A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in higher plants

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Osmoregulation is important for plant growth, development and response to environmental changes. SNF1-related protein kinase 2s (SnRK2s) are quickly activated by osmotic stress and are central components in osmotic stress and abscisic acid (ABA) signaling pathways; however, the upstream components required for SnRK2 activation and early osmotic stress signaling are still unknown. Here, we report a critical role for B2, B3 and B4 subfamilies of Raf-like kinases (RAFs) in early osmotic stress as well as ABA signaling in Arabidopsis thaliana. B2, B3 and B4 RAFs are quickly activated by osmotic stress and are required for phosphorylation and activation of SnRK2s. Analyses of high-order mutants of RAFs reveal critical roles of the RAFs in osmotic stress tolerance and ABA responses as well as in growth and development. Our findings uncover a kinase cascade mediating osmoregulation in higher plants.

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https://doi.org/10.1038/s41467-020-14477-9
Drought, high salinity, and low temperatures cause osmotic stress to plants, greatly limiting plant productivity1–3. Osmoregulation is essential for plants to cope with environmental challenges and is also required for plant growth and development. SNF1-related protein kinase 2s (SnRK2s) are critical for osmotic stress responses4–5. Three of the ten SnRK2 family members, SnRK2.2, SnRK2.3, and SnRK2.6, are core components in the signaling pathway of abscisic acid (ABA)6,7, a phytohormone that accumulates in plants subjected to osmotic stress8–11. In the absence of ABA, SnRK2.2/3/6 are inhibited by clade A protein phosphatase 2Cs (PP2Cs) through dephosphorylation12–14. Upon hyperosmotic stress, ABA accumulates and binds to its receptors, the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) family proteins, which subsequently inhibit PP2C activity, resulting in the release of SnRK2s from inhibition. Current models suggest that SnRK2.6 is self-activated by autophosphorylation after release from PP2C-mediated inhibition15. The activated SnRK2s phosphorylate downstream effectors to mediate stress responses16,17.

All members of the SnRK2 family, except SnRK2.9, are also activated by osmotic stress4. The snrk2.1/2/3/4/5/6/7/8/9/10 double mutant, which lacks all ten members of the SnRK2 family, is hypersensitive to osmotic stress5. Neither the snrk2.2/2/3/6 triple nor snrk2.1/4/5/7/8/9/10 septuple mutant has an obvious osmotic stress-sensitive phenotype, suggesting redundancy among SnRK2s in the osmotic stress response5. Osmotic-stress-mediated SnRK2 activation is independent of the ABA signaling pathway4,5,18,19. In ABA insensitive 1 (abi1-1), a dominant mutant where the ABI1 PP2C phosphatase cannot be inhibited by PYR/PYL/ACAR, or in high-order mutants of PYR/PYL/ACAR ABA receptors, osmotic stress-induced SnRK2 activation is not reduced18,20, while ABA-induced SnRK2 activation is abolished20,21. How the SnRK2s are activated by osmotic stress is a major unanswered question.

RAF-like protein kinases (RAFs) have been classified as mitogen activated protein kinase kinase kinases (MAPKKKs) in plants22,23. According to sequence similarity, RAF-like protein kinases are classified into four B and seven C subgroups22. In the moss Physcomitrella patens, ABA and abiotic stress-responsive RAF-like Kinase (ARK), a B3 subfamily RAF-like kinase, participates in the regulation of both ABA and hyperosmotic stress responses by phosphorylating PpSnRK224. The Arabidopsis thaliana genome contains 80 genes encoding RAF-like protein kinases, including four members of the B1 subgroup, six members of the B2 subgroup, six members of the B3 subgroup, and seven members of the B4 subgroup. One B4 subfamily member, Hydraulic Conductivity of Root 1 (HCR1), is involved in a potassium-dependent response to hypoxia25. In Arabidopsis, mutants of several members of the B2 and B3 families of RAF-like protein kinases such as ctr1, raf10 and raf11, are insensitive to ABA26,27, and the sis8 mutant is hypersensitive to salt stress28. The phosphorylation of a B4 RAF-like protein kinase, AT1G16270, is up-regulated by mannitol treatment in Arabidopsis29. However, whether RAF-like protein kinases function in SnRK2 activation, and in osmotic stress and/or ABA signaling in higher plants remains unknown.

Here, we report a critical role for some RAF-like kinases in early osmotic stress as well as ABA signaling in Arabidopsis thaliana. B2, B3 and B4 RAFs are very quickly activated by osmotic stress and are required for phosphorylation and activation of SnRK2s. Analyses of high-order mutants of RAfs reveal critical roles of these RAfs in osmotic stress tolerance and ABA responses as well as in plant growth and development. Our findings uncover an upstream kinase cascade mediating osmoregulation and ABA signaling in higher plants.

