Clade A PP2Cs negatively regulate SnRK1 signaling in *Arabidopsis*

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ABSTRACT

Plant survival under environmental stress requires the integration of multiple signaling pathways into a coordinated response, but the molecular mechanisms underlying this integration are poorly understood. Stress-derived energy deprivation activates the Snf1-Related protein kinases 1 (SnRK1s), triggering a vast transcriptional and metabolic reprogramming that restores homeostasis and promotes tolerance to adverse conditions. Here, we show that clade A type 2C protein phosphatases (PP2Cs), established repressors of the abscisic acid (ABA) hormonal pathway, interact with the SnRK1 catalytic subunit causing its dephosphorylation and inactivation. Accordingly, SnRK1 repression is abrogated in double and quadruple *pp2c* knockout mutants, provoking, likewise SnRK1 overexpression, sugar hypersensitivity during early seedling development. Reporter gene assays and SnRK1 target gene expression analyses further demonstrate that PP2C inhibition by ABA results in SnRK1 activation, promoting SnRK1 signaling during stress and once the energy deficit subsides. Consistent with this, SnRK1 and ABA induce largely overlapping transcriptional responses. Hence, the PP2C hub allows the coordinated activation of ABA and energy signaling, strengthening the stress response through the cooperation of two key and complementary pathways.

INTRODUCTION

Changes in water and nutrient availability, soil salinity and extreme temperatures, amongst others, generate signals in plants that need to be finely integrated with metabolic activity and development for optimal growth and survival (Smith and Stitt, 2007). One such signal is energy deficiency derived from impaired carbon assimilation and/or respiration in situations of stress, which triggers the activation of the SnRK1 protein kinases to restore homeostasis and elaborate adequate longer-term responses through a vast metabolic and transcriptional reprogramming (Radchuk et al., 2006; Schwachtje et al., 2006; Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008; Lee et al., 2009). The *Arabidopsis* (*Arabidopsis thaliana*) genome encodes 38 SnRKs, of which 3, SnRK1.1 (KIN10/AKIN10), SnRK1.2 (KIN11/AKIN11), and SnRK1.3 (KIN12/AKIN12), represent the orthologs of the budding yeast Sucrose-non-fermenting1 (Snf1) and mammalian AMP-activated Protein Kinase (AMPK) metabolic sensors (Halford et al., 2003; Polge and Thomas, 2007; Hardie, 2011). An increasing body of evidence suggests that SnRK1s act as convergence points for various metabolic, hormonal and stress signals during growth and development, linking it to key hormonal pathways and in particular to ABA (Nemeth et al., 1998; Bhalerao et al., 1999; Bradford et al., 2003; Radchuk et al., 2006; Baena-Gonzalez et al., 2007; Lu et al., 2007; Rosnoblet et al., 2007; Ananieva et al., 2008; Baena-Gonzalez and Sheen, 2008; Lee et al., 2008; Jossier et al., 2009; Radchuk et al., 2010; Coello et al., 2012; Tsai and Gazzarrini, 2012). SnRK1 is an heterotrimeric complex composed of an α-catalytic subunit (SnRK1.1/1.2/1.3 in *Arabidopsis*), and two regulatory subunits, β and γ (Polge and Thomas, 2007). Similarly to its mammalian and yeast counterparts SnRK1 activity requires phosphorylation of a highly conserved T-loop residue (T175 in SnRK1.1) (Estruch et al., 1992; Hawley et al., 1996; Stein et al., 2000; McCartney and Schmidt, 2001; Baena-Gonzalez et al., 2007; Shen et al., 2009; Crozet et al., 2010). Under normal energy conditions in mammalian cells Mg-ATP is bound to the γ-subunit of the AMPK complex resulting, through the joint action of the constitutively active upstream Liver Kinase B1 (LKB1) and the still unknown upstream phosphatase, into a basal T-loop phosphorylation:dephosphorylation cycle with no net AMPK activation (Hardie, 2011). Under energy deficiency conditions, the replacement of Mg-ATP by AMP/ADP triggers a conformational change that promotes AMPK phosphorylation and most importantly, protects AMPK from dephosphorylation by rendering it a poor substrate for

phosphatases (Oakhill et al., 2011; Xiao et al., 2011). Despite the rate of dephosphorylation being a primary determinant of AMPK activity, the identity of the AMPK phosphatase(s) remains unclear and may differ between tissues and conditions of cell stimulation (Steinberg and Kemp, 2009; Carling et al., 2012). In the budding yeast, Reg1, a regulatory subunit of the protein phosphatase 1 (PP1) Glc7 enzyme, interacts with Snf1 and is required to maintain Snf1 in an inactive state during growth on glucose (Sanz et al., 2000; Hong et al., 2005). The metabolic signal underlying Snf1 regulation remained enigmatic for a long time but recent work demonstrated that also Snf1 is regulated by ADP at the substrate level, preventing its dephosphorylation by phosphatases (Mayer et al., 2011). In plants, SnAK1/2 (GRIK2/1) have been identified as upstream SnRK1 kinases (Shen et al., 2009; Crozet et al., 2010), but the phosphatases responsible for resetting SnRK1 signaling are unknown.

