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LETTERS

In vitro reconstitution of an abscisic acid signalling pathway

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The phytohormone abscisic acid (ABA) regulates the expression of many genes in plants; it has critical functions in stress resistance and in growth and development[1–7]. Several proteins have been reported to function as ABA receptors[8–13], and many more are known to be involved in ABA signalling[3,4,14]. However, the identities of ABA receptors remain controversial and the mechanism of signalling from perception to downstream gene expression is unclear[15,16]. Here we show that by combining the recently identified ABA receptor PYR1 with the type 2C protein phosphatase (PP2C) ABI1, the serine/threonine protein kinase SnRK2.6/OST1 and the transcription factor ABF2/AREB1, we can reconstitute ABA-triggered phosphorylation of the transcription factor *in vitro*. Introduction of these four components into plant protoplasts results in ABA-responsive gene expression. Protoplast and test-tube reconstitution assays were used to test the function of various members of the receptor, protein phosphatase and kinase families. Our results suggest that the default state of the SnRK2 kinases is an autophosphorylated, active state and that the SnRK2 kinases are kept inactive by the PP2Cs through physical interaction and dephosphorylation. We found that in the presence of ABA, the PYR/PYL (pyrabactin resistance 1/PYR1-like) receptor proteins can disrupt the interaction between the SnRK2s and PP2Cs, thus preventing the PP2C-mediated dephosphorylation of the SnRK2s and resulting in the activation of the SnRK2 kinases. Our results reveal new insights into ABA signalling mechanisms and define a minimal set of core components of a complete major ABA signalling pathway.

Several ABA receptors have been reported[8–13], although many of them remain unconfirmed[15,16]. Recently, a family of novel START domain proteins, known as PYR/PYLs (also known as RCARs), were identified as ABA receptors. Several of the PYR/PYLs were shown to interact with and inhibit clade-A PP2Cs[11–13]. The PP2Cs (ABI1, ABI2, HAB1 and PP2CA/AHG3) negatively regulate ABA responses[13]. In contrast, a subfamily of ABA-activated SnRK2s are positive regulators of ABA signalling[17–21]. Through unknown mechanisms, the inhibition of the negatively acting PP2Cs leads to the successful activation of a subfamily of SnRK2 kinases (SnRK2.2, SnRK2.3 and SnRK2.6 in *Arabidopsis*), which phosphorylate the basic leucine zipper (bZIP) transcription factors called ABFs/AREBs[22,23]. The ABFs bind to ABA-responsive promoter elements (ABRE) to induce the expression of ABA-responsive genes[1].

The present study was aimed at defining the core components of the ABA response pathway that are both necessary and sufficient for ABA perception, signalling, and finally ABA-responsive gene expression. It has been suggested that ABA-dependent phosphorylation of ABF2 at amino-acid residues S26, S86, S94 and T135 is important for stress-responsive gene expression in *Arabidopsis*[23]. We used transient activation analysis with protoplasts from the *snrk2.2/2.3/2.6* triple mutant to determine the role of ABF2 phosphorylation and its dependence on SnRK2s for ABA-responsive gene expression. We have shown previously that the *snrk2.2/2.3/2.6* triple mutant is deficient in ABA responses[21]. As expected, transfection of *snrk2.2/2.3/2.6* protoplasts with ABF2 did not induce *RD29B–LUC* (luciferase reporter gene driven by the ABA-responsive *RD29B* promoter) expression even in the presence of ABA, but co-transfection of ABF2 with SnRK2.6 resulted in the induction of *RD29B–LUC* in an ABA-dependent manner (Fig. 1a). Furthermore, ABF2 with alanine substitutions at all of the four phosphorylation sites was inactive, whereas aspartic acid substitutions at these sites led to a constitutively active ABF2, resulting in the induction of *RD29B–LUC* expression even without ABA treatment (Fig. 1a). Co-transfection of Ala-substituted ABF2 with SnRK2.6 led to only a very low level of *RD29B–LUC* induction (Fig. 1a). Replacement of lysine 50, a conserved residue critical for ATP-binding and kinase activity, with asparagine (K50N) inactivates SnRK2.6 in phosphorylation assays *in vitro* (H.F. and J.-K.Z., unpublished observations). Co-transfection of ABF2 with SnRK2.6^{K50N} did not induce *RD29B–LUC* expression (Fig. 1a), demonstrating that the kinase activity is necessary for ABF2 activation. Transfection of ABF2 alone in wild-type protoplasts induced a low level of *RD29B–LUC* expression under ABA treatment, which is consistent with the presence of a low basal level of endogenous ABA-signalling components in the protoplasts (Supplementary Fig. 1a). These results show that SnRK2.6 mediates ABF2 activation in an ABA-dependent manner, and that ABF2 phosphorylation is sufficient for the induction of *RD29B–LUC* expression by ABA.

We next tested the effect of ABI1 and PYR1 on the induction of *RD29B–LUC* expression by ABA. Transfection of ABI1 together with ABF2 and SnRK2.6 resulted in inhibition of *RD29B–LUC* expression (Fig. 1b and Supplementary Fig. 1a). This shows that ABI1 negatively regulates the activation of *RD29B–LUC* expression that is dependent on SnRK2.6 and ABF2. Addition of PYR1 together with ABI1, SnRK2.6 and ABF2 enabled the ABA-dependent induction of *RD29B–LUC* expression (Fig. 1b and Supplementary Fig. 1a). However, addition of PYR1^{P88S}, which is defective in interaction with and inhibition of PP2Cs[12], did not enable the ABA-dependent induction of *RD29B–LUC* expression. The dominant *abi1-1* mutation (G180D) disrupts the interaction between ABI1 and PYR1 (ref. 12). Like the wild-type ABI1, ABI1^{G180D} also inhibited the effect of SnRK2.6 and ABF2 on *RD29B–LUC* expression in response to ABA, but this antagonistic effect could not be overcome by expression of PYR1 (Fig. 1b and Supplementary Fig. 1a). This suggests that the ABI1^{G180D} mutant protein retains the inhibitory activity but can no longer be regulated. Thus reconstitution with PYR1, ABI1, SnRK2.6 and ABF2 is sufficient

