Error bars indicate standard error of the mean. * $p < 0.05$ in the t-test ($n = 3$). **(F)** Gene expression with or without 10 μ M ABA treatment in WT and the mutant lines. Transient gene expression analysis was carried out using the construct of ARK fused to the rice actin promoter (*proAct*) or that without ARK (Vec). Error bars indicate standard error of the mean. * $p < 0.05$, ** $p < 0.01$ in the t-test ($n = 4$) compared with the values of WT (Vec, -ABA).

Figure 7: Working models showing ARK-mediated ABA responses in *Physcomitrella patens*. ABA stimulates ARK autophosphorylation and inhibits PP2C-A, causing activation of SnRK2 regulating downstream factors, in which phosphorylation of SnRK2 by ARK proceeds negative regulation by PP2C-A. ABA-stimulated activation of both ARK and SnRK2 occurs without PP2C-A, suggesting the presence an unidentified negative regulator (NR). Details of these models are shown in Fig. S8.

Parsed Citations

Amagai A, Honda Y, Ishikawa S, Hara Y, Takezawa D, Sakata Y, Shinozaki K, Umezawa T. 2018. Phosphoproteomic profiling reveals ABA-responsive phosphosignaling pathways in *Physcomitrella patens*. *Plant Journal* 94: 699-708.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Arif MA, Hiss M, Tomėk M, Busch H, Meyberg R, Tintelnot S, Reski R, Rensing SA, Frank W. 2019. ABA-Induced vegetative diaspore formation in *Physcomitrella patens*. *Frontiers in Plant Science* 10: 315.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Bhyan SB, Minami A, Kaneko Y, Suzuki S, Arakawa K, Sakata Y, Takezawa D. 2011. Cold acclimation in the moss *Physcomitrella patens* involves abscisic acid-dependent signaling. *Journal of Plant Physiology* 169: 137-145.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Boudsocq M, Barbier-Brygoo H, Laurie`re C. 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 279: 41758-41766.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Boudsocq M, Droillard MJ, Barbier-Brygoo H, Laurie`re C. 2007. Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Molecular Biology* 63: 491-503.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Bruce WB, Christensen AH, Klein T, Fromm M, Quail PH. 1989. Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proceedings of the National Academy of Sciences of the United States of America* 86: 9692-9696.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chater C, Gray JE. 2011. Stomatal closure: the old guard takes up the SLAC. *Current Biology* 25: R271-R273.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Cuning AC, Lane BG. 1979. Protein synthesis in imbibing wheat embryos. *European Journal of Biochemistry* 99: 217-224.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ding Y, Li H, Zhang X, Xie Q, Gong Z, Yang S. 2015. OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in *Arabidopsis*. *Developmental Cell* 32: 278-289.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ding Y, Jia Y, Shi Y, Zhang X, Song C, Gong Z, Yang S. 2018. OST1-mediated BTF3L phosphorylation positively regulates CBFs during plant cold responses. *EMBO Journal* 37: e98228.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Frye CA, Innes RW. 1998. An *Arabidopsis* mutant with enhanced resistance to powdery mildew. *Plant Cell* 10: 947-956.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fujita Y, Fujita M, Satoh R, Maruyama, K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K. 2005. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* 17: 3470-3488.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hiruma K, Nishiuchi T, Kato T, Bednarek P, Okuno T, Schulze-Lefert P, Takano Y. 2011. *Arabidopsis* ENHANCED DISEASE RESISTANCE 1 is required for pathogen-induced expression of plant defensins in nonhost resistance, and acts through interference of MYC2-mediated repressor function. *Plant Journal* 67: 980-992.