In *Arabidopsis* at least seven of the nine PP2Cs from clade A (Schweighofer et al., 2004) act as negative regulators of the ABA pathway (Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007; Rubio et al., 2009; Antoni et al., 2012) through their interaction with SnRK2s, more divergent members of the SnRK family and specific to plants (Halford et al., 2003; Cutler et al., 2010). *Arabidopsis* contains 10 SnRK2s of which three, SnRK2.2/2.3/2.6, are specifically activated by ABA and play a central role in the ABA pathway (Gomez-Cadenas et al., 1999; Li et al., 2000; Mustilli et al., 2002; Boudsocq et al., 2004; Yoshida et al., 2006; Boudsocq et al., 2007; Fujii et al., 2007; Fujii et al., 2009). Clade A PP2Cs regulate SnRK2.2/2.3/2.6 through physical obstruction and direct dephosphorylation of a conserved serine residue in the T-loop (S175 in SnRK2.6) (Umezawa et al., 2009; Vlad et al., 2009; Soon et al., 2012). In the presence of ABA, the Pyrabactin Resistance 1 (PYR1)/PYR1-Like (PYL)/Regulatory Components of ABA Receptors (RCAR) family of ABA receptors (hereafter PYL) inhibit PP2Cs, resulting in SnRK2 activation and downstream gene expression (Ma et al., 2009; Park et al., 2009; Soon et al., 2012).

Considering that clade A PP2Cs, through interaction with a wide array of targets, act as a regulatory hub for different abiotic stress responses (Sheen, 1996; Cherel et al., 2002; Guo et al., 2002; Himmelbach et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Umezawa et al., 2009; Vlad et al., 2009; Geiger et al., 2010) and taking into account the role of SnRK1 as a convergence point for multiple

types of stress (Baena-Gonzalez et al., 2007), we postulated that clade A PP2Cs might function as SnRK1 phosphatases. An additional hint came from data mining on a high-throughput proteomics screen for YFP-ABI1 interacting proteins, which inadvertently identified SnRK1s as putative ABI1-interacting proteins (Nishimura et al., 2010) (see below).

Here, we provide molecular, genetic and physiological evidence for the role of clade A PP2Cs as negative regulators of SnRK1 signaling in *Arabidopsis* through their direct interaction with the SnRK1 α-catalytic subunit, its dephosphorylation, and subsequent inactivation, hence contributing to resetting SnRK1 signaling upon the remittance of stress. On the other hand, PP2C inhibition allows ABA to promote SnRK1 activity, potentiating the stress response through the interplay of two complementary pathways and providing an explanation for the extensive genetic interactions reported between ABA and sugar signaling (Rolland et al., 2006).

RESULTS

Clade A PP2Cs interact with the SnRK1 catalytic subunit

A high-throughput screen employing GFP-affinity purification and mass-spectrometric analyses was carried out by Nishimura and colleagues to identify proteins interacting with YFP-ABI1 (Nishimura et al., 2010). Data mining of their results revealed the presence of peptides corresponding to both SnRK1s in several of their replicate experiments with YFP-ABI1 (SnRK1.1 in Experiments #1, #3, and #8 and SnRK1.2 in Experiments #1 and #3), whereas neither of the two SnRK1s were identified in any of the YFP control experiments.

As a first step to validate this data and investigate the possible regulation of SnRK1 by clade A PP2Cs, we tested in yeast-two-hybrid (Y2H) assays the interaction between the SnRK1 catalytic subunit and ABI1 or PP2CA, representative members of the two clade A branches in the PP2C family (Schweighofer et al., 2004). SnRK1.1 interacted with ABI1 and PP2CA in yeast cells, and deletion of its regulatory domain (RD) abolished this interaction (Figure 1A and Supplemental Figure 1). The N-terminus harbors the kinase catalytic domain (CD), whilst the C-terminus harbors the RD that binds the β- and γ-subunits (Polge and Thomas, 2007). The SnRK1 RD contains a subdomain of unknown function, the Kinase-Associated 1 (KA1) domain, that was reported in the SnRK3.11/SOS2 protein kinase to closely superimpose on the protein

phosphatase interaction (PPI) domain (Sanchez-Barrena et al., 2007), a docking site for the clade A PP2C ABI2 (Ohta et al., 2003). Modeling SnRK1.1 with the structures resolved for the KA1 domain in SnRK3.11 (Sanchez-Barrena et al., 2007), the AMPK-related Microtubule-Affinity Regulating Kinase 3 (MARK3) (Tochio et al., 2006), and for AMPKα (Xiao et al., 2011), revealed that in SnRK1.1 this subdomain spans residues 390-512 (Supplemental Figure 2). As shown in Figure 1A, the KA1 domain was both required and sufficient for the interaction with the phosphatase. Nevertheless, colony growth when using the KA1 domain alone was weaker than with SnRK1.1-RD or the full-length protein, suggesting that other regions may play a role in the PP2C-interaction.

To further validate the Y2H data, we performed an *in vitro* pull-down assay (Figure 1B). Purified recombinant His-SnRK1.1-CD or His-SnRK1.1-RD were incubated with GST-PP2CA or GST and the interacting proteins were pulled-down employing a glutathione-agarose matrix. SnRK1.1-RD was recovered only when using GST-PP2CA as bait, and a clear enrichment of SnRK1.1-CD was observed when using GST-PP2CA compared to GST alone, suggesting that even though not detected in the Y2H assay, PP2Cs interact also with the SnRK1.1-CD. To determine whether a SnRK1.1-PP2C interaction occurs also *in planta*, SnRK1.1 was transiently co-expressed in *Arabidopsis* protoplasts with control DNA or with a plasmid expressing ABI1-HA. Immunoprecipitation with an anti-HA antibody revealed a specific interaction between SnRK1.1 and ABI1-HA (Figure 1C), demonstrating that clade A PP2Cs interact with SnRK1.1 also *in vivo*.