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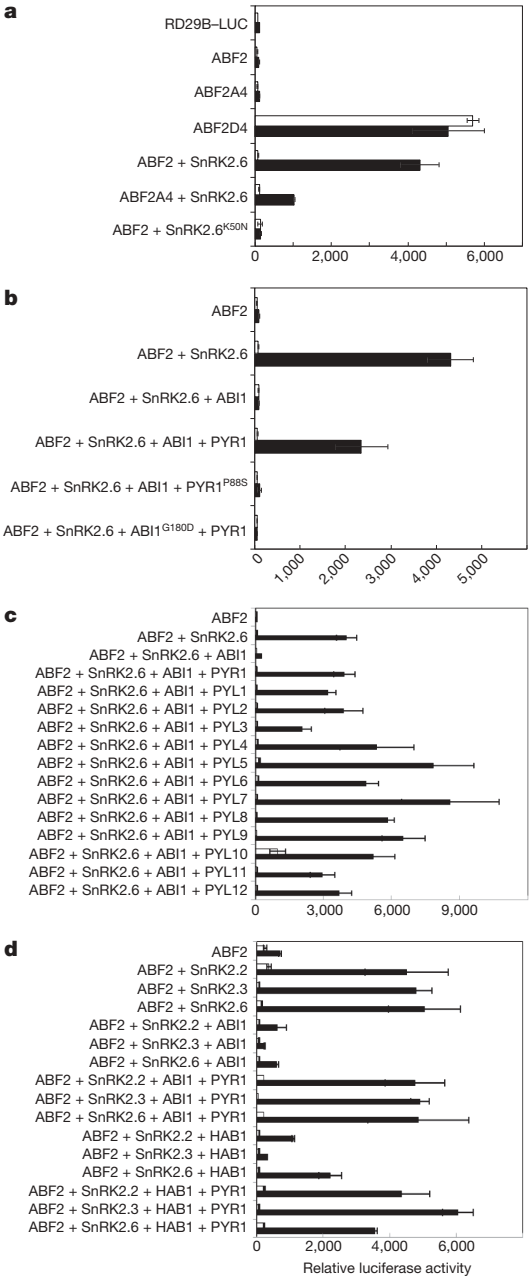

Figure 1 | Reconstitution of the ABA signalling pathway for stress-responsive gene expression in *Arabidopsis* protoplasts. **a**, SnRK2-mediated phosphorylation of ABF2 is sufficient for ABA-responsive gene expression. **b**, Reconstitution of ABA signalling pathway by co-expression of PYR1, ABI1, SnRK2.6 and ABF2. **c**, Reconstitution of ABA signalling pathway with different members of the PYR/PYL family. **d**, Reconstitution with different combinations of the core components. Protoplasts (2×10^4) from the *snrk2.2/3/6* triple mutant were used except in **d**, in which protoplasts from Col-0 wild-type plants were used. *RD29B*::*LUC* and *ZmUBQ*::*GUS* were used as the ABA-responsive reporter and internal control, respectively. After transfection, protoplasts were incubated for 5 h under light and in the absence of ABA (open bars) or in the presence of 5 μM ABA (filled bars). Error bars indicate s.e.m. ($n = 3$).

to enable ABA-mediated gene expression in protoplasts, providing *in vivo* evidence in favour of our previously proposed model of ABA signalling[12].

The PYR/PYL family consists of 14 members. Although genetic studies suggested redundancy in their function[12], it is not known whether all members can act as ABA receptors and transduce the ABA signal to induce gene expression. To address this matter, we reconstituted the ABA signalling pathway with different members of the PYR/PYL family. Our results show that all of the tested PYR/PYLs could antagonize the ability of ABI1 to inhibit the ABA-dependent induction of *RD29B–LUC* expression in *snrk2.2/2.3/2.6* protoplasts, although not all PYR/PYL members were equally effective (Fig. 1c). The results suggest that all of the PYR/PYLs are likely to function as ABA receptors. We also tested reconstitution of the ABA signalling pathway with different combinations of SnRK2 kinases, PP2Cs and receptors, and found that the SnRK2 kinases are inhibited by both the ABI1 and HAB1 PP2Cs, and that PYR1 or PYL2 can antagonize this inhibition. The inhibitory effect of ABI1 was stronger than that of HAB1 in the reconstituted ABA signalling system in protoplasts (Fig. 1b–d and Supplementary Fig. 1). Each of the three clade A PP2Cs (ABI1, ABI2 and HAB1) was capable of interacting with the three SnRK2 kinases (SnRK2.2, SnRK2.3 and SnRK2.6) in yeast two-hybrid (Y2H) assays, although with different intensities. For example, the ABI1 interaction was stronger than that of ABI2 or HAB1 (Supplementary Fig. 2a), which correlates with the level of inhibitory effect of ABI1 and HAB1 in the protoplast assay (Fig. 1d and Supplementary Fig. 1b). A carboxy-terminally truncated SnRK2.6 lacking amino acids 280–362 did not interact with ABI1 (Supplementary Fig. 2a), which is consistent with previous studies demonstrating that deletion of a short C-terminal domain abrogates the interaction between ABI1 and SnRK2.6 in yeast[19]. Bimolecular fluorescence complementation (BiFC) assays in tobacco show that ABI1 interacts with the SnRK2s in the nucleus as well as in the cytosol and that the C-terminal region of SnRK2.6 is required for the interaction with ABI1 (Supplementary Fig. 2b). Expression of the fusion proteins was verified by immunoblot analysis (Supplementary Fig. 2c). The interaction between ABI1 and SnRK2.6 *in vivo* was further confirmed by a co-immunoprecipitation assay using the tobacco protein extracts (Supplementary Fig. 2c).

PYR/PYLs inactivate clade A PP2Cs in an ABA-dependent manner[11–13]. In protoplast transactivation assays, we showed that PYR/PYLs can reverse the inhibitory effect of PP2Cs (Fig. 1 and Supplementary Fig. 1). We speculated that the PYR/PYLs might prevent the inhibitory effect of the PP2Cs by disrupting the interaction between the PP2Cs and the SnRK2s. We used yeast triple-hybrid assays to test whether co-expression of PYLs might disrupt the interaction between PP2Cs and SnRK2s. First, we reproduced the interaction of the ABI1, ABI2 and HAB1 PP2Cs (fused to the Gal4 activation domain (GAD)) with SnRK2.6 (fused to the Gal4 DNA-binding domain (GBD)) by using the pBridge triple-hybrid vector (Supplementary Fig. 3). Next, we cloned PYL5 and PYL8 into the SnRK2.6–pBridge construct; these proteins have been shown to act as potent inhibitors of the PP2Cs[13]. Nuclear localization of PYL5 and PYL8 in yeast is driven by fusion with a nuclear localization sequence present in the pBridge vector. Co-expression of PYL8 with GBD-SnRK2.6 led to an abrogation or decrease (depending on the dilution of the yeast culture) in the interaction with GAD–ABI1 (Supplementary Fig. 3). Similar results were obtained when GBD–SnRK2.6 and GAD–ABI2 or GAD–HAB1 were tested with either PYL8 or PYL5, respectively (Supplementary Fig. 3). These results show that co-expression of a PYL impairs the interaction of ABI1, ABI2 and HAB1 PP2Cs with SnRK2.6.

