

Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*

Taishi Umezawa^a, Naoyuki Sugiyama^b, Masahide Mizoguchi^{a,c}, Shimpei Hayashi^d, Fumiyoshi Myouga^e, Kazuko Yamaguchi-Shinozaki^c, Yasushi Ishihama^{b,f}, Takashi Hirayama^{d,g,1}, and Kazuo Shinozaki^{a,1}

^aGene Discovery Research Team, RIKEN Plant Science Center, 3-1-1 Kouyadai, Tsukuba, Ibaraki 305-0074, Japan; ^bInstitute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0017, Japan; ^cGraduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; ^dInternational Graduate School of Arts and Sciences, Yokohama City University, 1-7-29 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045 Japan; ^eR&D program, RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan; ^fPRESTO, Japan Science and Technology Agency, 5-Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan; and ^gRIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan

Edited by Joseph R Ecker, Salk Institute for Biological Studies, La Jolla, CA, and approved August 24, 2009 (received for review June 25, 2009)

Abscisic acid (ABA) signaling is important for stress responses and developmental processes in plants. A subgroup of protein phosphatase 2C (group A PP2C) or SNF1-related protein kinase 2 (subclass III SnRK2) have been known as major negative or positive regulators of ABA signaling, respectively. Here, we demonstrate the physical and functional linkage between these two major signaling factors. Group A PP2Cs interacted physically with SnRK2s in various combinations, and efficiently inactivated ABA-activated SnRK2s via dephosphorylation of multiple Ser/Thr residues in the activation loop. This step was suppressed by the RCAR/PYR ABA receptors in response to ABA. However the *abi1-1* mutated PP2C did not respond to the receptors and constitutively inactivated SnRK2. Our results demonstrate that group A PP2Cs act as 'gatekeepers' of subclass III SnRK2s, unraveling an important regulatory mechanism of ABA signaling.

protein phosphorylation | LC-MS | SnRK2 | PYR1

As sessile organisms, plants have to rapidly recognize and adapt to environmental changes. The phytohormone abscisic acid (ABA) plays a central role in such responses (1, 2), and is also involved in many developmental processes (3) and defense systems (4). Thus, ABA functions as a key molecule that unifies and regulates biotic and abiotic stress responses and the developmental status of the plant. Hence, the biological and agricultural importance of ABA has led to extensive studies on its signaling mechanism, and many putative signal transducers have been reported (5). Although it has been difficult to integrate all of the current findings, significant progress was recently made by two independent research groups. They identified the RCAR/PYR family proteins as ABA receptors that inhibit protein phosphatase 2C (PP2C) in an ABA-dependent manner (6, 7). Among plant PP2Cs, a group A subfamily (e.g., ABI1 and ABI2) is annotated as negative regulators of the ABA response in seeds through to adult plants (5). Such PP2C-dependent negative regulation can be canceled by RCAR/PYR in response to ABA, leading to activation of some positive regulatory pathways (6, 7). Previously, we demonstrated that ABI1 interacts with a protein kinase, SRK2E (OST1/SnRK2.6) (8). SRK2E belongs to the SNF1-related protein kinase 2 (SnRK2) family, which is activated by ABA or osmotic stress and positively regulates the ABA response in various tissues (9–11). Furthermore, ABA-dependent activation of SRK2E was repressed in an *abi1-1* mutant, suggesting that SnRK2 functions downstream of PP2C (8, 9). Based on these findings, a model was hypothesized in which RCAR/PYR and PP2C negatively regulate SnRK2 (7). However, there is no direct evidence demonstrating how PP2C regulates SnRK2, and the molecular process between them remains a question in ABA signaling. Our presented data clearly demonstrated the biochemical relation between PP2C and SnRK2 and elucidated an important regulatory mechanism of ABA signaling.