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Huang Y, Li CY, Qi Y, Park S, Gibson SI. 2014. SIS8, a putative mitogen-activated protein kinase kinase kinase, regulates sugar-resistant seedling development in *Arabidopsis*. *Plant Journal* 77:577-588.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Jahan A, Komatsu K, Wakida-Sekiya M, Hiraide M, Tanaka K, Ohtake R, Umezawa T, Toriyama T, Shinozawa A, Yotsui I, Sakata Y, Takezawa D. 2019. Archetypal roles of the PYR/PYL/RCAR-like receptor in drought and sugar responses in liverworts. *Plant Physiology* 179: 317-328.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6: 3901-3907.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Kannan N, Neuwald AF. 2005. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? Journal of Molecular Biology 351: 956-972.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Khandelwal A1, Cho SH, Marella H, Sakata Y, Perroud PF, Pan A, Quatrano RS. 2010. Role of ABA and ABI3 in desiccation tolerance. Science 327: 546.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell 72: 427-441.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, Coates D, Quatrano RS, Bahadur S, Stockley PG, Cuming AC. 1995. Molecular responses to abscisic acid and stress are conserved between moss and cereals. Plant Cell 7: 499-506.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Köhler M, Brummer T. 2016. B-Raf activation loop phosphorylation revisited. Cell Cycle 15: 1171-1173.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Komatsu K, Nishikawa Y, Ohtsuka T, Taji T, Quatrano RS, Tanaka S, Sakata Y. 2009. Functional analyses of the ABI1-related protein phosphatase type 2C reveal evolutionarily conserved regulation of abscisic acid signaling between Arabidopsis and the moss Physcomitrella patens. Plant Molecular Biology 70:327-340.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Komatsu K, Suzuki N, Kuwamura M, Nishikawa Y, Nakatani M, Ohtawa H, Takezawa D, Seki M, Tanaka M, Taji T et al. 2013. Group A PP2Cs evolved in land plants as key regulators of intrinsic desiccation tolerance. Nature Communications 4: 2219.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Kornev AP, Haste NM, Taylor SS, Ten Eyck LF. 2006. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proceedings of the National Academy of Sciences of the United States of America. 103:17783-17788.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Koster KL, Balsamo RA, Espinoza C, Oliver MJ. 2010. Desiccation sensitivity and tolerance in the moss Physcomitrella patens: assessing limits and damage. Plant Growth Regulation 62: 293-302.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Lin, Z, Li, Y., Zhang, Z, Liu X, Hsu C-C, Du Y, Sang T, Zhu C, Wang Y, Satheesh V et al. 2020. A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in higher plants. Nature Communications 11, 613.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Ma Y, Szostkiewicz, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science 324:1064-1068.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Marcotte WR Jr, Russell SH, Quatrano RS. 1989. Absciscic acid-responsive sequences from the Em gene of wheat. Plant Cell 1: 969-976.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Marella HH, Sakata Y, Quatrano RS. 2006. Characterization and functional analysis of ABSCISIC ACID INSENSITIVE3-like genes from Physcomitrella patens. Plant Journal 46: 1032-1044.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only Title Only Author and Title](#)

Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, Kolch W. 2011. Raf family kinases: old dogs have learned new tricks. Genes and Cancer 2: 232-260.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Minami A, Nagao M, Arakawa K, Fujikawa S, Takezawa D. 2003. Absciscic acid-induced freezing tolerance in the moss *Physcomitrella patens* is accompanied by increased expression of stress-related genes. *Journal of Plant Physiology* 160: 475-483.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Minami A, Nagao M, Ikegami K, Koshiba T, Arakawa K, Fujikawa S, Takezawa D. 2005. Cold acclimation in bryophytes: low-temperature-induced freezing tolerance in *Physcomitrella patens* is associated with increases in expression levels of stress-related genes but not with increase in level of endogenous absciscic acid. *Planta* 220: 414-423.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Minami A, Togawa S, Nagao M, Takezawa D. 2006. Altered freezing tolerance in the *Physcomitrella patens* mutant with reduced sensitivity to absciscic acid. *Cryobiology and Cryotechnology* 52: 135-139.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nishimura N, Sarkeshik A, Nito K, Park SY, Wang A, Carvalho PC, Lee S, Caddell DF, Cutler SR, Chory J et al. 2010. PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in *Arabidopsis*. *Plant Journal* 61: 290-299.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M. 2000. Tagged mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle mutagenesis. *DNA Research* 7: 9-17.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Niwa Y. 2003. A synthetic green fluorescent protein gene for plant biotechnology. *Plant Biotechnology* 20: 1-11.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nolen B, Taylor S, Ghosh G. 2004. Regulation of protein kinases: controlling activity through activation segment conformation. *Molecular Cell* 15: 661-675.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Tsz-fung FC et al. 2009. Absciscic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324:1068-1071.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud PF, Lindquist EA, Kamisugi Y et al. 2008. The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319: 64-69.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rock CD, Sakata Y, Quatrano RS. 2010. Stress Signaling I: The Role of Absciscic Acid (ABA). *Abiotic Stress Adaptation in Plants*. Springer, 33-73.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rodrigues A, Adamo M, Crozet P, Margalha L, Confraria A, Martinho C, Elias A, Rabissi A, Lumberras V, González-Guzmán et al. 2013. ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in *Arabidopsis*. *Plant Cell* 25: 3871-3884.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sakata Y, Komatsu K, Takezawa D. 2014. ABA as a universal plant hormone. *Progress in Botany* 75: 58-96.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sakata Y, Nakamura I, Taji T, Tanaka S, Quatrano RS. 2010. Regulation of the ABA-responsive Em promoter by ABI3 in the moss *Physcomitrella patens*: role of the ABA response element and the RY element. *Plant Signaling and Behavior* 5: 1061-1066.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Saruhashi M, Ghosh TK, Arai K, Ishizaki Y, Hagiwara K, Komatsu K, Shiwa Y, Izumikawa K, Yoshikawa H, Umezawa T et al. 2015. Plant Raf-like kinase integrates absciscic acid and hyperosmotic stress signaling upstream of SNF1-related protein kinase2. *Proceedings of the National Academy of Sciences of the United States of America* 112: E6388-6396.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sasaki K, Christov NK, Tsuda S, Imai R. 2013. Identification of a novel LEA protein involved in freezing tolerance in wheat. *Plant and Cell Physiology* 55:136-147.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shi Y, Ding Y, Yang S. 2018. Molecular regulation of CBF signaling in cold acclimation. Trends in Plant Science 23: 623-637.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shinozawa A, Otake R, Takezawa D, Umezawa T, Komatsu K, Tanaka T, Amagai A, Ishikawa S, Hara Y, Kamisugi Y et al. 2019. SnRK2 protein kinases represent an ancient system in plants for adaptation to a terrestrial environment. Communications Biology 2: 30.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Stevenson SR, Kamisugi Y, Trinh CH, Schmutz J, Jenkins JW, Grimwood J, Muchero W, Tuskan GA, Rensing SA, Lang D et al. 2016. Genetic analysis of *Physcomitrella patens* identifies ABSCISIC ACID NON-RESPONSIVE, a regulator of aba responses unique to basal land plants and required for desiccation tolerance. Plant Cell 28:1310-1327.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sun Y, Harpazi B, Wijerathna-Yapa A, Merilo E, de Vries J, Michaeli D, Gal M, Cuming AC, Kollist H, Mosquana A. 2019. A ligand-independent origin of abscisic acid perception. Proceedings of the National Academy of Sciences of the United States of America 116: 24892-24899.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Suzuki K, Shinshi H. 1995. Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. Plant Cell 7, 639-647.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Takahashi Y, Zhang J, Hsu P, Ceciliato PHO, Zhang L, Dubeaux G, Munemasa S, Ge C, Zhao Y, Hauser F, Schroeder JI. 2020. MAP3Kinase-dependent SnRK2-kinase activation is required for abscisic acid signal transduction and rapid osmotic stress response. Nature Communications 11, 12.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Thomashow MF. 2010. Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. Plant Physiology 154: 571-577.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Tougane K, Komatsu K, Bhyan SB, Sakata Y, Ishizaki K, Yamato KT, Kohchi T, Takezawa D. 2010. Evolutionarily conserved regulatory mechanisms of abscisic acid signaling in land plants: characterization of ABSCISIC ACID INSENSITIVE1-like type 2C protein phosphatase in the liverwort *Marchantia polymorpha*. Plant Physiology 152: 1529-1543.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K. 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 106: 17588-17593.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences of the United States of America 97: 11632-11637.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Laurière C, Merlot S. 2009. Plant cell protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. Plant Cell 21:3170-3184.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yasumura Y, Pierik R, Kelly S, Sakuta M, Voesenek LA, Harberd NP. 2015. An ancestral role for CONSTITUTIVE TRIPLE RESPONSE1 proteins in both ethylene and abscisic acid signaling. Plant Physiology 169:283-298.

Pubmed: [Author and Title](#)
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

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