We have reconstituted the apparent entire ABA signalling pathway for stress-responsive gene expression by the co-expression of PYR/PYLs, PP2Cs, SnRK2s and ABF2 in *Arabidopsis* protoplasts (Fig. 1b–d and Supplementary Fig. 1). To verify whether these are the minimal signalling components that are both necessary and sufficient for ABA signalling in the absence of other cellular components, we attempted to reconstitute the pathway *in vitro*. We constructed recombinant maltose-binding protein (MBP)-tagged SnRK2.6 and found that it is capable of phosphorylating an ABF2 fragment as well as showing autophosphorylation (Fig. 2a, b). Incubation of glutathione *S*-transferase (GST)-tagged ABI1 but not GST with SnRK2.6 before the kinase assay substantially decreases ABF2 phosphorylation by the recombinant SnRK2.6 (Fig. 2a). SnRK2.6 pulled down from extracts of

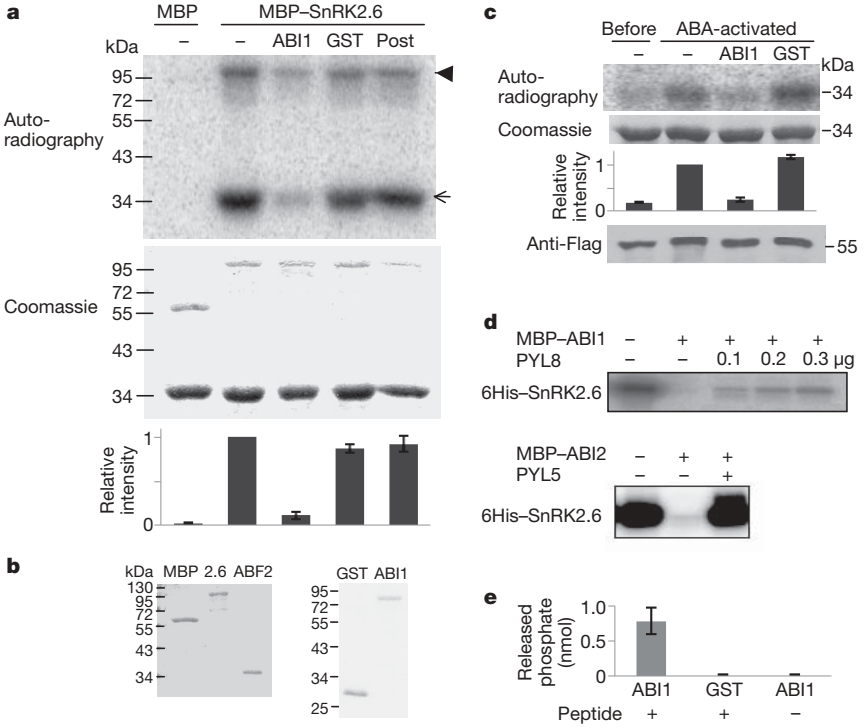

Figure 2 | ABI1 and ABI2 inhibit SnRK2.6 by dephosphorylation. a, SnRK2.6 is deactivated by ABI1. MBP or MBP–SnRK2.6 treated without (−) or with GST–ABI1 or GST was incubated with a GST–ABF2 fragment (Gly 73 to Gln 119) in the presence of [γ-^{32}P]ATP. In the rightmost lane (Post), GST–ABI1 was added after phosphorylation of the GST–ABF2 fragment by MBP–SnRK2.6. Bands of GST–ABF2 fragment and MBP–SnRK2.6 are indicated by an arrow and an arrowhead, respectively. Radioactivities of GST–ABF2 fragment bands were measured with a phosphoimager and were normalized, taking as unity the radioactivity of the band by MBP–SnRK2.6 without ABI1 treatment. Error bars indicate s.e.m. ($n = 5$). **b**, Coomassie staining of purified MBP, SnRK2.6, ABF2, GST and ABI1. **c**, Flag-tagged SnRK2.6 extracted from transgenic plants before and after ABA treatment was used instead of the MBP–SnRK2.6 in **a**. Coomassie staining, autoradiography and relative radioactivities (error bars indicate s.e.m.; $n = 5$) of the GST–ABF2 fragment are shown. Western blotting with anti-Flag antibody shows the amount of Flag–SnRK2.6 protein. **d**, Autoradiography of autophosphorylated SnRK2.6 showing dephosphorylation of SnRK2.6 by MBP–ABI1 and MBP–ABI2 and the effect of PYL8 and PYL5, respectively, in the presence of 1 μM ABA. **e**, Release of phosphate from the synthetic peptide HSQPKpSTVGTP, corresponding to residues 170–180 of SnRK2.6. Error bars indicate s.e.m. ($n = 3$).

ABA-treated plants is also active in phosphorylating ABF2, but SnRK2.6 from untreated plants is not. This phosphorylation is also inhibited by GST–ABI1 (Fig. 2c). ABI1 added after ABF2 phosphorylation by SnRK2.6 is not as effective in decreasing the level of phosphorylation (Fig. 2a), suggesting that ABI1 inhibits ABF2 phosphorylation by dephosphorylating SnRK2.6 (Fig. 2a). Indeed, we found that both ABI1 and ABI2 efficiently dephosphorylated SnRK2.6 (Fig. 2d). The autophosphorylated Ser 175 is essential for the kinase activity of SnRK2.6 *in vitro*[24]. We found that ABI1 can dephosphorylate a synthetic phosphopeptide corresponding to amino-acid residues His 170–Pro 180 of SnRK2.6, which is phosphorylated at Ser 175 (HSQPKpSTVGTP; Fig. 2e). These results suggest that ABI1 may deactivate SnRK2.6 by dephosphorylating Ser 175.