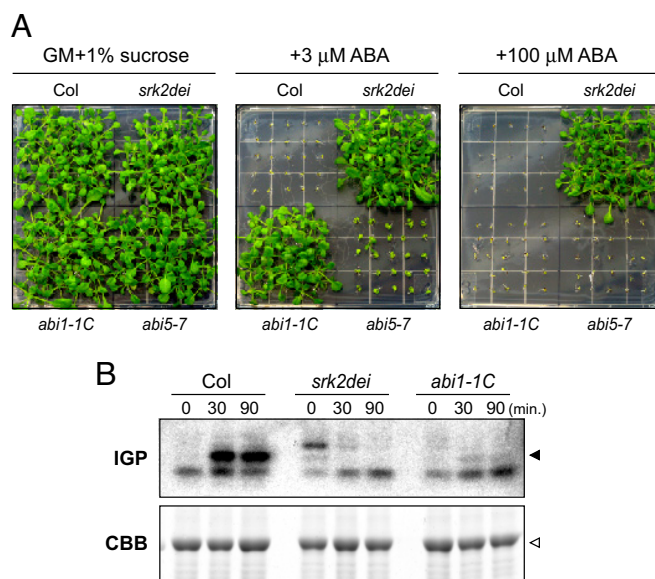


Fig. 1. Subclass III SnRK2s are essential for ABA responses. (A) Germination test to determine ABA sensitivity in the *srk2dei* triple mutant. *Arabidopsis* seeds of Col, *srk2dei*, *abi1-1C*, and *abi5-7* were sown and grown for 2 weeks on GM agar medium containing 0, 3, or 100 μ M ABA. (B) ABA-activated protein kinases in Col, *srk2dei*, and *abi1-1C* plants. Three-week-old plants were treated with 50 μ M ABA for 0, 30, or 90 min. Upper panel shows in-gel phosphorylation (IGP) assay using histone as a substrate. Black arrow indicates ABA-activated protein kinase (\approx 40 kDa) overlapping with SnRK2s, and the open arrow indicates the RubisCo large subunit (\approx 50 kDa) in a CBB-stained gel. The results were confirmed through several replications.

Results and Discussion

Requirement of SnRK2 Activity for ABA Responses. In *Arabidopsis*, subclass III of the SnRK2 family is composed of three proteins, namely SRK2D (SnRK2.2), SRK2I (SnRK2.3), and SRK2E (OST1/SnRK2.6). To further investigate the functions of SnRK2s, we established a triple knockout mutant for subclass III SnRK2s (*srk2dei*). Recently, some researchers independently established the same triple mutant, and its phenotype was

Author contributions: T.U., T.H., and K.S. designed research; T.U., N.S., M.M., S.H., F.M., and T.H. performed research; N.S., K.Y.-S., and Y.I. contributed new reagents/analytic tools; T.U., N.S., S.H., and F.M. analyzed data; and T.U., Y.I., and T.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹To whom correspondence may be addressed. E-mail: takashih@riken.jp or sinozaki@rtc.riken.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0907095106/DCSupplemental.