Clade A PP2Cs dephosphorylate and inactivate SnRK1.1

To evaluate whether the detected PP2C-SnRK1.1 interaction results in SnRK1.1 dephosphorylation and inactivation, we immunoprecipitated SnRK1.1 from plants overexpressing an HA-tagged version (*35S::SnRK1.1-HA*) (Baena-Gonzalez et al., 2007) and treated with recombinant His-PP2CA. PP2CA treatment caused a clear dephosphorylation of SnRK1.1, as assessed by a faster mobility in a Phos-tag SDS-PAGE that selectively retards phosphorylated proteins (Kinoshita et al., 2009) (Figure 2A). To investigate the effect of this dephosphorylation on SnRK1 activity, we performed *in vitro* kinase assays. In agreement with previous reports, active SnRK1.1 could efficiently autophosphorylate and phosphorylate the ABF2 transcription factor *in*

178 *vitro* (Bhalerao et al., 1999; Zhang et al., 2008; Shen et al., 2009) (Figure 2B, lane 1).
179 However, addition of PP2CA to the reaction caused a substantial decrease in the
180 phosphorylation of both SnRK1.1 and ABF2 (Figure 2B, lane 2). The PYL receptors
181 inhibit clade A PP2Cs in the presence of ABA, resulting in SnRK2 activation (Fujii et
182 al., 2009; Ma et al., 2009; Park et al., 2009). Adding the PYL4 receptor in the absence
183 of ABA did not change the ability of PP2CA to inactivate SnRK1 (Figure 2B, lane 3),
184 whilst in the presence of ABA, PYL4 fully blocked SnRK1.1 inactivation by PP2CA
185 (Figure 2B, lane 4). The repressive effect of PP2CA was at least partly due to a direct
186 effect on SnRK1.1, since a similar inactivation was observed when SnRK1.1 was pre-
187 incubated with PP2CA and PYL4 (PP2CA active) prior to ABA and ABF2 addition
188 (PP2C inactive) (Supplemental Figure 3, lanes 2 and 3).

189 SnRK1 requires phosphorylation of the T-loop T175 residue (S175 for
190 SnRK2.6) for activity (Baena-Gonzalez et al., 2007; Shen et al., 2009; Crozet et al.,
191 2010). To test whether T175 could be a substrate for clade A PP2Cs, we first performed
192 *in vitro* dephosphorylation experiments. Recombinant SnRK1.1 is not phosphorylated
193 and hence barely active, but can be strongly activated by the upstream kinases SnAK1/2
194 (GRIK2/1) through the specific phosphorylation of T175 (Shen et al., 2009; Crozet et
195 al., 2010). PP2CA treatment of recombinant GST-SnRK1.1, pre-phosphorylated with
196 GST-SnAK2, resulted in significant T175 dephosphorylation, as detected with an anti-
197 phospho-AMPK(T172) antibody (Sugden et al., 1999; Baena-Gonzalez et al., 2007)
198 (Figure 2C). A similar effect was observed when SnRK1.1 was immunoprecipitated
199 from *35S::SnRK1.1-HA* plants and treated with GST-PP2CA (Figure 2D), altogether
200 showing that T175 is efficiently dephosphorylated by PP2Cs *in vitro*.

201 To determine whether T175 is a PP2C substrate *in vivo*, we used *Arabidopsis*
202 mesophyll protoplasts to transiently express SnRK1.1-GFP alone or in combination
203 with various PP2Cs. As shown in Figure 2E, co-expression of SnRK1.1-GFP with either
204 ABI1 or PP2CA (from clade A) resulted in a significant reduction in T175
205 phosphorylation levels, whilst co-expression with the unrelated PP2C6-6 from clade E
206 (Schweighofer et al., 2004) did not have an impact on T175 phosphorylation (Figure
207 2F). These results suggest T175 is a substrate for clade A PP2Cs also *in vivo*.

208

209 **Clade A PP2Cs repress SnRK1 signaling**

To further explore the functional implications of SnRK1 regulation by PP2Cs, we employed a transient cell-based assay that uses luciferase (LUC) induction from the *DIN6::LUC* reporter as readout of SnRK1 activity (Baena-Gonzalez et al., 2007). In transfected mesophyll protoplasts, SnRK1.1 overexpression is sufficient to induce strong LUC activity under control conditions (Figure 3A) (Baena-Gonzalez et al., 2007). Co-expression with the ABI1 or PP2CA phosphatases reduced SnRK1.1-mediated *DIN6::LUC* induction more than 60% without affecting SnRK1.1 levels (Figure 3A). Importantly, the ability of these phosphatases to repress reporter gene induction by SnRK1.1 was strongly diminished in the corresponding catalytically inactive variants (D177A and D142A, respectively), suggesting that repression of SnRK1 signaling by ABI1 and PP2CA occurs to a large extent through dephosphorylation. As a negative control, co-expression with the unrelated PP2C6-6 from clade E (Schweighofer et al., 2004) had no effect on the ability of SnRK1.1 to induce the reporter (Figure 3B), altogether supporting the specific repressive role of clade A PP2Cs on the SnRK1 pathway.