When His-tagged PYR1 is incubated together with GST–ABI1 and MBP–SnRK2.6, SnRK2.6-mediated phosphorylation of ABF2 is significantly recovered in the presence of 2 μM (+)-ABA (Fig. 3a, b). Without ABA, His-PYR1 cannot reverse the inhibitory effect of ABI1 on SnRK2.6-mediated phosphorylation of ABF2 (Fig. 3a). PYR1^{P88S}, which cannot bind to and inhibit ABI1 (ref. 12), is not capable of reversing the inhibitory effect of ABI1 even in the presence of ABA (Fig. 3a). We found that in the presence of ABA, PYL8 or PYL5 can prevent the dephosphorylation of SnRK2.6 by ABI1 or ABI2 (Fig. 2d). These data are consistent with results from the protoplast transactivation assays, and they show that it is possible to reconstitute the activation of ABF2 phosphorylation by ABA *in vitro*. The ABF2 phosphorylation status in this reconstituted *in vitro* system responds to ABA in a concentration-dependent manner (Fig. 3c). The apparent concentration of ABA giving a half-maximal response is 0.8 μM, which is similar to the concentration of ABA giving a half-maximal inhibition of seed germination[11] and falls within the physiological range of ABA concentrations in plants. Similar responses to ABA were observed when ABA-activated SnRK2.6 isolated from plants instead of recombinant SnRK2.6 was used in the reconstitution assay (Supplementary Fig. 4a). Furthermore, reconstitution was also achieved when the PP2C protein HAB1 was used instead of ABI1 (Supplementary Fig. 4b). Our protoplast and *in vitro* reconstitution results support a model in which PYR1 (and PYLs) binds ABA, and then interacts with and is able to inactivate the PP2Cs. The ABA-bound receptors also disrupt the interaction between the PP2Cs and the SnRK2 kinases. These actions of the receptors prevent the dephosphorylation and thereby relieve inhibition of the SnRK2s by the PP2Cs. The relieved SnRK2s can then phosphorylate ABFs to activate ABA-responsive genes.

Consistent with our model was our previous observation[12] that the SnRK2s are substantially less activated by ABA in the *pyr1pyl1pyl2pyl4* mutant than in the wild type. The model also predicts that the SnRK2s may be constitutively activated in mutant plants deficient in the PP2Cs. Indeed, the PP2C triple mutant *abi1-2hab1-1pp2ca-1* shows a constitutive activation of 42 and 45-kDa kinases, which correspond to SnRK2.2/2.3 and SnRK2.6, respectively (Fig. 4a). This mutant displays a constitutive ABA response phenotype in germination and early seedling development (Fig. 4b, c), as reported previously[25]. In contrast, the PP2C triple mutant *abi1-2hab1-1abi2-2* does not have a constitutive ABA response as strong as that in *abi1-2hab1-1pp2ca-1* (Fig. 4b, c) and does not show a strong constitutive activation of the SnRK2s (Fig. 4a).

We have achieved the *in vitro* reconstitution of a phytohormone signal transduction pathway using recombinant proteins. The *in vitro* reconstitution results are supported by the reconstitution assays in

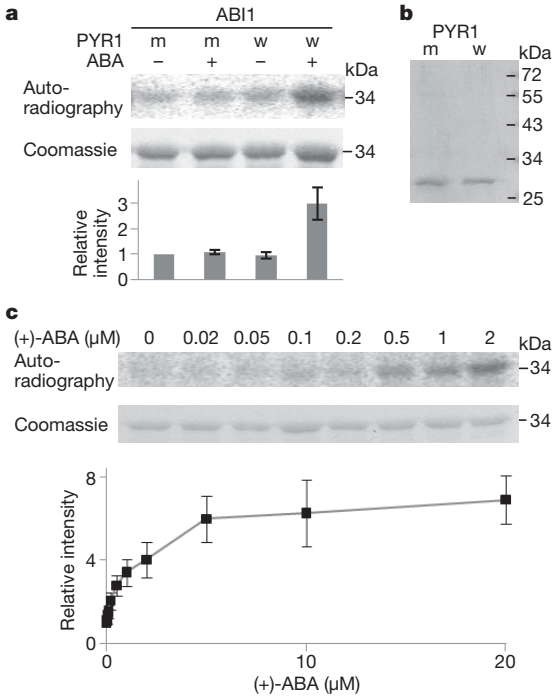

Figure 3 | The combined effect of ABA, PYR1 and ABI1 on the phosphorylation of the GST–ABF2 fragment by SnRK2.6 *in vitro*. **a**, Reconstitution of ABA regulation of ABF2 phosphorylation. MBP–SnRK2.6 treated with GST–ABI1 and His-tagged wild-type PYR1 (w) or mutated PYR1^{P88S} (m) in the absence (−) or presence (+) of 2 μM (+)-ABA was incubated with a GST–ABF2 fragment (Gly 73 to Gln 119) in the presence of [γ-^{32}P]ATP. Coomassie staining, autoradiography and relative radioactivities of GST–ABF2 fragment are shown. Radioactivities of the GST–ABF2 fragment were normalized, taking as unity the radioactivity of the band with PYR1^{P88S} in the absence of ABA. Error bars indicate s.e.m. ($n = 5$). **b**, Coomassie staining of PYR1 (w) and PYR1^{P88S} (m). **c**, ABA dose response. MBP–SnRK2.6, GST–ABI1 and His-PYR1 were incubated with different concentrations of (+)-ABA before the kinase assay, using the GST–ABF2 fragment as substrate. Coomassie staining, autoradiography and relative radioactivities of the GST–ABF2 fragment are shown, taking as unity the radioactivity of the band in the absence of ABA. Error bars indicate s.e.m. ($n = 9$ for less than 5 μM, $n = 4$ for 5 μM or more).