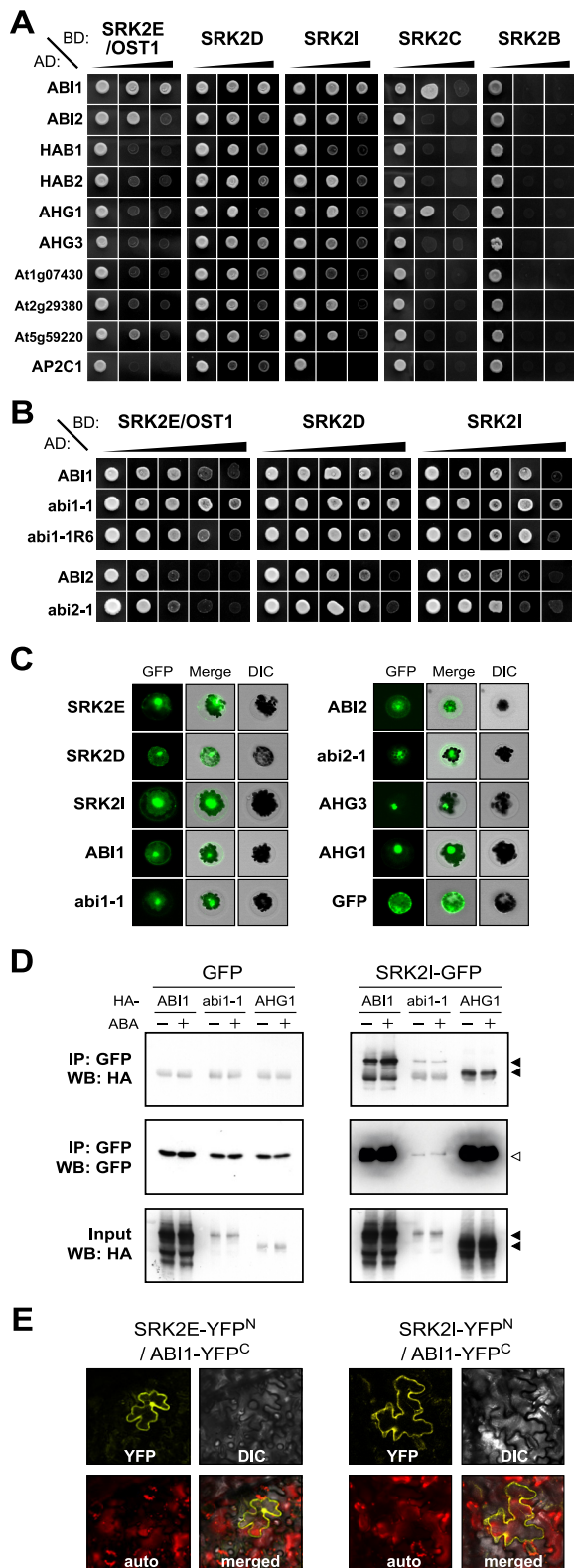


Fig. 2. Group A PP2Cs interact with subclass III SnRK2s. (A) Yeast two-hybrid analysis of SnRK2s and PP2Cs. GAL4BD:SnRK2s and GAL4AD:PP2Cs were introduced to yeast cells as indicated. Black slopes indicate screening stringency of SD media supplemented with: left, -LW; center, -LWH + 10 mM 3-AT; right, -LWHA + 30 mM 3-AT. (B) Yeast two-hybrid analysis of PP2C mutations. ABI1, *abi1-1*, *abi1-1R6*, ABI2, and *abi2-1* were tested against subclass III SnRK2s. The black slope demonstrates stringency of screening media: SD -LW, SD -LWH + 10 mM 3-AT, SD -LWHA + 30, 60, or 100 mM 3-AT from left to right. (C) Subcellular localization of GFP-tagged SnRK2 or PP2C proteins that were transiently

thoroughly analyzed (12, 13). They showed the triple mutation completely impaired the ABA response, suggesting critical roles of subclass III SnRK2s and their fundamental redundancy in ABA signaling. In our study, we tested ABA responsiveness in the *srk2dei* mutant to compare with other ABA-insensitive mutants, *abi1-1C* and *abi5-7*. Consistent with a previous report, *srk2dei* showed marked ABA insensitivity, even in the presence of 100 μ M ABA (Fig. 1A). Furthermore, in-gel phosphorylation assay demonstrated the loss of ABA-activated kinase activity (approx. 40 kDa) in *srk2dei*, and a low level of activity in *abi1-1C* (Fig. 1B), showing a correlation with their ABA sensitivity. Although some kinase activities were observed in *srk2dei*, they might be unknown kinases that were non-functional in wild-type plants. These data imply that all subclass III SnRK2s are strongly inhibited by *abi1-1* mutation, and the SnRK2 activity should be major determinant of the ABA response in *Arabidopsis*.

Physical Interaction Between PP2C and SnRK2. Our observations of *srk2dei* suggested that regulation of SnRK2 activity is a key to understanding how ABA signals are transmitted. As described above, we identified PP2C as a potential regulator of SnRK2, because ABI1 interacted with the domain II of SRK2E in the yeast two-hybrid (Y2H) system (8). In this study, we further tested five SnRK2s and 10 PP2Cs by Y2H assay. Consistent with our previous study, ABI1 strongly bound to SRK2E, and some other PP2Cs (e.g., ABI2 and At5g59220) also interacted with SRK2E (Fig. 2A). Compared with SRK2E, SRK2D and SRK2I showed a wide preference for various group A PP2Cs. Other SnRK2s (not subclass III) or an outgroup of PP2C, AP2C1 (group B), showed limited or no interaction (Fig. 2A). These results indicate that group A PP2Cs interact specifically with SnRK2s in various combinations. It should be noted that some of the Y2H results were not consistent with our previous study (8). This discrepancy may be related to technical problems, for which reason we confirmed our results through several replicates.