**Results**

Osmotic stress activates protein kinase OKs. To investigate the phosphorylation events in early osmotic stress signaling, we used in-gel kinase assays to measure kinase activation upon hyperosmotic stress caused by mannitol treatment22,30. Four groups of protein kinases were activated by mannitol treatment and ABA (Fig. 1a). SnRK2.2/3/6 (approximately 40 to 42 kDa) were strongly activated by both ABA and osmotic stress, while the ABA-independent SnRK2.1/4/5/9/10 (37 to 40 kDa) were strongly activated only by osmotic stress (Fig. 1b). In addition to the SnRK2s, we found two groups of protein kinases of approximately 130 and 100 kDa that were strongly activated by osmotic stress but not ABA (Fig. 1a). We termed these kinases osmotic stress-activated protein kinases (OKs). Strong activation of the 130-kDa OKs (OK130) was observed at 2.5 min after mannitol treatment, peaking at 5 min (Fig. 1a). Activation of the 100-kDa OKs (OK100) was clearly detectable after 5 min of mannitol treatment (Fig. 1a). Rapid OK activation in response to osmotic stress suggests a role for these kinases in early osmotic stress signaling. Activation of the OKs did not require SnRK2s and was independent of ABA signaling, since OK activation by mannitol treatment was still observed in the snrk2.2/2/3/6 triple (snrk2-triple) mutant, which is deficient in ABA signaling, and in snrk2.1/4/5/7/8/9/10 septuple...
Identification of OKs by quantitative phosphoproteomics. To determine the identity of the OKs, we used phosphoproteomic analysis to examine phosphopeptides from both the wild-type and snrk2-dec mutant plants after 30 min of mannitol treatment (Fig. 2a, Supplementary Fig. 1, Supplementary Data 1 to 5), since we expected that the OKs would be autophosphorylated upon activation. Twenty-one phosphosites in 18 protein kinases were found to be up-regulated by mannitol treatment in both the wild-type and snrk2-dec mutant (Fig. 2a, Supplementary Data 4 and 5). These included several phosphosites in Raf-like protein kinases. B4 Raf-like kinases (117 to 140 kDa, see Fig. 2b) have an N-terminal PB1 domain and a C-terminal kinase domain. Phosphopeptides from six of the seven B4 Raf-like kinases were significantly up-regulated by osmotic stress, in both the wild-type and snrk2-dec mutant (Fig. 2c, see also Supplementary Data 4 and 5). Several phosphosites in members of the B2 and B3 subfamilies of the Raf-like kinases, RAF4 (AT1G18160), RAF5/Sugar Insensitive 8(SIS8), RAF2/Enhanced Disease Resistance 1(EDR1), RAF11, and RAF10, were also present in the list of mannitol-induced phosphopeptides (Fig. 2d, Supplementary Data 4). Members of the B2 and B3 groups have molecular weights from 75 to 112 kDa. Some of the phosphosites from the RAFs were located in the activation loop of these kinases. Phosphorylation in the activation loop is a conserved mechanism of protein kinase activation. Taking these results together, we hypothesized that members of the B4 Raf-like kinases may correspond to the OK130, and that members of the B2 and B3 Raf-like kinases may be the OK100.

Mutational analyses identify the OKs as Raf-like kinases. To validate our hypothesis, we first determined the activation of OKs

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**Fig. 2 Identification of OKs by phosphoproteomics.** a Heat map showing relative intensity of phosphosites from protein kinases in control and mannitol-treated wild-type and snrk2-dec mutant seedlings. b Phylogenetic tree of B2, B3, and B4 RAFs. c Quantitative analysis of phosphopeptides from B4 Raf-like kinases in seedlings with or without mannitol treatment. Error bars, SEM (n = 3). Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001. d Quantitative analysis of phosphopeptides from B2 and B3 Raf-like kinases in seedlings with or without mannitol treatment. Two-tailed paired t-tests, **p < 0.01, ***p < 0.001. N.D., not identified in the samples. Source data are provided in Supplementary Data 4 and 5.
and SnRK2s in single mutants of the B4 Raf-like kinases. The overall signals of OKs in the single mutants raf16, raf40/hcr1, raf24, raf18, raf20, raf35 and raf42, were comparable to those in the wild-type (Supplementary Fig. 2a). Using Clusters of Regularly Interspaced Short Palindromic Repeats/CRISPR-associated 9 (CRISPR/Cas9)-mediated genome editing in a T-DNA insertion mutant line raf16 (salk_007884), we generated a high-order mutant, OK130-weak, containing frameshift/early termination mutations in raf40/hcr1, raf18, raf20 and raf35, and 30 and 18 bp deletions in raf24 and raf42, respectively (Fig. 3a, Supplementary Fig. 2b, c). OK130 activation was strongly, although not completely, impaired in the OK130-weak mutant (Supplementary Fig. 2d, f), supporting our hypothesis that the B4 subfamily represents OK130. The mannitol-induced activation of SnRK2.1/4/5/9/10 was markedly, but not completely abolished in OK130-weak (Fig. 3c), suggesting that the activation of SnRK2.1/4/5/9/10 is dependent on OK130. OK100 activation was also weakened or delayed in the OK130-weak mutant, when compared to that in the wild-type (Supplementary Fig. 2d).