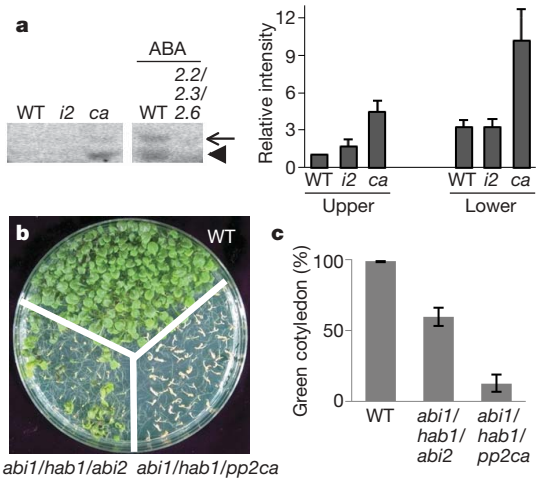

Figure 4 | Effect of PP2C mutations on ABA response phenotypes and kinase activities of SnRK2s. **a**, In-gel kinase assay showing the activities of SnRK2s in the *abi1/hab1/abi2* (*i2*) and *abi1/hab1/pp2ca* (*ca*) triple mutants. *snrk2.2/2.3/2.6* was used as a control. A GST-fused ABF2 fragment (Gly 73 to Gln 119) was used as the phosphorylation substrate. The expected positions of SnRK2.6 and SnRK2.2/2.3 are indicated by an arrow and an arrowhead, respectively. Radioactivities of the upper and lower bands were normalized, taking as unity the radioactivity of the upper band in the wild type (WT). Error bars indicate s.e.m. ($n = 3$). **b**, The PP2C triple mutants show hypersensitivity to ABA during germination and early seedling development. The photograph shows plants of the indicated genotypes grown for 14 days on MS medium containing 3% sucrose. **c**, The percentage of seedlings with green cotyledons 6 days after the end of stratification. Error bars indicate s.e.m. ($n = 3$).

the protoplasts and by genetic analysis. The protoplast reconstitution assays enabled us to test the functions of nearly all members of the PYR/PYL family. Our results indicate that all members of the family can function as ABA receptors in inducing gene expression. Although each of the proteins used in the reconstitution assays has been studied previously, it was not known how these components might connect to form a signalling pathway. Our study has revealed insights into the mechanisms of action of these components. Our results suggest that the default state of the SnRK2 protein kinases is an autophosphorylated, active state, and that the SnRK2 kinases are kept inactive by the PP2Cs through physical interaction and dephosphorylation. We found that, on binding to ABA, the PYR/PYL receptor protein can disrupt or decrease the interaction between the SnRK2s and PP2Cs and prevent the PP2C-mediated dephosphorylation of the SnRK2s, thus resulting in the activation of the SnRK2 kinases.

Successful reconstitution with the recombinant proteins implies that we have identified all essential core components of an ABA response pathway from hormone perception to phosphorylation of ABFs. Although ABA signalling in plants has been considered to be very complicated with numerous other proteins involved, our study reveals a surprising simplicity of the pathway and demonstrates that the PYR/PYLs, clade-A PP2Cs, SnRK2s and ABFs are the only core components to complete the ABA regulation of gene expression. Because there are multiple family members for each of these core components, many combinations of them are possible. The functions of the family members may overlap, but their unique spatial and temporal expression patterns may confer some distinct functions in specific tissues. Extensive genetic analysis will be necessary to determine the *in planta* importance of specific combinations of the core components.

We suggest that the other proteins previously identified as being involved in ABA responses may function to modulate the expression and/or activities of one or more of the core components defined here. Calcium and reactive oxygen signalling, RNA metabolism and protein degradation are known to be important in regulating ABA sensitivity[2–4,14,26,27]. It will be of great interest to determine how these processes may connect to one or more of the core components to affect ABA responses. It will also be interesting to determine whether other ABA response pathways such as ABA regulation of ion channels in guard cells[2,3,6] also use components of the PYR/PYLs–PP2C–SnRK2 regulatory module and whether additional receptors and core signalling components are involved.

METHODS SUMMARY

Transient activity assays were performed in *Arabidopsis* mesophyll protoplasts from Columbia wild-type or *snrk2.2/2.3/2.6* (ref. 21) plants as described previously (http://genetics.mgh.harvard.edu/sheenweb)[28]. Transfected protoplasts were incubated for 5 h in light in the absence of ABA or the presence of 5 μM ABA, and then used for the measurement of luciferase (LUC) and β-glucuronidase (GUS) activities as described previously[28]. Yeast two-hybrid and triple-hybrid assays, co-immunoprecipitation and BiFC assays were similar to those described previously[13]. Purification of GST–HAB1, His–PYR1 and His–PYR1^{P88S} was performed as described previously[12]. GST, GST–ABI1, GST–ABF2 fragment, MBP and MBP–SnRK2.6 constructs were transformed into *Escherichia coli* Rosetta cells (Novagen) and the recombinant proteins were isolated by affinity purification. Purification of MBP–ABI1, MBP–ABI2, His–PYL8, His–PYL5 and His–SnRK2.6 was as described previously[13]. In-gel kinase assays were performed as described previously[20] with the modification that 300 μg of protein was loaded for samples without ABA treatment. For germination assays, seeds were plated on MS (Murashige and Skoog) nutrient medium containing 3% sucrose. In each experiment, at least 50 seeds per genotype were stratified at 4 °C for 3 days, and the presence of green cotyledons was scored after incubation for 6 days at 23 °C.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Zhu, J. K. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **53,** 247–273 (2002).
2. Hetherington, A. M. Guard cell signaling. *Cell* **107,** 711–714 (2001).
3. Schroeder, J. I., Kwak, J. M. & Allen, G. J. Guard cell abscisic acid signalling and engineering of drought hardiness in plants. *Nature* **410,** 327–330 (2001).
4. Finkelstein, R. R., Gampala, S. S. & Rock, C. D. Abscisic acid signaling in seeds and seedlings. *Plant Cell* **14** (Suppl), S15–S45 (2002).
5. Koornneef, M., Bentsink, L. & Hilhorst, H. Seed dormancy and germination. *Curr. Opin. Plant Biol.* **5,** 33–36 (2002).
6. Roelfsema, M. R. G. & Hedrich, R. In the light of stomatal opening—new insights into 'the Watergate'. *New Phytol.* **167,** 665–691 (2005).
7. Nemhauser, J. L., Hong, F. & Chory, J. Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* **126,** 467–475 (2006).
8. Shen, Y. Y. *et al.* The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443,** 823–826 (2006).
9. Liu, X. *et al.* G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science* **315,** 1712–1716 (2007).
10. Pandey, S., Nelson, D. C. & Assmann, S. M. Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. *Cell* **136,** 136–148 (2009).
11. Ma, Y. *et al.* Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324,** 1064–1068 (2009).
12. 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).
13. Santiago, J. *et al.* Modulation of drought resistance by the abscisic acid-receptor PYL5 through inhibition of clade A PP2Cs. *Plant J.* 10.1111/j.1365–313X.2009.03981.x (2009).
14. Li, S., Assmann, S. M. & Albert, R. Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. *PLoS Biol.* **4,** e312 (2006).
15. McCourt, P. & Creelman, R. The ABA receptors–we report you decide. *Curr. Opin. Plant Biol.* **11,** 474–478 (2008).
16. Pennisi, E. Stressed out over a stress hormone. *Science* **324,** 1012–1013 (2009).
17. Mustilli, A. C., Merlot, S., Vavasseur, A., Fenzi, F. & Giraudat, J. *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 (2002).
18. Yoshida, R. *et al.* ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol.* **43,** 1473–1483 (2002).
19. Yoshida, R. *et al.* 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 (2006).
20. Fujii, H., Verslues, P. E. & Zhu, J. K. 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 (2007).
21. Fujii, H. & Zhu, J. K. *Arabidopsis* mutant deficient in three abscisic acid-activated protein kinases reveals critical roles in growth, reproduction and stress. *Proc. Natl Acad. Sci. USA* **106,** 8380–8385 (2009).
22. Johnson, R. R., Wagner, R. L., Verhey, S. D. & Walker-Simmons, M. K. The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant Physiol.* **130,** 837–846 (2002).
23. Furihata, T. *et al.* Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc. Natl Acad. Sci. USA* **103,** 1988–1993 (2006).
24. Belin, C. *et al.* Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol.* **141,** 1316–1327 (2006).
25. Rubio, S. *et al.* Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. *Plant Physiol.* **150,** 1345–1355 (2009).
26. Kuhn, J. M. & Schroeder, J. I. Impacts of altered RNA metabolism on abscisic acid signaling. *Curr. Opin. Plant Biol.* **6,** 463–469 (2003).
27. Hirayama, T. & Shinozaki, K. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci.* **12,** 343–351 (2007).
28. Yoo, S.-D., Cho, Y.-H. & Sheen, J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2,** 1565–1572 (2007).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Plant culture. For protoplast isolation, *Arabidopsis thaliana* ecotype Columbia and *snrk2.2/2.3/2.6* triple mutant plants were grown on Jiffy7 soil (Jiffy Products) in an environment-controlled chamber (illumination of 75 μmol m^{-2} s^{-1}, photoperiod of 13 h light/11 h dark, 22 °C).