The physical interaction between SnRK2s and PP2Cs observed in the Y2H assay was partly supported by gene expression patterns and subcellular localizations. SRK2E and ABI1 were co-expressed in guard cells (Figs. S1 and S2) and were localized in the cytosol or nucleus (Fig. 2C). SRK2D and SRK2I were significantly expressed in seeds together with AHG1 or AHG3 which were localized in the nucleus predominantly (Fig. 2C and Fig. S1). These data were consistent with their regulatory roles in guard cells or seeds (11, 14, 15), showing a correlation between physiological function and Y2H results. To confirm these interactions *in vivo*, we performed co-immunoprecipitation (Co-IP) experiments using protoplasts expressing SRK2I-GFP and HA-tagged ABI1 or AHG1. As shown in Fig. 2D, these PP2Cs were co-immunoprecipitated with SRK2I in an ABA-independent manner, suggesting that they interact constantly *in vivo*. The co-expression of some pairs, for example, SRK2E and ABI1, was almost undetectable in protoplasts, making it impossible to demonstrate their interaction in Co-IP experiments. However, BiFC analysis confirmed the *in planta* interaction between SRK2E and ABI1 in the cytosol and nucleus, similar to SRK2I and ABI1 (Fig. 2E). The *abi1-1* mutation causes a substitution of Gly to Asp (G180D) in the PP2C catalytic domain, which

expressed in *Arabidopsis* mesophyll protoplasts. (D) Co-immunoprecipitation of GFP-tagged SnRK2s and HA-tagged PP2Cs was performed using *Arabidopsis* protoplasts. Immunoprecipitates against anti-GFP antibody (IP) or crude extracts (input) were analyzed via Western blotting (WB) using anti-GFP or -HA antibodies. (E) BiFC analyses were performed for the pairs of SRK2E-YFP^N/ABI1-YFP^C or SRK2I-YFP^N/ABI1-YFP^C in epidermal cells of fava bean leaves. The photos were YFP, DIC, autofluorescence (auto) and merged images. The results were confirmed through several replications.