To eliminate the remaining OK130 activity, we introduced additional mutations into OK130-weak using a second CRISPR/Cas9 construct containing additional guide RNAs targeting the S regions of raf24 and raf42 and isolated an OK130-weak/null mutant with frameshift/null mutations in all seven members of the subfamily (Fig. 3a and Supplementary Fig. 2e). As expected, osmotic stress-activation of OK130 and SnRK2.1/4/5/9/10 was completely abolished in the OK130-null mutant (Supplementary Fig. 2f). These results further support that the B4 subfamily Raf-like kinases correspond to the OK130, and show that the OK130 genes redundantly control the activation of SnRK2.1/4/5/9/10 upon osmotic stress. By contrast, the activation of SnRK2.2/3/6 by ABA and osmotic stress in the OK130-null mutant was comparable to that in the wild-type (Supplementary Fig. 2f), and the activation of the OK100 upon osmotic stress was only partially impaired in the OK130-null mutant (Supplementary Fig. 2f). This suggests that osmotic stress-induced activation of OK100 and SnRK2.2/3/6 is not dependent on OK130.

To further identify the OK100 and study how osmotic stress activates SnRK2.2, SnRK2.3 and SnRK2.6, we generated high-order mutants by introducing mutations into B2 and B3 subfamily members in wild-type and OK130 mutant backgrounds (Fig. 3a). As OK130-null plants produce few seeds (Supplementary Fig. 2g, h), we had to use the OK130-weak plants to generate higher order mutants. Gene editing in the wild-type background resulted in OK100-quin (raf2/edr1;raf4;raf5/sis8;raf10;raf11) (Fig. 3a, Supplementary Fig. 3a, b). The phosphopeptides from the five protein kinases showed mannitol up-regulation (Fig. 2a). Gene editing in the OK130-weak background produced an OK-quintdec mutant (raf16;raf40;RAF2/EDR1;raf18;raf20;raf35;RAF42,raf3;raf4;raf5/sis8;raf7;raf8;raf9;raf10) and an OK-quintdec mutant (raf16;raf40;RAF2/EDR1;raf18;raf20;raf35;RAF42,raf3;raf4;raf5/sis8;raf7;raf8;raf9;raf10) (Fig. 3a and Supplementary Fig. 3a, c, d). OK100-quin showed strong growth inhibition phenotypes and OK-quintdec showed extremely arrested growth (Fig. 3b, Supplementary Fig. 4a). OK-quadtdec, which differed from OK-quintdec by having wild-type RAF2/EDR1, showed only a slightly inhibited growth (Fig. 3b). The mannitol-triggered activation of the OK100 and SnRK2.2/3/6 was almost completely abolished in the OK-quintdec mutant (Fig. 3c). ABA still activated SnRK2.2/3/6 in the OK-quintdec seedlings, but the activation was much weaker than that in the wild type (Fig. 3c). Together, our results show that B4 RAfs correspond to the OK130 and that members of the B2 and B3 Raf-like kinases are the OK100.

We examined the expression patterns of RAfs in different tissues and stress conditions from an online eFP Browser database (http://bar.utoronto.ca/efp2). The various RAfs were expressed in different tissues and some RAfs were highly expressed in dry seeds and mature pollens (Supplementary Fig. 4b), consistent with the observation that OK130-null produces fewer seeds than the wild type (Supplementary Fig. 2g, h). Interestingly, the expression of
some RAFs, especially in the root, was highly induced by osmotic stress caused by mannitol and high salt (Supplementary Fig. 4c). ABA also up-regulated the expression of RAF35, RAF6 and RAF12 (Supplementary Fig. 4d). By generating transgenic lines expressing GFP fusions, we examined the subcellular localization of RAF proteins. GFP-RAF20, GFP-RAF12 and GFP-RAF7 were localized to the cytosol, while RAF11 was localized to small spots in the cytosol (Supplementary Fig. 5).

RAFs are required for osmotic stress tolerance. We examined the responses of high-order mutants of RAFs to osmotic stress and found that OK\textsuperscript{130-null} but not OK\textsuperscript{130-weak} was hypersensitive to osmotic stress caused by mannitol, NaCl, or polyethylene glycol (PEG) treatment, in assays of seed germination, seedling growth, and electrolyte leakage (Fig. 4a–c and Supplementary Fig. 6c, d). OK\textsuperscript{-quatdec}, containing weak alleles of RAF24 and RAF42, showed only mild hypersensitivity to osmotic stresses (Fig. 4a–c and Supplementary Fig. 6a, b), consistent with the mild osmotic stress sensitivity of OK\textsuperscript{130-weak} (Supplementary Fig. 6c, d).

Upon ABA treatment, the activation of SnRK2.6 depends on the inhibition of the A clade PP2C by ABA-PYL\textsubscript{14,21,31}. Consistent with this notion, ABA-induced SnRK2.2, SnRK2.3 and SnRK2.6 activation was enhanced in the abi1/abi2/pp2ca triple mutant, but strongly impaired in abi1-1 (Supplementary Fig. 7a). By contrast, the osmotic stress-induced activation of SnRK2s was not, or only slightly, affected by mutations in the PP2Cs (Supplementary Fig. 7a). The notion that osmotic stress-triggered activation of SnRK2s does not require ABA signaling is supported by our previous work\textsuperscript{20}, as well as studies from other groups\textsuperscript{18,32}. Interestingly, the activation of both OK\textsuperscript{130} and OK\textsuperscript{100} by osmotic stress is also not impaired in the abi1-1 mutant (Supplementary Fig. 7a), suggesting that the activation is not due to inhibition of the clade A PP2Cs.