Plasmid constructs for protoplast transient assay. The RD29B promoter region[11] was amplified by PCR from Columbia genomic DNA and cloned by replacing the CBF3 promoter in the CBF3–LUC protoplast expression vector. CBF3–LUC, UQ10–GUS, ABI1 and ABI1^{G180D} protoplast expression plasmid vectors were provided by J. Sheen. ABI1 was replaced with His–PYR1/His–PYLs/His–PYR^{P88S}, HAB1–Myc, ABF2–haemagglutinin (HA)/ABF2 mutant versions, SnRK2–Flag/SnRK2.6^{K50N} sequences. All the plasmids were sequenced to confirm the sequence and avoid cloning errors. The GUS reporter plasmid was used as an internal control to normalize transfection efficiency in protoplast assays. Primers used for preparing protoplast expression vectors were as follows: PRD29B-F, 5′-CGGGATCCGTTGAATCTTGCGGAAGCA-3′; PRD29B-R, 5′-CATGCCATGGTTCAAGTGAATCAATCAAAC-3′; HAB1-F, 5′-CATGCCATGGAGGAGATGACTCCCGCAG-3′; HAB1-R1, 5′-CAGATCCTCCTCAGAAATCAGCTTTTGCTCAGGCCTGGTTCTGGTCTTGAACTTTC-3′; HAB1-R2, 5′-GCCTGCAGTCATAAGTCTTCTTCGCTTATTAATTTCTGTTCCAGATCCTCCTCAGAAA-3′; ABF2-F, 5′-CGGGATCCATGGATGGTAGTATGAATTTGG-3′; ABF2-R, 5′-GTCAAGGCCTCCAAGGTCCCGACTCTGTCCT-3′; SnRK2.2-F, 5′-CATGCCATGGATCCGGCGACTAATTCAC-3′; SnRK2.2–Flag-R, 5′-GTCCTTGTAGTCAGAAGGCCTGAGAGCATAAACTATCTC-3′; SnRK2.2–Flag–Pst-R, 5′-GCCTGCAGTCACTTGTCATCGTCGTCCTTGTAGTCAGAAG-3′.

In the SnRK2.2–Flag plasmid, SnRK2.2 was replaced with SnRK2.3 or SnRK2.6: SnRK2.3-F, 5′-CATGCCATGGATCGAGCTCCGGTGACCA-3′; SnRK2.3-R, 5′-GTCAAGGCCTGAGAGCGTAAACTATCTCTCCGCT-3′; SnRK2.6-F, 5′-CATGCCATGGATCGACCAGCAGTGAGT-3′; SnRK2.6-R, 5′-GTCAAGGCCTCATTGCGTACACAATCTCTC-3′.

His–PYR1 was constructed with following primers: His–PYR1-F1, 5′-CACCATCACCCATGGATGCCTTCGGAGTTAACACCAG-3′; His–PYR1-F2, 5′-CGGGATCCATGAAACATCACCATCACCATCACCCATGGATG-3′; PYR1-R, 5′-GTCAAGGCCTTCACGTCACCTGAGAACCACTTC-3′.