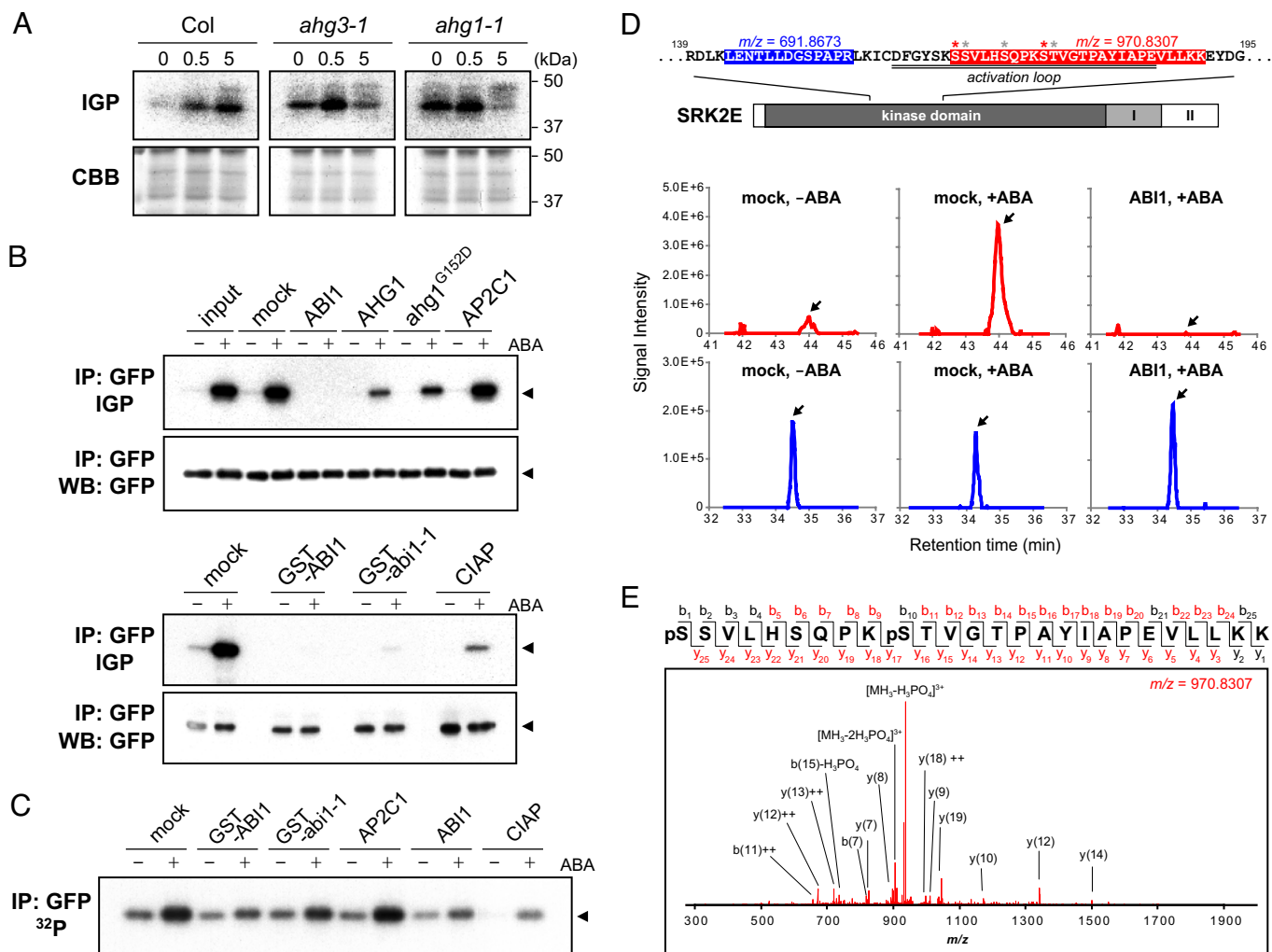


Fig. 3. PP2C directly regulates SnRK2. (A) ABA-activated protein kinase activity in imbibed seeds of Col, *ahg3-1*, and *ahg1-1* plants treated with 25 μ M ABA for 0, 0.5, or 5.0 h. Upper panels show in-gel phosphorylation assay (IGP) using histone as a substrate. Lower panels are CBB-stained gel images. (B) Inactivation assay for SnRK2 by in vitro reaction with PP2Cs. SRK2E-GFP proteins were prepared by immunoprecipitation (IP) against an anti-GFP antibody from *Arabidopsis* cultured cells with or without ABA treatment. After incubation with recombinant PP2C proteins as indicated, SRK2E activity was monitored with an in-gel phosphorylation assay (IGP) using histone as a substrate. Immunoprecipitates were analyzed via Western blotting (WB) using an anti-GFP antibody. Arrows indicate SRK2E-GFP. (C) Dephosphorylation of 32 P-labeled SRK2E-GFP by PP2Cs. Immunoprecipitated SRK2E-GFP was prepared from untreated or ABA-treated cultured cells labeled with 32 P-phosphate, and incubated with PP2Cs as indicated. The phosphorylation level of SRK2E-GFP was monitored by autoradiography. (D) Immunoprecipitated SRK2E-GFP was analyzed with a nanoLC-MS/MS system. As examples, a non-phospho-peptide of $m/z = 691.8673$ (blue) and a phospho-peptide of $m/z = 970.8307$ (red) are found in the structure of SRK2E and in extracted ion current (XIC) chromatograms. Arrows in XICs indicate the target peaks. Asterisks in red and black indicate probable phosphorylation sites detected in this study with the highest and less PTM scores, respectively. (E) An MS/MS spectrum derived from a phospho-peptide of $m/z = 970.8307$. Fragmented ions detected in this analysis are shown in red, and the top 15 ions were annotated in the spectrum. The results were confirmed through several replications.

confers a dominant ABA-insensitivity by unknown mechanisms (16, 17). Although very little *abi1-1* was produced in protoplasts, the data revealed that *abi1-1* was able to bind to SRK2I constantly (Fig. 2D). This result was supported by the Y2H assay, in which *abi1-1* and *abi2-1* showed some enhanced interaction with SnRK2s, whereas *abi1-1R6*, a loss-of-function mutation for ABI1 (18), had no effect (Fig. 2B).