RAFs interact with and phosphorylate SnRK2s. Our findings that B4 Raf-like kinases were activated earlier than SnRK2s and were required for the SnRK2 activation suggested that the B4 Raf-like kinases may directly activate SnRK2s by phosphorylation. To test this hypothesis, we first used immunoprecipitation-mass
spectrometry (IP-MS) to detect possible interactions between SnRK2s and B4 Raf-like kinases in plants (Supplementary Fig. 7b). We found several peptides from SnRK2.1/2/4/5/9/10 in anti-GFP immunoprecipitates from GFP-RAF40, GFP-RAF20, or GFP-RAF35, but not from empty GFP plants, implying that the B4 Raf-like kinases and SnRK2s are associated in vivo in plants. A split luciferase complementation assay on RAF35 and several SnRK2s supported the association and suggested direct physical interactions between RAF35 and the tested SnRK2s (Supplementary Fig. 7c).

We then tested whether recombinant B4 Raf-like kinase proteins may phosphorylate SnRK2s. The recombinant kinase domains (KDs) of RAF40/HCR1 and RAF24 displayed detectable kinase activities in vitro. Recombinant RAF24-KD strongly phosphorylated the full-length SnRK2.1K33R, SnRK2.4K33R, and SnRK2.10K33R, the “kinase-dead” mutant versions of the osmotic stress-activated SnRK2s lacking autophosphorylation (Fig. 4d, lane 3, 5, and 7). The RAF24-KD also weakly phosphorylated SnRK2.6K50R, a “kinase-dead” mutant of the ABA-activated SnRK2.6 (Fig. 4d, lane 9). Similarly, RAF40/HCR1-KD strongly phosphorylated SnRK2.4K33R and SnRK2.10K33R, and weakly phosphorylated SnRK2.6K50R (Supplementary Fig. 7d).

To identify the RAF target sites in the SnRK2s, we used mass spectrometry to identify phosphoryptides from the above in vitro kinase reactions. We identified 23 and 6 phosphopeptides from SnRK2.4K33R and SnRK2.6K50R, respectively, after in vitro kinase reactions with different B4 Raf-like kinases using 32P-ATP as the phosphate donor (Supplementary Fig. 8, Supplementary Data 6). Six putative RAF target sites, Ser158, Ser162, Ser166, Thr167, Thr170 and Ser180, were found in phosphopeptides coming from the activation loop of SnRK2.4 (Supplementary Fig. 8a–d, Supplementary Data 6), and two putative target sites, Ser171 and Ser175, were found in the same region in SnRK2.6 (Supplementary Fig. 8e, Supplementary Data 6). The phosphorylation of a highly conserved site in SnRK2s, corresponding to Ser175 in SnRK2.6, is essential for SnRK2.6 activation. Another conserved site corresponding to Ser171 in SnRK2.6 is also crucial for osmotic stress- and ABA-mediated SnRK2 activation. Ser to Ala mutations of these sites partially reduced but did not abolish the phosphorylation signal in the in vitro kinase assay (Supplementary Fig. 8f), which is consistent with our mass spectrometry results showing that multiple sites in addition to the activation loop residues in SnRK2.4 and SnRK2.6 are phosphorylated by Raf-like kinases (Supplementary Data 6).

Since phosphor-mimicking and non-phosphorylatable mutations in the activation loop render SnRK2 inactive, we could not evaluate the contribution of these sites to SnRK2 activation by directly mutating them. So, we performed an in vitro kinase assay with the phosphorylation of an ABA-responsive element-Binding Factor 2 (ABF2) fragment, a well-defined SnRK2 substrate2,30, as an indicator of SnRK2 activity. Recombinant RAF24-KD itself did not phosphorylate ABF2 (Fig. 4e, lane 2). Adding RAF24-KD did not enhance the existing activity of SnRK2.4 (Fig. 4e, lane 4), which might be because the recombinant SnRK2.4 was already highly auto-phosphorylated and fully activated in E. coli. After being dephosphorylated by ABF1 in vitro, the full-length SnRK2.4 showed very weak kinase activity (Fig. 4e, lane 6). This suggests that dephosphorylated SnRK2.4 has no or only very weak self-activation activity, which contrasts with the previous hypothesis that SnRK2s have strong auto-phosphorylation activity and self-activate when they are not inhibited by PP2C15,31. Interestingly, co-incubating with RAF24-KD, but not with the “kinase-dead” form RAF24-KD1001R, substantially increased the kinase activity of dephosphorylated SnRK2.4 (Fig. 4e, lane 7 and 8). Finally, by immunoblotting using an anti-SnRK2.6-pS175 antibody20, which recognizes the phosphorylated serine residue in the activation loop of multiple SnRK2s corresponding to Ser175 in SnRK2.6, we found that the Ser175 phosphorylation triggered by osmotic stress was abolished in OK130-null seedlings (Fig. 4f). Together with the in-gel kinase assay result (Fig. 3c), our findings suggest that the phosphorylation of SnRK2s, especially the conserved serine residue in the activation loop, by Raf-like kinases is required for osmotic stress-triggered SnRK2 activation.