To investigate the influence of clade A PP2Cs on endogenous SnRK1 signaling, we treated detached leaves of wild-type (WT), the double *abi1-2 pp2ca-1* (Rubio et al., 2009), and two different quadruple *pp2c* knockout mutants (*hai1-1 pp2ca-1 hab1-1 abi1-2*, hereafter Q*hai1-1,* and *abi2-2 pp2ca-1 hab1-1 abi1-2,* hereafter Q*abi2-2*; Supplemental Figure 4; (Antoni, 2013)) under control (3h light, *L*), activating (3h darkness, *D*) and inactivating conditions (3h darkness followed by 1h darkness in 50 mM glucose, *DG*), and analyzed SnRK1 target gene expression (Baena-Gonzalez et al., 2007) by quantitative RT-PCR (qRT-PCR). Exposure to darkness triggered a strong induction of SnRK1 target genes in all genotypes (Figure 3C), in agreement with the current view that the conformation adopted by AMPK and Snf1 under conditions of low energy renders the kinases resistant to phosphatase action (Mayer et al., 2011; Oakhill et al., 2011; Xiao et al., 2011). In marked contrast, SnRK1 inactivation in response to subsequent glucose addition was deficient in *abi1-2 pp2ca-1* plants (for *DIN6*) and completely blocked in the quadruple *pp2c* mutants (Figure 3C), demonstrating that clade A PP2Cs are essential components for the post-stress inactivation of SnRK1.

To assess whether these differences in SnRK1 target regulation between WT and quadruple *pp2c* mutants were correlated with differences in T175 phosphorylation, we monitored phospho-T175 levels in WT and Q*abi2-2* leaves under the same conditions

as for the gene expression analyses. In agreement with previous work (Baena-Gonzalez et al., 2007), we did not observe differences in T175 phosphorylation between control, inducing, and inactivating conditions nor between WT and Q*abi2-2* leaves (Supplemental Figure 5), suggesting that even though T175 phosphorylation is required for SnRK1 activity, other phosphorylation events or additional mechanisms play a role in the regulation of SnRK1.

Altered sugar responses in *pp2c* mutants

High concentrations of sugars (6% glucose, ~330 mM) induce a developmental arrest characterized *e.g.* by repression of cotyledon greening and expansion (Rolland et al., 2006). WT seedlings grow well on plates containing 4% glucose but cotyledon greening and expansion are clearly impaired on higher sugar concentrations (Figure 4). Such adverse conditions trigger SnRK1 activation leading to sugar hypersensitivity in *35S::SnRK1.1* seedlings (Jossier et al., 2009) (Figure 4). The *abi1-2 pp2ca-1* double mutant displays glucose hypersensitivity visible only in 6% glucose, but this is markedly enhanced in the quadruple *pp2c* mutants, which exhibit a clear phenotype in 4% glucose (Figure 4). Even though the high ABA hypersensitivity of these mutants (Supplemental Figure 4) renders them more sensitive to increased osmolarity in the 4% sorbitol control plates (Antoni et al., 2012), a clear impact on development can be observed on 4% glucose plates. In 6% sorbitol and glucose plates the growth of these mutants is so compromised that a distinction between osmotic and sugar effects is not possible. Consistent with the loss-of-function phenotype, plants overexpressing PP2CA are sugar insensitive (Figure 4), altogether genetically supporting the role of PP2Cs as negative regulators of SnRK1 signaling.

ABA promotes SnRK1 signaling via PP2Cs

We next wanted to assess whether PP2C regulation of the SnRK1 pathway could allow ABA to modulate SnRK1 activity. The transient co-expression of PYL receptors with ABI1 in ABA-treated mesophyll protoplasts is enough to efficiently repress ABI1 action and to trigger the activation of an ABA signaling reporter (Fujii et al., 2009). Similarly, co-expression of ABI1 with PYL4 in the presence of ABA fully restored SnRK1.1 ability to induce the *DIN6::LUC* reporter in protoplasts (Figure 5A), presumably through ABI1 sequestration in the ABA-PYL-PP2C ternary complex. We

observed an overall 2-fold increase in LUC activity when comparing mock- and ABA-treated samples (Supplemental Figure 6), further suggesting that ABA can induce SnRK1 signaling. To further explore this possibility and to examine the ABA effect on other SnRK1 target genes (Baena-Gonzalez et al., 2007), we treated *Arabidopsis* leaf discs with or without ABA (100 µM) for 5h and quantified downstream gene expression changes by qRT-PCR. ABA treatment did result in SnRK1 activation, albeit to an extent one order of magnitude lower than that triggered by darkness (Figure 5B). Most importantly, the impact of ABA on SnRK1 target genes was clearly reduced in plants overexpressing PP2CA (*35S::PP2CA*, Figure 5C) (Antoni et al., 2012), indicating that the effect of ABA on SnRK1 activity is *via* PP2C inhibition. To investigate this connection at the whole genome level, we compared the transcriptional profile associated with SnRK1.1 activation in protoplasts (Baena-Gonzalez et al., 2007) with that of seedlings treated with ABA (http://Arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp, AtGenExpress Consortium) (Nemhauser et al., 2006). Despite differences in tissue type and developmental stage in the two datasets, there was a significant overlap between the transcriptional changes triggered by SnRK1.1 and by ABA (Figure 5D). More than 22% and 28% of the total number of genes upregulated and downregulated by SnRK1.1, respectively, were similarly regulated by ABA, in marked contrast with the negligible overlap with other hormone treatments or when comparing genes oppositely regulated in the SnRK1.1 and ABA datasets (Supplemental Figure 7). Despite the wide impact of both SnRK1 and ABA on the transcriptome, the probability of obtaining such an overlap of similarly regulated genes by chance is very low (hypergeometric test, $p<9.2^{-42}$).

We next analyzed SnRK1 target gene expression from WT leaf discs adding ABA in the beginning of the dark treatment to test the combined effect of ABA and energy stress, or 2h prior to glucose addition to test the impact of ABA on the sugar-induced inactivation of SnRK1. Addition of ABA could clearly enhance SnRK1 activation by darkness (Figure 5E, samples *D*, *DA*). Moreover, adding ABA prior to glucose diminished SnRK1 inactivation in response to sugar (Figure 5E, samples *DG*, *DGA*). Collectively, these results show that ABA positively regulates SnRK1 signaling through inhibition of clade A PP2Cs, promoting SnRK1 signaling during stress and once energy deficiency remits.