In His–PYR1 plasmid, PYR1 was replaced with PYLs with following primers: PYL1-F, 5′-CATGCCATGGATGGCGAATTCAGAGTCCTCCTC-3′; PYL1-R, 5′-GTCAAGGCCTTTACCTAACCTGAGAAGAGTTG-3′; PYL2-F, 5′-CATGCCATGGATGAGCTCATCCCCGGCCGTG-3′; PYL2-R, 5′-GTCAAGGCCTTTATTCATCATCATGCATAGGTG-3′; PYL3-F, 5′-CATGCCATGGATGAATCTTGCTCCAATCCATG-3′; PYL3-R, 5′-GTCAAGGCCTTCAGGTCGGAGAAGCCGTGGA-3′; PYL4-F, 5′-CATGCCATGGATGCTTGCCGTTCACCGTCCTTC-3′; PYL4-R, 5′-GTCAAGGCCTTCACAGAGACATCTTCTTCTTG-3′; PYL5-F, 5′-CATGCCATGGATGAGGTCACCGGTGCAACTC-3′; PYL5-R, 5′-GTCAAGGCCTTTATTGCCGGTTGGTACTTCGAG-3′; PYL6-F, 5′-CATGCCATGGATGCCAACGTCGATACAGTTTC-3′; PYL6-R, 5′-GTCAAGGCCTTTACGAGAATTTAGAAGTGTTCTC-3′; PYL7-F, 5′-CATGCCATGGATGGAGATGATCGGAGGAGAC-3′; PYL7-R, 5′-GTCAAGGCCTTCAAAGGTTGGTTTCTGTATGA-3′; PYL8-F, 5′-CATGCCATGGATGGAAGCTAACGGGATTGAGAAC-3′; PYL8-R, 5′-GTCAAGGCCTTTAGACTCTCGATTCTGTCGTG-3′; PYL9-F, 5′-CATGCCATGGATGATGGACGGCGTTGAAGG-3′; PYL9-R, 5′-GTCAAGGCCTTCACTGAGTAATGTCCTGAGAAG-3′; PYL10-F, 5′-CATGCCATGGATGAACGGTGACGAAACAAAGAAG-3′; PYL10-R, 5′-GTCAAGGCCTTCATATCTTCTTCTCCATAGATTCTG-3′; PYL11-F, 5′-CATGCCATGGATGGAAACTTCTCAAAAATATC-3′; PYL11-R, 5′-GTCAAGGCCTTTACAACTTTAGATGAGCCACC-3′; PYL12-F, 5′-CATGCCATGGATGAAAACATCTCAAGAACAGCA-3′; PYL12-R: 5′-GTCAAGGCCTTTAAGTGAGCTCCATCATCTTC-3′.

***In vitro* mutagenesis.** ABF2–HA plasmid was used for *in vitro* mutagenesis by using the GeneTailor Site-Directed Mutagenesis System (catalog no. 12397-014; Invitrogen) with the following mutagenic primers: ABF2S26-R, 5′-ACCTTGTCTAGTCAACCCTCCACCTCCACCAC-3′; ABF2S26A-F, 5′-AGGGTTGACCTAGACAAGGTGCTATATACTCGTTG-3′; ABF2S26D-F, 5′-AGGGTTGACTAGACAAGGTGATATATACTCGTTG-3′; ABF2S86-R, 5′-GCCTTGCCTCTGCAGCTGCAAACCCTCTTG-3′; ABF2S86A-F, 5′-CAGCTGCAGAGGCAAGGCGCTTTGACTCTGCCTC-3′; ABF2S86D-F, 5′-CAGCTGCAGAGGCAAGGCGATTTGACTCTGCCTC-3′; ABF2S94-R, 5′-AAGCGTTCGAGGCAGAGTCAACGAGCCTTG-3′; ABF2S94A-R1, 5′-AAGCGTTCGAGGCAGAGTCAAAGCGCCTTG-3′; VC-ABF2S94D-R1, 5′-AAGCGTTCGAGGCAGAGTCAAATCGCCTTG-3′; ABF2S94A-F, 5′-ACTCTGCCTCGAACGCTTGCTCAGAAGACGGTTG-3′; ABF2S94D-F, 5′-ACTCTGCCTCGAACGCTTGATCAGAAGACGGTTG-3′; ABF2T135-R, 5′-TTGCTGCCTCTGACTCTGACTCTGACTCTG-3′; ABF2T135A-F, 5′-TCAGAGTCAGAGGCAGCAAGCTTTAGGTGAAGTA-3′; ABF2T135D-F, 5′-TCAGAGTCAGAGGCAGCAAGATTTAGGTGAAGTA-3′.

Protoplast isolation and transactivation assay. Plants about 4 weeks old were used for protoplast isolation as described previously (http://genetics.mgh.harvard.edu/sheenweb)[28]. All the chemicals used in protoplast isolation were obtained from Sigma. Leaf strips about 0.5–1 mm in width cut from the middle part of leaves were vacuum infiltrated with enzyme solution containing 20 mM MES pH 5.7, 1.5% (w/v) cellulase R10 (Yakult Pharmaceutical Industry), 0.4% (w/v) macerozyme R10 (Yakult Pharmaceutical Industry), 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 1 mM 2-mercaptoethanol (optional) and 0.1% BSA. After infiltration for 30 min, leaf strips in enzyme solution were incubated in the dark for 3 h. Protoplasts were diluted with W5 (2 mM MES pH 5.7 containing 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl) solution to a final concentration of 2×10^5 cells ml^{-1}. After 30 min resting, W5 solution was removed and protoplasts were resuspended in MMg solution (4 mM MES pH 5.7 containing 0.4 M mannitol and 15 mM $MgCl_2$). For transfection, 100 μl of protoplasts (2×10^4 cells) were mixed with plasmid constructs (in 10 μl) and 110 μl of PEG solution (40% w/v PEG-4000 in doubly distilled water containing 0.2 M mannitol and 100 mM $CaCl_2$). After incubation for 10 min, transfection was stopped by the addition of 440 μl of W5 solution. Protoplasts were harvested by centrifugation at 100*g* for 2 min at room temperature (22–25 °C) and resuspended in about 100 μl of W5 solution. Protoplasts were then incubated in WI solution (4 mM MES pH 5.7 containing 0.5 M mannitol and 20 mM KCl) with 0 or 5 μm (+)-ABA for 5 h. Protoplasts were harvested by centrifugation at 100*g* for 2 min, frozen in liquid N_2 and stored at −80 °C until further use. The frozen protoplasts were resuspended in 100 μl of protoplast lysis buffer (2.5 mM Tris-phosphate pH 7.8 containing 1 mM dithiothreitol, 2 mM DACTAA, 10% (v/v) glycerol and 1% (v/v) Triton X-100). LUC mix (Promega) (100 μl) was added to 10 μl of the lysate, and LUC activity was measured with a plate reader (Wallac VICTOR2 plate reader). Protoplast lysate (2 μl) was mixed with 10 μl of 4-methylumbelliferyl β-D-glucuronide (MUG) substrate mix (10 mM Tris-HCl pH 8 containing 1 mM MUG and 2 mM $MgCl_2$) and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 100 μl of 0.2 M Na_2CO_3, and the fluorescence of 4-methylumbelliferone) was quantified with a plate reader (Wallac VICTOR2 plate reader).