Group A PP2C Inactivates Subclass III SnRK2. Previous studies showed that the ABA-responsive activation of SRK2E was inhibited in the *abi1-1* mutant (8, 9). In addition, our data further demonstrated that all subclass III SnRK2s were inhibited by *abi1-1* mutation (Fig. 1B), implying that subclass III SnRK2s function downstream of PP2C. To clarify this regulation, we examined SnRK2 activity in imbibed seeds from *ahg1-1* and *ahg3-1* which were loss-of-function mutations of PP2C causing

ABA-hypersensitive germination (14, 15). The results revealed that approximately 40 kDa of protein kinase activity involving SnRK2 were hyperactivated in *ahg1-1* and *ahg3-1* (Fig. 3A), suggesting that PP2C negatively regulates SnRK2 activity. Next, we examined whether PP2C directly regulates SnRK2 activity using an in vitro phosphatase assay with recombinant PP2C proteins and GFP-tagged SnRK2s from *Arabidopsis* cells. SnRK2 activity was determined with an in-gel phosphorylation assay in which SnRK2-GFP was clearly activated by ABA (Fig. 3B and Fig. S3A). The activity of SRK2E or SRK2I was efficiently or significantly reduced by ABI1 or AHG1, and, surprisingly, ABI1-mediated inactivation was stronger than that of CIAP (Fig. 3B and Fig. S3A). Dominant-type PP2C mutants such as *abi1-1* and *ahg1^{G152D}* inactivated SnRK2 significantly (Fig. 3B and Fig. S3A), implying they are capable of regulating SnRK2 negatively as well as wild-type proteins. Because it was

previously proposed (7), and simplify the molecular process of ABA signaling to just four steps from perception to gene expression (Fig. 4B). First, PP2C interacts with domain II at the C terminus of SnRK2 (8); this interaction is constant and independent of ABA. In the absence of ABA, PP2Cs repress the ABA signaling pathway via the dephosphorylation and inactivation of SnRK2s. After the induction of free ABA by environmental or developmental cues, RCAR/PYR binds to PP2C (6, 7) and SnRK2 is released from PP2C-dependent negative regulation. This allows SnRK2 to phosphorylate downstream substrates, including bZIP transcription factors (AREB/ABFs) (26) etc., to activate ABA-responsive gene expression or other responses. In contrast, dominant mutations in PP2C, such as *abi1-1*, allow the protein to avoid RCAR/PYR-binding (6, 7), resulting in the constitutive inactivation of SnRK2s. At present, the mechanisms underlying the internal phosphorylation of SnRK2 remain unclear, although autophosphorylation is one of possible regulations (2). Further analysis will be required to clarify this issue.

We argue that the RCAR/PYR-PP2C-SnRK2 complex is the primary framework for ABA signaling, and that a conventional protein phosphorylation/dephosphorylation system, PP2C-SnRK2, provides a reversible regulatory module. Recently, group A PP2Cs and AREB/ABF bZIP transcription factors were found in *Physcomitrella patens* (27–29). Furthermore, our computational analysis detected subclass III SnRK2s in *P. patens*, but not in *Chlamydomonas reinhardtii* (Fig. S7). Thus, the PP2C-SnRK2-mediated signal transduction system may be conserved from bryophytes to higher plants, suggesting this system is an important acquired characteristic in the evolution of land plants with respect to drought tolerance.

Materials and Methods

Plant Materials. The *srk2dei* triple mutant was generated from *srk2e* (SALK_008068), *srk2d* (GABI-Kat.807G04), and *srk2i* (SALK_096546), which were obtained from public sources (ABRC or GABI-Kat). The *abi1-1C* was isolated from a genetic screen of Col background lines. The ABA response test was performed as described previously (15).

Plasmids. In this study, a series of vector plasmids, such as pGBKT7 and pGADT7 (Clontech), pGEX6P (GE Healthcare), and the pGreen series (30), were converted to Gateway destination vectors. Gateway entry clones were prepared in the pENTR/D-TOPO or pDONR207 vector (Invitrogen) by insertion of cDNAs, SRK2E, SRK2D, SRK2I, SRK2C, SRK2B, ABI1, ABI2, HAB1, HAB2, AHG1, AHG3, At1g07430, At3g29380, At5g59220, AP2C1, and PYR1. Mutated PP2Cs [i.e., *abi1-1* (G180D), *abi1-1R6* (G307D), *abi2-1* (G168D), and *ahg1^{G152D}*] were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with entry clones as templates.