**RAFs are required for ABA-mediated SnRK2 activation.** The strong reduction in ABA-triggered SnRK2 activation in OK-quindec (Fig. 3c) suggested that Raf-like kinases may regulate ABA responses. Since the OK-quindec plants produced very few seeds for subsequent experiments, we tested ABA responses in OK-quindec mutant plants and found that the mutant was insensitive to ABA during seed germination and post-germination seedling growth (Fig. 5a, b, and Supplementary Fig. 9a, b). In addition, OK-quindec mutant seedlings showed higher water loss than the wild type and the other tested RAF high-order mutants, phenocopying the snrk2-triple mutant (Fig. 5c). These results suggest that the RAFs also participate in ABA-triggered SnRK2.2/3/6 activation. This notion is supported by the observation that all five tested kinase domains of B2 and B3 RAFs, RAFTS/SIS8-KD, RAFT2/EDR1-KD, RAFT6-KD, RAFT10-KD and RAFT7-KD, could phosphorylate SnRK2.6K50R (Supplementary Fig. 9c). Interestingly, RAFT6-KD and RAFT10-KD showed a stronger capability to phosphorylate ABA-dependent SnRK2s (SnRK2.6 and SnRK2.8 in our assay) than ABA-independent SnRK2s (SnRK2.1, SnRK2.4, and SnRK2.10 in the assay) (Fig. 5d and Supplementary Fig. 9d). A yeast-two-hybrid assay showed that B2 and B3 subgroup RAFs interact with SnRK2.6 but not with SnRK2.4 (Supplementary Fig. 9e). We also found that, like SnRK2.4, the dephosphorylated-SnRK2.6 (i.e., pretreated with ABF1) was incapable of self-activation (Fig. 5e, lane 7). However, adding RAFT5-KD, RAFT6-KD, or RAFT10-KD strongly increased the phosphorylation of the dephosphorylated SnRK2.6 and therefore increased the phosphorylation of ABF2 (Fig. 5e, lane 8–10). RAF40-KD and RAF24-KD had almost no effect on the phosphorylation of dephosphorylated-SnRK2.6 and ABF2 (Fig. 5e, lane 11, 12), further indicating specificity between subgroups of RAF-like kinases and SnRK2s. Consistent with this, the ABA-induced phosphorylation of Ser175 was only abolished in the OK-quindec mutant but not in the OK130-weak or OK130-null allele (Supplementary Fig. 9f).

Consistent with the strong ABA-insensitive phenotypes of OK-quindec mutant plants, the osmotic stress- and ABA-induced transcript accumulation of several ABA-responsive genes, like Responsive to Desiccation 29B (RD29B), Responsive to ABA 18 (RAB18), and Cold-Regulated 15 A (COR15A), was dramatically impaired in the OK-quindec mutant (Fig. 5f). The expression of some ABA-responsive transcription factors, e.g., ABF2 and ABF4, was also partially impaired in the OK-quindec mutant when compared to the wild type (Supplementary Fig. 9g).

**Discussion** Our results show that the B2, B3, and B4 subfamilies of Raf-like protein kinases are upstream kinases that phosphorylate and activate SnRK2s and are critical in mediating osmotic stress and ABA responses. The RAFs are likely also important for osmoregulation during growth and development, as the OK130-null and OK-quindec mutants show strong growth and developmental defects. The plant RAFs are presumed to be MAPKKks22, although their ability to phosphorylate MAPKks in plants has not been characterized biochemically or genetically. Our results suggest that the 19 group B Raf-like protein kinases together with 10 SnRK2s form a kinase cascade in early osmotic stress and ABA signaling. The OK130/B4 Raf-like kinases prefer to phosphorylate Ser residue in the
ABA-independent SnRK2s, while OK100/B2&3 Raf-like kinases favor phosphorylation of ABA-dependent SnRK2s (Figs. 3c, 4d, 5d, Supplementary Figs. 7d, 9c, d). Our discovery of the group B Raf-like kinases as the upstream kinases for osmotic stress-triggered activation of SnRK2s advances our understanding of osmoregulation in plants. In addition, our results suggest that transphosphorylation of the ABA-dependent SnRK2s by group B2 and B3 Raf-like kinases is a pre-requisite for the activation of SnRK2s in ABA signaling, which provides an important update on our current understanding of the ABA core signaling pathway. Since SnRK2.6 purified from *E. coli* can autophosphorylate and can be activated in vitro by ABA in a test tube reconstitution of the core ABA signaling pathway with PYR/PYL/ACAR and clade A PP2C12,15,30, it has been assumed that SnRK2.6 autophosphorylation is sufficient for its activation. Our results here with dephosphorylated SnRK2.6 show that transphosphorylation by group B Raf-like kinases is necessary, suggesting that previous in vitro assay results were affected by some kinase(s) in *E. coli* that can transphosphorylate SnRK2.6.