DISCUSSION

Despite the central role of SnRK1 kinases in the plant stress response, the regulatory mechanisms underlying SnRK1 function are poorly understood. We have demonstrated here that clade A PP2Cs are *bona fide* SnRK1 phosphatases that contribute to resetting SnRK1 activity upon restoration of energy levels and that allow ABA to induce and potentiate SnRK1 signaling during stress (Figure 6).

A clear interaction between SnRK1.1 and PP2Cs was observed both *in vitro* and *in vivo* (Figure 1), demonstrating that PP2Cs act through direct binding to the SnRK1 α-catalytic subunit, probably using the C-terminal regulatory domain of SnRK1 as a docking site, albeit interacting also with the catalytic region that harbors the T175 target residue. Based on Y2H experiments the KA1 domain of SnRK1 may play a key role in the PP2C-SnRK1 interaction (Figure 1A). As previously noted (Sanchez-Barrena et al., 2007), the KA1 domain can be closely superimposed on the phosphatase interaction domain of SOS2/SnRK3.11 and, given its presence also in the related AMPK and MARK3 kinases, has been suggested to represent an ancient highly conserved scaffold for interaction with PP2Cs (Sanchez-Barrena et al., 2007) (Supplemental Figure 2). SnRK2.2/2.3/2.6 also require their C-terminal region, namely the ABA box, for PP2C binding (Vlad et al., 2009; Soon et al., 2012), and additional regions of interaction exist within the N-terminal catalytic domain (Soon et al., 2012), some of which, such as the T- loop and the αG helix, correspond to conserved features of the protein kinase canonical fold (Hanks and Hunter, 1995) (Supplemental Figure 2). Similarly to SnRK2s, our *in vitro* pull-down assays suggested that the SnRK1.1-PP2CA interaction is mediated through regions both in the regulatory and the catalytic domains (Figure 1B). Interestingly, a high-throughput screen for YFP-ABI1 interactors employing affinity purification and LC-MS/MS identified SnRK1s as candidate ABI1-interacting proteins, whilst peptides corresponding to SnRK2.6 were not retrieved and the ABI1-SnRK2.6 interaction could only be confirmed by co-immunoprecipitation of the transiently overexpressed proteins in tobacco (Nishimura et al., 2010)).

As an outcome of the interaction with clade A PP2Cs, SnRK1 is dephosphorylated and inactivated (Figures 2 and 3). Nevertheless, mutation of the catalytic site in the ABI1_D177A and PP2CA_D142A mutants did not fully restore SnRK1 activity (Figure 3A), suggesting that, although dephosphorylation plays a major

role in SnRK1 inactivation, physical blockage may, similarly to SnRK2s (Soon et al., 2012), also be important for SnRK1 repression.

PP2CA was able to efficiently dephosphorylate T175 *in vitro* and *in vivo* (Figures 2, 3D, and 3E), consistent with the *in vitro* dephosphorylation of this residue by mammalian PP2C (Sugden et al., 1999). In agreement with previous work (Baena-Gonzalez et al., 2007), we could not detect any differences in the phosphorylation levels of T175 under various conditions (Supplemental Figure 5). It is thus likely that, in contrast to mammals (Hardie, 2011), and despite being a requirement for kinase activity (Baena-Gonzalez et al., 2007; Shen et al., 2009; Crozet et al., 2010), T175 phosphorylation is not the final switch between SnRK1 activation and inactivation and additional modifications may be involved, similarly to what has been reported for several SnRK2s (Vlad et al., 2010). For example, while S175 phosphorylation is necessary for the catalytic activity of SnRK2.6, full kinase activation by ABA requires phosphorylation at a second site, S171 (Vlad et al., 2010), and both of these residues are substrates of ABI1 (Umezawa et al., 2009). Additional phosphorylated residues in the T-loop and the C-terminal regulatory region have indeed been identified in SnRK1.1 (Wang et al., 2012), and the phosphorylation level of one of these (S364) appears to be increased in response to ABA and in particular to dehydration (Umezawa et al., 2013), suggesting they might be involved in the activation of SnRK1.1 under these conditions. Whether S364 and other residues are, likewise T175, dephosphorylated by clade A PP2Cs and whether they are differentially phosphorylated in the conditions used in our study remains to be determined. It is also plausible that this level of regulation applies only to a small fraction of the SnRK1 cellular pool and thus differential phosphorylation may remain undetected when assaying total protein extracts.

Our results employing reporter gene assays and gene expression analyses in WT, *pp2c* knockout mutants and PP2CA overexpressors show that PP2Cs are negative regulators of SnRK1 signaling (Figures 3 and 4). Transient co-expression of PP2Cs with SnRK1 in protoplasts reduced more than 60% the ability of SnRK1 to activate gene expression (Figure 3). Using a similar approach Fujii and colleagues showed that the extent of repression by ABI1 was nearly 100% when co-expressing SnRK2.6 and its downstream ABF2 transcription factor to activate an ABA reporter (Fujii et al., 2009). However, the ability of PP2Cs to repress kinase activity varied depending on the SnRK2 and PP2C combination employed, and in the case of SnRK2.6 and HAB1 the

repression was only 30%. Since some clade A PP2Cs have been shown to dephosphorylate ABF2 (Antoni et al., 2012), it is also possible that the difference in the extent of repression is due to a simultaneous effect of ABI1 on the kinase and on the transcription factor.