Yeast Two-Hybrid Analysis. Yeast two-hybrid analysis was carried out using the MatchMaker GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer's instructions. A yeast strain (AH109) was transformed with various pairs of pGBKT7 vectors harboring SnRK2s and pGADT7 vectors harboring PP2Cs. Then, the transformants were tested on SD screening medium as indicated in Fig. 2.

In-Gel Phosphorylation Assay for Seed Proteins. Dry seeds (50 mg) of Col, *ahg1-1*, and *ahg3-1* were imbibed in water and placed at 4 °C for 3 days. Then imbibed seeds were treated with 25 μM (±)-ABA (Sigma) for 0, 1, and 5 h. Seeds were crushed by a mortar and pestle with silica sand in liquid nitrogen and homogenized in 100 μL extraction buffer containing 50 mM HEPES pH 7.5,

5 mM EDTA, 5 mM EGTA, 1 mM Na₃VO₄, 25 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 2 mM DTT, and proteinase inhibitor mixture (Sigma). Crude extracts containing 50 μg total protein were loaded in 10% SDS-polyacrylamide and analyzed by in-gel kinase assay to check ABA-activated protein kinases as described previously (31).

Transient Expression in Arabidopsis Mesophyll Protoplasts. The preparation of protoplasts from *Arabidopsis* rosette leaves and PEG-calcium transfection were performed according to ref. 32. We prepared pGreen0029 vectors harboring GFP, SRK2E-GFP, SRK2I-GFP, ABI1-HA, *abi1-1*-HA, or AHG1-HA. For subcellular localization, 20 μg plasmid DNAs were transfected into 3 × 10⁴ protoplasts, and GFP signals were observed with a laser-scanning confocal fluorescence microscopy. For co-immunoprecipitation (Co-IP) assay, a total of 2 mg plasmid DNA mixture with SnRK2-GFP and PP2C-HA was transfected into 3 × 10⁶ protoplasts. Crude extracts were obtained by homogenizing protoplasts in 1 mL extraction buffer supplemented with 100 mM KCl, 50 mM NaCl, and 0.1% Nonidet P-40 (IP buffer). Co-IP was performed with 100 μL Protein G Sepharose (GE Healthcare) and 1 μL polyclonal anti-GFP antibody (MBL), and aliquots were analyzed by Western blot analysis using monoclonal anti-GFP antibody (MBL) or anti-HA antibody (Covance).

Bimolecular Fluorescence Complementation (BiFC) in Fava Bean Leaves. The cDNAs of SRK2E, SRK2I, and ABI1 were cloned into pUC-SPYNE^{GW} or pUC-SPYCE^{GW} (33). The constructs were delivered into leaf cells of fava bean (*Vicia faba*) by particle bombardment, as previously described (34), with modifications.

In Vitro Phosphatase Reaction. Bacterial recombinant PP2C proteins and *Arabidopsis* T87 cells expressing SRK2E- or SRK2I-GFP were prepared as previously described (8, 14). T87 cells were treated with or without 50 μM ABA for 30 min, and IP was performed as described above. After washing, the buffer was exchanged for phosphatase reaction buffer (8), and then aliquots (30 μL) were mixed with 100 ng recombinant PP2C proteins, ABI1, AHG1, *ahg1^{G152D}*, AP2C1, or 300 ng GST-ABI1 and GST-*abi1-1*, or 1 U CIAP (Toyobo). After incubation at 30 °C for 1 h, half aliquots were analyzed with an in-gel phosphorylation assay or Western blotting.

In Vivo Labeling of Arabidopsis Cells. To check the phosphorylation status of SnRK2, *Arabidopsis* T87 cells expressing SRK2E- or SRK2I-GFP were labeled with 30 MBq/ml ³²P-phosphate (NEX053H, Perkin-Elmer) for 2 h and then treated with or without 100 μM ABA for 30 min. IP and in vitro phosphatase reactions were performed as described above. After SDS/PAGE, radioactivity was detected by autoradiography.

In Vitro Reconstitution Assay of ABA Signaling Components. Immunoprecipitated SRK2E- or SRK2I-GFP (bed vol. 30 μL) were incubated with 500 ng GST-ABI1 or GST-*abi1-1*, 100 ng ABI1, and 800 ng PYR1 proteins in the presence or absence of 10 μM ABA at 30 °C for 1 h. Recombinant PP2Cs and PYR1 were preincubated with ABA for 10 min in advance. Aliquots were analyzed by in-gel phosphorylation assay or Western blotting as described above.