Fig. 5 B2 and B3 Raf-like kinases mediate ABA signaling by phosphorylating SnRK2s. a Photographs of seedlings 7 days after transfer to and growth on 1/2 MS medium containing 20 μM ABA. b Photographs of seeds after 7 days of germination on 1/2 MS medium containing 1 μM ABA. c Water loss of the 4-week-old wild type and high-order RAF mutants. Error bars, SD (n = 5). Two-tailed paired t-tests, *p < 0.05, **p < 0.01, ***p < 0.001. d RAF10-KD phosphorylates SnRK2s in vitro. Autoradiograph (upper) and Coomassie staining (bottom) show phosphorylation and loading of purified RAF10-KD and SnRK2s, respectively. e Adding RAF5-KD, RAF6-KD or RAF10-KD, but not RAF40-KD or RAF24-KD, strongly increases the phosphorylation of pre-dephosphorylated SnRK2.6 and ABF2 fragment. Autoradiograph (upper) and Coomassie staining (lower) show phosphorylation and loading of purified RAF-KD and SnRK2.6, respectively. f Expression of stress-responsive genes in wild type, OK130-null, and OK-quatdec seedlings after 6 h of mannitol or ABA treatments. Error bars, SEM (n = 3). Two-tailed paired t-tests, *p < 0.05, **p < 0.01. Source data are provided as Source Data file.
Although group B2 and B3 Raf-like kinases are required for SnRK2.2/3/6 activation in both ABA and osmotic stress, the mechanisms of SnRK2 activation might differ. ABA-induced SnRK2 activation is impaired in the ab1-1 mutant, whereas osmotic stress-induced SnRK2.2/3/6 activation is not affected (Supplementary Fig. 7a). We noticed that RAFs phosphorylate SnRK2.6 on both Ser171 and Ser175 (Supplementary Fig. 8), which are known as direct target sites of the PP2C phosphatases\(^1\). Additional RAF phosphosites exist in SnRK2.6 besides Ser171 and Ser175 (Supplementary Data 6). The phosphorylation of these additional phosphosites may circumvent the PP2C-mediated inhibition to cause activation of the SnRK2s under osmotic stress. Further studies are needed to determine the detailed biochemical mechanisms that differentiate the SnRK2 activation by ABA and osmotic stress in plants.

Due to the large number of kinases in the RAF-SnRK2 cascade and the functional redundancy between the members, it will be challenging to dissect the unique functions of each RAF. Furthermore, we suspect coordination between the RAFs and feed-forward activation by ABA and osmotic stress in plants.

In-gel kinase assay. For in-gel kinase assays, 20 µg extract of total proteins was used for SDS/PAGE analysis with histone embedded in the gel matrix as the kinase substrate. After electrophoresis, the gel was washed three times at 23 °C under a 16 h light/8 h dark photoperiod. Photographs of seedlings were taken at indicated times after transfer to light. For growth assays, sterilized seeds were grown vertically on 0.85% agar and 0.25% methionine, photoperiod. Photographs of seedlings were taken at indicated times after transfer to light. For growth assays, sterilized seeds were grown vertically on 0.85% agar and 0.25% methionine, photoperiod. Photographs of seedlings were taken at indicated times after transfer to light.

Protein extraction and digestion. Protein extraction and digestion was performed as previously described\(^8\). Plants were lysed in lysis buffer (6 M guanidine hydrochloride in 100 mM Tris-HCl, pH 8.5, with 10 mM NaF, EDTA-free proteinase, and phosphatase inhibitor cocktails (Sigma-Goldrich, St. Louis, MO). Disulfide bonds in proteins were reduced and alkylated with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride and 40 mM N-2-mercaptoethanol at 56 °C for 30 min. Protein lysate was precipitated with 100 mM Na3VO4, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100 and incubated at 4 °C overnight with three changes of the wash solution.

In-gel kinase assay. For in-gel kinase assays, 20 µg extract of total proteins was used for SDS/PAGE analysis with histone embedded in the gel matrix as the kinase substrate. After electrophoresis, the gel was washed three times at 23 °C under a 16 h light/8 h dark photoperiod. Photographs of seedlings were taken at indicated times after transfer to light. For growth assays, sterilized seeds were grown vertically on 0.85% agar and 0.25% methionine, photoperiod. Photographs of seedlings were taken at indicated times after transfer to light.
PCR Detection System (Bio-Rad) was used to detect ampli-
were set up with iQ SYBR Green Supermix (Bio-Rad). A CFX96 Touch Real-Time
Browser
stress and hormonal treatments were downloaded from the Arabidopsis eFP
fi
4 sgRNAs was used to transform
transform Col-0 wild type. The fourth vector pCAMBIA-2300-11RAFs containing
OK-quin dec
OK130-weak
OK130-null

Electrolyte leakage assay. To measure ion leakage in seedlings induced by PEG
treatment, 5-day-old wild type, OK130-null and OK-quince seedlings were rinsed
brieﬂy in distilled water, and placed in a solution containing 30% PEG for 5 h. After
treatment, seedlings were rinsed brieﬂy in distilled water and placed immediately
in a tube with 5 mL of water. The tube was agitated gently for 3 h before the elec-
trolyte content was measured. Three replicates of each treatment were conducted.