Most importantly, constitutive PP2C depletion in the quadruple *pp2c* mutants abrogates SnRK1 inactivation and downstream target gene repression after stress-derived energy deprivation subsides (Figure 3C, *DG* samples). However, the impact of PP2C depletion is less obvious under activating stress conditions (Figure 3C, *D* samples) presumably because, likewise AMPK and Snf1 (Mayer et al., 2011; Oakhill et al., 2011; Xiao et al., 2011), the kinase is protected from dephosphorylation when energy levels are low (Sugden et al., 1999). Similarly to plants overexpressing SnRK1.1, double and quadruple *pp2c* knockout mutants showed to varying degrees a sugar hypersensitive phenotype, whilst PP2CA overexpressors displayed an opposite phenotype (Figure 4), all consistent with the conclusions from the molecular data that PP2Cs negatively regulate SnRK1.

Our results indicate that the ABA and energy signaling pathways interact through PP2Cs and that ABA can induce SnRK1 signaling through PP2C inhibition (Figure 5). This is in agreement with a recent study reporting enhanced SnRK1 activity in wheat roots in response to ABA (Coello et al., 2012), and provides a molecular explanation for the extensive interactions observed between ABA and sugar signaling in genetic screens (Rolland et al., 2006). SnRK1s were never identified amongst ABA-activated kinases, most probably because the extent of SnRK1 activation by ABA is one order of magnitude lower than that by energy stress (darkness; Figure 3C), and would probably remain masked by the much stronger activities of SnRK2s. On the other hand, these studies relied on in-gel kinase assays for detection of kinase activities (Yoshida et al., 2002; Furihata et al., 2006; Fujii et al., 2007). Despite our current lack of knowledge regarding the exact subunit composition of functional SnRK1, and despite the fact that the catalytic subunit alone is active (Bhalerao et al., 1999; Shen et al., 2009; Crozet et al., 2010), *in vivo* SnRK1 most likely operates, similarly to Snf1 and AMPK, as a heterotrimeric complex (Polge and Thomas, 2007; Hedbacker and Carlson, 2008; Hardie, 2011; Ramon et al., 2013), whose dissociation under the denaturing conditions employed in the in-gel kinase assays may result in loss of kinase activity.

In addition to the interaction through PP2Cs other points of crosstalk are likely to exist between ABA and energy signaling, and *e.g.* SnRK1 may regulate ABA transcription factors like ABF2 (Figure 2B) or FUS3 (Zhang et al., 2008; Tsai and Gazzarrini, 2012) that can also be directly dephosphorylated by PP2Cs (Antoni et al., 2012). It is conceivable that in plants with altered SnRK1 signaling, aberrant PP2C:SnRK1 ratios, as well as the possible PP2C/SnRK1 co-regulation of downstream factors, could account for the altered ABA sensitivity and ABA-related phenotypes of these plants (Radchuk et al., 2006; Lu et al., 2007; Rosnoblet et al., 2007; Jossier et al., 2009; Radchuk et al., 2010; Tsai and Gazzarrini, 2012).

We propose a dual role for the regulation of SnRK1 by clade A PP2Cs (Figure 6). On one hand, activation of the SnRK1 pathway through alternative signals like ABA, could support the ABA response with a more general one directed towards a metabolic and transcriptional reprogramming to cope with energy deficiency. Activation of SnRK1 by ABA could also serve to prime the SnRK1 system, potentiating a subsequent response to energy imbalance derived from stress. On the other hand, PP2C regulation appears to be an integral part of the SnRK1 signaling pathway, resetting the system once stress subsides or an energy balance is attained through the appropriate metabolic readjustments. Persistence of ABA under these conditions would in turn promote the maintenance of SnRK1 in an active state, similarly to how elevated interleukin-6 (IL-6) sustains high AMPK activity in skeletal muscle when energy levels are presumably no longer altered after exercise (Ruderman et al., 2006). With this scenario in mind, one could envision that in tissues directly exposed to stress SnRK1 activation would be mainly dictated by the energy-dependent branch, whereas in distant tissues this activation could be mediated by ABA. In addition to IL-6, AMPK responds to other inflammatory mediators and hormones, but the precise mechanisms underlying this regulation are in most cases unknown (Steinberg and Kemp, 2009; Lim et al., 2010). Interestingly, chronic TNFα treatment in muscle cells suppresses the AMPK pathway through the induction of the repressor PP2C (Steinberg et al., 2006), suggesting that a connection between hormone signals and energy signaling through the inhibitory PP2Cs might be conserved in multicellular eukaryotes.

In summary, we have identified clade A PP2Cs as the upstream phosphatases of SnRK1 uncovering also a mechanism through which ABA can stimulate SnRK1 action. Future work to further understand SnRK1 regulation and to unravel the interplay of

these two central pathways may offer new insight not only into the mechanisms of stress tolerance, but also into fundamental developmental processes like seed maturation and germination.

METHODS

Primers, gene identifiers and constructs

A list of all primers, gene identifiers, cloning steps and vectors is provided in Supplemental Table 1.

Plant material and growth conditions

All used *Arabidopsis thaliana* (*Arabidopsis*) plants are in the Columbia (Col-*0*) background, except *35S::SnRK1.1-HA* (Landsberg *erecta*) (Baena-Gonzalez et al., 2007). The *35S::SnRK1.1* (*35S::SnRK1.1-2)* (Jossier et al., 2009), *35S::PP2CA* (Antoni et al., 2012), and *abi1-2 pp2ca-1* (Rubio et al., 2009) lines have been described. Quadruple *pp2c* knockout mutants were generated from *pp2ca-1 hai1-1* (Antoni et al., 2012) and the corresponding triple *pp2c* mutants (Rubio et al., 2009).