The RD29B promoter fused with the LUC coding sequence was used as an ABA-responsive reporter gene (7 μg of plasmid per transfection). *ZmUBQ*::*GUS* was included in each sample as an internal control (3 μg per transfection). ABF2–HA and its mutant versions, SnRK2.6–Flag and SnRK2.6^{K50N}–Flag, His–PYR/PYLs and HAB1–Myc plasmid constructs were used at 3 μg per transfection, and ABI1 was used at 2 μg per transfection.

***In vitro* phosphatase and kinase assays.** MBP–SnRK2.6 (1 μg), MBP (1 μg) or Flag–SnRK2.6 on beads was incubated for 20 min with GST–ABI1 (1 μg), GST (1 μg), His–PYR1 (1 μg) and/or His–PYR1^{P88S} (1 μg), as indicated, in 40 μl of phosphatase buffer (50 mM Tris-HCl pH 7.0, 60 mM magnesium acetate, 0.1 mM EGTA and 0.1% 2-mercaptoethanol) containing the indicated concentration of (+)-ABA (Biosynth AG) at 30 °C. After removal of the solution, GST–ABF2 fragment (10 μg) was added. The reaction mixture (20 μl) contained 25 mM Tris pH 7.4, 12 mM $MgCl_2$, 1 μM ATP, 5 μCi of [γ-^{32}P]ATP, 1 mM dithiothreitol, 1 mM Na_3VO_4 and 5 mM NaF. The reaction mixtures were incubated for 30 min at 30 °C. The reaction was stopped by the addition of Laemmli sample buffer, and the mixtures were then subjected to SDS–PAGE. Radioactivity was detected with a Typhoon phosphoimager (GE Healthcare). SnRK2.6 kinase deactivation assays (Fig. 2d) were performed by previous incubation with protein phosphatase for 30 min at room temperature. Assays to test the recovery of SnRK2.6 activity were performed by incubation of the protein phosphatase for 10 min with the receptors in the presence of 1 μM (+)-ABA.

To measure phosphate release from synthetic phosphopeptide, the phosphopeptide (10 nmol) from Biomatik was incubated for 10 min with 1 μg of ABI1 or GST in phosphatase buffer (40 μl) at 30 °C. Released phosphate was measured as described previously[29].

Construction of plasmids for yeast two-hybrid and triple-hybrid analysis. The coding sequence of SnRK2.6, SnRK2.2 and SnRK2.3 was amplified by PCR with the following pairs of primers: F4g33950 (5′-ATGGATCGACCAGCAGTGAGT-3′) and R4g33950 (5′-TTTGTCGACTCACATTGCGTACACAATCT-3′), F3g50500 (5′-ATGGATCCGGCGACTAATTCA-3′) and R3g50500 (5′-TTTGTCGACTCAGAGAGCATAAACTATCT-3′), and F5g66880 (5′-ATGGATCGAGCTCCGGTGACC-3′) and R5g66880 (5′-TTTGTCGACTTAGAGAGCGTAAACTATCT-3′), respectively.

The PCR product was initially cloned into pCR8/GW/TOPO, double-digested with EcoRI–SalI and cloned into pGBT9, where the kinase coding sequences were fused to the GAL4 DNA-binding domain (GBD). pGBT9 constructs in which SnRK2s acted as baits were faced in two-hybrid assays with the following pGADT7 constructs: ABI1, ABI2 and HAB1, where the PP2C coding sequences were fused to the GAL4 activation domain (GAD).

To perform triple-hybrid experiments, the SnRK2.6 EcoRI–SalI fragment described above was cloned into the pBridge vector (Clontech), in which the kinase coding sequence is fused to GBD. Next, the coding sequences of PYL8 and PYL5 were cloned into the NotI site of pBridge–SnRK2.6. To reproduce the yeast two-hybrid interaction described above, yeast host AH109 was co-transformed with one of the following plasmids, pGADT7–ABI1/pGADT7–ABI2/pGADT7–HAB1, encoding GAD–PP2C fusions, and pBridge–SnRK2.6, encoding a GBD–SnRK2.6 fusion; to test the interference of ABA receptors on this interaction, pBridge–SnRK2.6 + PYL8 and pBridge–SnRK2.6 + PYL5 were employed.

BiFC. Experiments were performed as described previously[13]. Constructs were made in the pSPYNE-35S[30] as well as the Gateway vector pYFP^{C}43 (provided by A. Ferrando). The ABI1 coding sequence was excised from pCR8/GW/TOPO as a BamHI–SmaI fragment and cloned into pSPYNE-35S. The coding sequences of SnRK2.6, SnRK2.2, SnRK2.3 and ΔCSnRK2.6, previously cloned into pCR8/GW/TOPO, were recombined by LR reaction (recombination reaction between an *attL* attachment site present in the entry vector and *attR* present in the destination vector, catalysed by a host of recombination proteins) into pYFP^{C}43 destination vector.

Immunoprecipitation. Protein extracts for immunodetection experiments were prepared from *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* C58C1 (pGV2260) transformed with different fusion proteins containing SnRK2.6/OST1, the C-terminal deletion of SnRK2.6, the amino-terminal deletion, and full-length ABI1. Protein extracts from the transfected plants were used for immunoprecipitation at 4 °C with super-paramagnetic MACS microbeads coupled to monoclonal anti-c-Myc antibody (Miltenyi Biotec). The antigen–antibody complex was eluted, fractionated on an 8% SDS–PAGE gel and transferred to Immobilon-P membranes (Millipore), and probed either with monoclonal anti-c-Myc (clone 9E10; Roche) or anti-GFP^{C} (clone JL-8; Clontech) as primary antibodies and enhanced chemiluminescence anti-mouse peroxidase (GE Healthcare) as secondary antibody. Detection was performed with the enhanced chemiluminescence advance western blotting detection kit (GE Healthcare).

To immunoprecipitate SnRK2.6, 3-week-old seedlings of transgenic plants expressing Flag–SnRK2.6 treated with 100 μM ABA were used. Protein extracts were incubated for 2 h with anti-Flag antibody beads (Sigma) at 4 °C. The beads were washed six times with pre-chilled extraction buffer.

29. Van Veldhoven, P. P. & Mannaerts, G. P. Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **161**, 45–48 (1987).
30. Walter, M. *et al.* Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**, 428–438 (2004).