Gene expression. Total RNA was extracted from wild type, OK130-null and OK-quince seedlings
siRNAs to target RAFs were designed according to the protocol described previously36. The sgRNAs for

Protein expression, purification, and in vitro kinase assay. cDNA fragments encoding full length SnRK2s were cloned into pET-28a vector with the coding sequence of 6 × HIS-tag, a thrombin cleavage site, and a T7 tag fused. cDNA fragments encoding kinase domains of the RAfs were cloned into pGEX-AT-J and pMal-c2x vectors with the primer listed in Supplementary Data 7. The resulting plasmids were transformed into BL21 or ArcticExpression cells. The recombinant proteins were expressed and puriﬁed using standard protocols. For the phos-
phorylation assay, recombinant full-length SnRK2s and kinase domains of RAfs
were expressed and puriﬁed using standard protocols. For the phos-

Water loss measurement. For the measurement of water loss, detached rosette
leaves of 4-week-old plants were placed in weighing dishes and left on the
laboratory bench with light. Fresh weight was monitored at the indicated time. Water loss was expressed as a percentage of initial fresh weight.

Split luciferase (LUC) complementation assay. The coding sequence of RAF35
and SnRK2s was ampliﬁed by PCR, cloned into pENTR vectors and transferred to
pEarley-nLUC/cLUC vectors through LR reactions. Split-LUC complementation
assay was performed by transient expression in tobacco leaves through agrobacterium-mediated infection. Two days after infection, luciferase activity was detected with a CCD camera by applying ﬁrew ﬂuorescin (Nanolight).

Immunoblotting. 30 mg samples were ground into ﬁne powder in liquid N2, and
dissolved in 100 µL protein extract buffer (100 mM HEPES, pH 7.5, 5 mM EDTA,
5 mM EGTA, 10 mM DTT, 10 mM Na2VO4, 10 mM NaF, 50 mM β-glycerophos-
phate, 1 mM PMSF, 5 µg/mL leupeptin, 5 µg/mL antipain, 5 µg/mL aprotinin, and

5% glycerol) followed by centrifugation at for 40 min at 4 °C. The supernatants
were separated by 12% SDS/PAGE. After electrophoresis, the proteins were
transferred to PVDF membrane and immunoblotted with antibodies against
SnRK2.2/3/6 and SnRK2.6-p-S175. Immunoblot with anti-actin was used as the
loading control.

Yeast two hybrid assay. To detect protein interactions between RAfs and
SnRK2s, pGADT7 plasmids containing RAfs were co-transformed with wild-type
or mutated pGBK7-SnRK2s into Saccharomyces cerevisiae AH109 cells. Suc-
cessfully transformed colonies were identiﬁed on yeast SD medium lacking Leu
and Trp. Colonies were transferred to selective SD medium lacking Leu, Trp, His, and
in the presence of 3-Amino-1,2,4-Triazol (3-AT). To determine the intensity of
protein interaction, saturated yeast cultures were diluted to 10−1, 10−2, and 10−3
and spotted onto selection medium. Photographs were taken after 4 days incubation.

Confocal microscopy. Seven-day-old seedlings of RAF-GFP were imaged using a
Leica TCS SP8 laser scanning confocal microscope at 488 nm laser excitation and
500 to 550 nm emission for GFP.

Quantitative and statistical analysis. Student’s t-test was used to determine
the statistical signiﬁcance between wild type and mutants in assays related to germi-
nation, root length, fresh weight, relative intensity, or relative abundance.

Reporting Summary. Further information on research design is available in the
Nature Research Reporting Summary linked to this article.

Data availability
The phosphoproteomic data were deposited to the ProteomeXchange Consortium via the
PRIDE partner repository with the dataset identiﬁer PXD014435. Source data underlying
Figs. 1a–b, 3c–f, 5–f, as well as Supplementary Figs. 2a, d, i–d, 6a, b, 7a, d, 8i, 9a–d,
i–g, 10 are provided as a Source Data File. Source data underlying Figs. 2a, c, d are also
available in Supplementary Data 4 and 5. Other data supporting the ﬁndings of this study
are available within the manuscript and its supplementary ﬁles or are available from the
Corresponding Authors upon request.

Received: 19 August 2019; Accepted: 8 January 2020;
Published online: 30 January 2020