Plants were grown in soil under short day conditions (12 h light 100 µE/12 h dark). For *in vitro* culture, sterilized seeds were stratified in the dark at 4ºC for 2 days, and sowed on plates containing Murashige and Skoog medium with 0.1% MES, 0.8% phytoagar, and glucose (4% or 6%) or sorbitol (4% or 6%). Plates were sealed and incubated at 23ºC under continuous light.

Antibodies and protein expression analyses

The SnRK1.1 antibody was purchased from Agrisera (anti-AKIN10, AS10919). Phospho-SnRK1.1(T175) was detected with an anti-phospho-AMPK(T172) antibody (referred as αP-AMPK; Cell Signaling #2532), detecting also phospho-SnRK1.2(T175) as a lower band (Baena-Gonzalez et al., 2007). An anti-GST polyclonal antibody (Sigma G7781), and anti-HA- (Roche, 11583816001), and anti-T7- (Novagen, 69522-3) monoclonal antibodies were used for the detection of the corresponding tagged proteins.

For analyses of protein expression from protoplast pellets and leaf tissue, the material was directly ground in 2X Laemmli solubilization buffer to maintain the phosphorylation status during protein extraction.

Protoplast transient expression assays

Vectors for protoplast transient expression and assays were as described (Yoo et al., 2007), using the UBQ10-GUS reporter as transfection efficiency control. For constructs for overexpression of SnRK1.1-GFP, ABI1-HA, PP2CA-HA, PP2C6-6-HA, and FLAG-PYL4 the corresponding coding sequences were cloned into a pHBT95 vector harboring the indicated C- or N-terminal tag. SnRK1 signaling was monitored using a *DIN6::LUC* reporter (Baena-Gonzalez et al., 2007). ABA and glucose were added to a final concentration of 5 µM and 30 mM, respectively.

For co-immunoprecipitation assays untagged SnRK1.1 was expressed with ABI1-HA or mER7 control DNA (Yoo et al., 2007) in 3 ml of protoplasts under standard conditions.

Frozen cell pellets were lysed in 500 µl of lysis buffer [50 mM Tris-HCl pH8.0, 50 mM NaCl, 10 mM EDTA, 10% Glycerol, 0.5% Triton X-100, Complete Protease inhibitor cocktail (Roche), 20 mM NaF, 1 mM Orthovanadate, 1/500 (v/v) Phosphatase inhibitor 2 (Sigma P044), 1/500 (v/v) Phosphatase inhibitor 3 (Sigma P5726)], incubated at 4°C for 10min and diluted to a final volume of 1.5 ml with lysis buffer without Triton X-100. The cleared lysate was incubated with 40 µl of anti-HA affinity matrix (Roche 11815016001) for 3h at 4ºC. Agarose beads containing immunoprecipitated proteins were washed 5 times with lysis buffer containing 0.05% Triton, eluted with 4X Laemmli solubilization buffer, and analyzed by Western blot with an anti-SnRK1.1 antibody.

Recombinant protein production

The coding sequence of PP2CA was cloned into pGEX-4T1. Recombinant GST-PP2CA was produced in *E. coli* (BL21:DE3) and purified through GSH affinity chromatography as recommended by the manufacturer (Sigma G4510).

N- (residues 1-293, CD) and C-terminal (residues 294-512, RD) SnRK1.1 were cloned into pET28a (Novagen). Recombinant proteins were produced in *E. coli* (BL21:DE3) and purified using IMAC (TALON, Clontech #635502) following

manufacturer's instructions. Successful protein production and purification was verified by Western blotting with anti-GST and anti-T7 antibodies. Recombinant His-PYL4, His-PP2CA and His-ΔC ABF2 (residues 1–173) were produced as described in (Antoni et al., 2012), and recombinant GST-SnRK1.1 and GST-SnAK2 as in (Crozet et al., 2010).

In vitro pull-down assays

Proteins (3µg of each) were incubated 1h at room temperature in 100µL of buffer A (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Triton-X100, 1/500 (V/V) plant-specific protease inhibitor cocktail (Sigma P9599)), mixed with 30 µl of GSH-agarose beads and incubated one more hour. Beads were washed 4 times with buffer A, and bound proteins were analyzed by Western blotting using anti-T7 antibodies.

SnRK1.1 immunoprecipitation, phosphatase treatment and *in vitro* kinase assays

SnRK1.1 was immunoprecipitated from leaves of *35S::SnRK1.1-HA* plants treated for 1h in darkness. Plant material (~1g) was extracted in 3 volumes of 1XPBS supplemented with 1mM EDTA, 0.05% Triton X-100, and 1/500 (V/V) plant-specific protease inhibitor cocktail (Sigma). After centrifugation (16000g, 4ºC, 15min) the supernatant was recovered and 1mg of total protein was incubated O/N at 4ºC with 30 µl Anti-HA affinity matrix. The matrix was washed 3 times with extraction buffer and resuspended in a total volume of 66 µl of buffer (50 mM Tris-HCl pH 7.6, 250 mM KCl, 10 % glycerol, 0.1 % Tween-20), of which 3 µl were used for each reaction.

To assess dephosphorylation of immunoprecipitated SnRK1.1 by PP2CA, SnRK1.1 was incubated with His-PP2CA (2 µg) in a 50µl reaction containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT. The reaction was stopped with Laemmli solubilization buffer and analyzed employing a Phos-tag SDS-PAGE (50 µM Phos-tag ligand (Wako) and 100 µM $MnCl_2$) (Kinoshita et al., 2009) and Western blot with an anti-HA antibody. The Phos-tag ligand selectively retards phosphorylated proteins.