NanoLC-MS/MS Analysis. Immunoprecipitated SnRK2-GFP proteins were prepared as described above and analyzed with a nanoLC-MS/MS system (LTQ-Orbitrap, Thermo) according to ref. 35. The details of experiments are described in *SI Text*.

ACKNOWLEDGMENTS. We thank Mrs. Hiroko Kobayashi, Saho Mizukado (RIKEN), and Sumiko Onuma (Keio University) for technical assistance. We are grateful to ABRC and GABI-Kat project for providing *Arabidopsis* T-DNA-tagged mutants. We also thank Prof. Masaru Tomita at Keio University for his support. This work was partly supported by the RIKEN Plant Science Center, Grants-in-Aid for Scientific Research for Young Scientists (B) to T.U. and Grants-in-Aid for Scientific Research (C) to T.H. from MEXT, and research funds from Yamagata prefecture and Tsuruoka City to Keio University.

1. Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol* 57:781–803.
2. Sirichandra C, Wasilewska A, Vlad F, Valon C, Leung J (2009) The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. *J Exp Bot* 60:1439–1463.
3. Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* 59:387–415.
4. Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. *Curr Opin Plant Biol* 8:409–414.
5. Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: Keys to the function of the versatile plant hormone ABA. *Trends Plants Sci* 12:343–351.
6. Ma Y, et al. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324:1064–1068.
7. Park S, et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324:1068–1071.
8. Yoshida R, et al. (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in *Arabidopsis*. *J Biol Chem* 281:5310–5318.

9. Mustilli A, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14:3089–3099.
10. Yoshida R, et al. (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol* 43:1473–1483.
11. Fujii H, Verslues PE, Zhu J (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* 19:485–494.
12. Fujii H, Zhu J (2009) *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc Natl Acad Sci USA* 106:8380–8385.
13. Nakashima K, et al. (2009) Three *Arabidopsis* SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1, and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol* 50:1345–1363.
14. Yoshida T, et al. (2006) ABA-Hypersensitive Germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among *Arabidopsis* protein phosphatase 2Cs. *Plant Physiol* 140:115–126.
15. Nishimura N, et al. (2007) ABA-Hypersensitive Germination1 encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in *Arabidopsis* seed. *Plant J* 50:935–949.
16. Leung J, et al. (1994) *Arabidopsis* ABA response gene ABI1: Features of a calcium-modulated protein phosphatase. *Science* 264:1448–52.
17. Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* 264:1452–5.
18. Gosti F, et al. (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* 11:1897–1910.
19. Kobayashi Y, Yamamoto S, Minami H, Kagaya Y, Hattori T (2004) Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* 16:1163–1177.
20. Burza AM, et al. (2006) *Nicotiana tabacum* osmotic stress-activated kinase is regulated by phosphorylation on Ser-154 and Ser-158 in the kinase activation loop. *J Biol Chem* 281:34299–34311.
21. Belin C, et al. (2006) Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol* 141:1316–1327.
22. Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 67:821–855.
23. Schweighofer A, et al. (2007) The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in *Arabidopsis*. *Plant Cell* 19:2213–2224.
24. Park C, et al. (2008) Rice XB15, a protein phosphatase 2C, negatively regulates cell death and XA21-mediated innate immunity. *PLoS Biol* 6:e231.
25. Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: Emerging functions in stress signaling. *Trends Plants Sci* 9:236–243.
26. Furihata T, et al. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci USA* 103:1988–1993.
27. Knight CD, et al. (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. *Plant Cell* 7:499–506.
28. Rensing SA, et al. (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319:64–69.
29. Komatsu K, et al. (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 Mol Biol* 70:341–357.
30. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 42:819–832.
31. Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 101:17306–17311.
32. Yoo S, Cho Y, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat Protoc* 2:1565–1572.
33. Walter M, et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40:428–438.
34. Myouga F, et al. (2008) A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. *Plant Cell* 20:3148–3162.
35. Sugiyama N, et al. (2007) Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Mol Cell Proteomics* 6:1103–1109.