References
1. Hasegawa, P. M., Bressan, R. A., Zhu, J. K. & Bohnert, H. J. Plant cellular
and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol.
51, 463–499 (2000).
2. Thomasow, M. F. PLANT COLD ACCLIMATION: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol.
50, 571–599 (1999).
3. Assmann, S. M. & Jegl, T. Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO2. Cur. Opin. Plant Biol. 33, 157–167 (2016).
4. Boudsocq, M., Barbier-Brygoo, H. & Christiane, L. Identiﬁcation of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic
and saline stresses in Arabidopsis thaliana. J. Biol. Chem. 279, 41758–41766 (2004).
5. Fuji, H., Verslues, P. E. & Zhu, J. K. Arabidopsis decuple mutant reveals the
importance of SnRK2 kinases in osmotic stress responses in vivo. Proc. Natl
Acad. Sci. USA 108, 1717–1722 (2011).
6. Fuji, H. & Zhu, J. K. Arabidopsis decuple 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 (2009).
7. Fujita, Y. et al. Three SnRK2 protein kinases are the main positive regulators of
abscisic acid signaling in response to water stress in Arabidopsis. Plant Cell
Physiol. 50, 2123–2132 (2009).
8. Raghavendra, A. S., Gonugunta, V. k., Christmann, A. & Grill, E. ABA
phosphorylation and signaling. Trends Plant Sci. 15, 395–401 (2010).
9. Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. & Abrams, S. R. Abscisic acid:
emerging from a core signaling network. Annu. Rev. Plant Biol. 61, 651–679 (2009).
10. Hubbard, K. E., Nishimura, N., Hitomi, K., Getzoff, E. D. & Schroeder, J. I.
Early abscisic acid signal transduction mechanisms: newly discovered
components and newly emerging questions. Genes Dev. 24, 1695–1708 (2010).
11. Umezawa, T. et al. Molecular basis of the core regulatory network in ABA
responses: sensing, signaling and transport. Plant Cell Physiol. 51, 1821–1839 (2010).
12. Fujii, H. et al. In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 522, 660–664 (2009).
13. Ma, Y. et al. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324, 1064–1068 (2009).
14. Park, S.-Y. et al. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324, 1068–1071 (2009).
15. Ng, L. M. et al. Structural basis for basal activity and autoactivation of abscisic acid (ABA) signaling SnRK2 kinases. *Proc. Natl Acad. Sci. USA* 108, 21259–21264 (2011).
16. Umezawa, T. et al. Genomics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in Arabidopsis thaliana. *Sci. Signal.* 6, rs8 (2013).
17. Wang, P. et al. Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effects of abscisic acid action. *Proc. Natl Acad. Sci. USA* 110, 11205–11210 (2013).
18. Vlad, F. et al. Phospho-site mapping, genetic and in planta activation studies reveal key aspects of the different phosphorylation mechanisms involved in activation of SnRK2s. *Plant J.* 63, 778–790 (2010).
19. Kobayashi, Y., Yamamoto, S., Minami, H., Kagaia, Y. & Hattori, T. Differential activation of the rice Sucrose Nonfermenting1-Related Protein Kinase2 family by hyperosmotic stress and abscisic acid. *Plant Cell* 16, 1163–1177 (2004).
20. Zhao, Y. et al. Arabidopsis duodecuple mutant of PYL ABA receptors reveals PYL repression of ABA-independent SnRK2 activity. *Cell Rep.* 23, 3340–3351 (2018).
21. Vlad, F. et al. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OSTI by abscisic acid in Arabidopsis. *Plant Cell* 21, 3170–3184 (2009).
22. Ichimura, K. et al. Mitogen-activated protein kinase cascade in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308 (2002).
23. Rao, K. P., Richa, T., Kumar, K., Raghuram, B. & Sinha, A. K. In silico analysis reveals ABA regulation of mitogen-activated protein kinase gene family in rice. *DNA Res.* 17, 139–153 (2010).
24. Saruhashi, M. et al. Plant Raf-like kinase integrates abscisic acid and hyperosmotic stress signaling upstream of SNF1-related protein kinase2. *Proc. Natl Acad. Sci. USA* 112, E6388–E6396 (2015).
25. Shahzad, Z. et al. A potassium-dependent oxygen sensing pathway regulates plant root haustorics. *Cell* 167, 87–98 (2016).
26. Beaudoin, N., Serizet, C., Gosti, F. & Giraudat, J. Interactions between abscisic acid and ethylene signaling cascades. *Plant Cell* 12, 1103–1116 (2000).
27. Lee, S.-j, Lee, M. H., Kim, J. I. & Kim, S. Y. Arabidopsis putative MAP Kinase Kinase Kinase Raf10 and Raf11 are positive regulators of seed dormancy and ABA response. *Plant Cell Physiol.* 56, 84–97 (2014).
28. Gao, L. & Xiang, C.-B. The genetic locus At1g35660 encodes a putative MAPKKK and negatively regulates salt tolerance in Arabidopsis. *Plant Mol. Biol.* 67, 125–134 (2008).
29. Stecker, K. E., Minkoff, B. B. & Sussman, M. R. Phosphoproteomic analyses reveal early signaling events in the osmotic stress response. *Plant Physiol.* 165, 1171–1187 (2014).
30. Wang, P. et al. Reciprocal regulation of the TOR kinase and ABA receptor balances plant growth and stress response. *Mol. Cell* 69, 100–112 (2018). e106.
31. Soon, F. F. et al. Molecular mimicry regulates ABA signaling by SnRK2 kinases and PP2C phosphatases. *Science* 335, 85–88 (2012).
32. Boudsocq, M., Drolillard, M.-J., Barbier-Brygoo, H. & Laurière, C. Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Mol. Biol.* 63, 491–503 (2007).
33. Yuan, F. et al. OSCA1 mediates osmotic-stress-evoked Ca2+ increases vital for osmoosmotic signaling in Arabidopsis. *Nature* 514, 367–371 (2014).
34. Tsai, C. F. et al. Sequential phosphoproteomic enrichment through complementary metal-directed immobilized metal ion affinity chromatography. *Anal Chem.* 86, 685–693 (2014).
35. Hsu, C. C. et al. Universal plant phosphoproteomics workflow and its application to tomato signaling in response to cold stress. *Mol Cell Proteomics* 17, 2068–2080 (2018).
36. Zhang, Z. et al. A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in Arabidopsis. *Plant Cell Rep.* 35, 1519–1533 (2016).