For *in vitro* kinase assays immunoprecipitated SnRK1.1 was preincubated (for 10 min) or not with His-PP2CA (0.6µg) and His-PYL4 (2.0µg) in 30 µl kinase buffer (20 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 2 mM $MnCl_2$) ± ABA (30 µM) and further incubated with GST-ΔC ABF2 (0.5µg) for 1h at room temperature in the presence of 3.5 µCi of γ^{32}P-ATP. Reaction products were resolved in an 8% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), and detected using a Phosphorimage system (FLA5100, Fujifilm) (Antoni et al., 2012).

For pre-activation of SnRK1.1, GST-SnRK1.1 and GST-SnAK2 (1µg of each) were incubated in 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 100µM ATP, 1 mM DTT, 1/1000 protease inhibitor cocktail (Sigma P9599) at 30°C for 30 min. After adding or not GST-PP2CA (1µg) the mix was further incubated for 30 min, and analyzed by Western blot employing anti-P-AMPK(T172) and anti-SnRK1.1 antibodies.

Yeast-two-hybrid assays

Y2H assays were performed as described (Saez et al., 2008). The full-length coding sequence of SnRK1.1 and the various deletions, cloned into pGBKT7, were faced with constructs harboring full-length PP2CA and ABI1 in fusion with the GAL4 activation domain (GAD). To generate the GAD-PP2CA fusion the PP2CA coding sequence was cloned into pGADT7. The pGADT7-ABI1 construct has been previously described (Vlad et al., 2010).

Gene expression analyses

Fully expanded leaves of 5-week old plants were used as such or to cut leaf discs (Ø 9mm) and incubated on sterile MilliQ water in Petri dishes. For examining SnRK1 regulation in the WT and *pp2c* mutants, leaves were incubated for 3h in light (control; *L;* 100 µE) or darkness (*D*), or 3h in darkness followed by 1h in darkness with glucose (*DG*). Unexpected darkness is perceived as stress and activates SnRK1 (Baena-Gonzalez et al., 2007). For assessing the effect of ABA leaf discs of WT or *35S::PP2CA* plants were incubated ± ABA under light for 5h. For the effect of ABA on SnRK1 activation by stress and inactivation by sugar, leaf discs of WT plants were

incubated 3h in light (*L*), in darkness with (*DA*) or without ABA (*D*), or 1h in darkness followed by 2h in darkness with ABA and 1h in darkness with ABA and glucose (*DGA*). Glucose and ABA were added to a final concentration of 50 mM and 100μM, respectively.

Following the indicated treatments, total RNA was extracted using TRIzol® reagent (Life Technologies), treated with RNase-Free DNase (Promega) and reverse transcribed (1.5 μg) using SuperScript III Reverse Transcriptase (Life Technologies). qRT–PCR analyses were performed using a 7900HT fast real time PCR System (Applied Biosystems) employing the Eva-Green fluorescent stain (Biotium), and the $2^{-\Delta CT}$ or comparative CT method (Livak and Schmittgen, 2001). Expression levels were normalized using the CT values obtained for the *EIF4* gene. Efficient ABA uptake and signaling was confirmed by monitoring the induction of the ABA marker genes *RAB18* and *RD29.*

Microarray dataset comparisons

The dataset for the SnRK1.1-induced transcriptional profile corresponds to Table S3 in (Baena-Gonzalez et al., 2007). The hormone treatment datasets, as compared in (Nemhauser et al., 2006), are from the *Arabidopsis* AtGenExpress consortium (http://*Arabidopsis*.org/portals/expression/microarray/ATGenExpress.jsp). A two-fold change filter was applied to all the hormone datasets and, given the 6h incubation of the SnRK1.1 overexpression dataset, only the 3h (and not the 1h) time points were considered for the comparisons. Overlap between the compared datasets was revealed using the Venny Venn diagram on-line application (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The dataset for the SnRK1.1-induced transcriptional profile corresponds to Table S3 in (Baena-Gonzalez et al., 2007). For determining the significance of overlap between the two experiments, hypergeometric testing was applied using the *dhyper* function in R.

Statistical analyses

All statistical analyses were performed with the GraphPad Prism software. For analyses of qPCR data, the statistical significance of the indicated changes was assessed employing log2-transformed relative expression values (Rieu and Powers, 2009).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following Accession numbers: SnRK1.1, At3g01090; ABI1, At4g26080; PP2CA, At3g11410; ABI2, At5g57050; HAB1, At1g72770; HAI1, At5g59220; PYL4, At2g38310; PP2C6-6, At1g03590; DIN6, At3g47340; SEN5, At3g15450; AXP, At2g33830.

Supplemental data

Supplemental Data includes Supplemental Figures 1-7 and Supplemental Table 1.

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Author Contributions

AR and EBG conceived the project. AR, PC, PLR, and EBG designed research. AR, MA, PC, LM, AC, CM, AE, MGG, RA, and EBG performed research. AR, MA, PC, LM, AC, CM, AE, MGG, RA, PLR, and EBG analyzed data. AR, PC, PLR, and EBG wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Clade A PP2Cs and SnRK1.1 interact *in vitro* and *in vivo*. **(A)** SnRK1.1 interacts with ABI1 and PP2CA in yeast-two-hybrid assays. CD and RD, catalytic and regulatory domain, respectively. **(B)** *In vitro* interaction between GST-PP2CA and His-T7-SnRK1.1 detected by GST-pull-down and T7-immunodetection of SnRK1.1 preys. **(C)** HA-immunoprecipitation pulls down SnRK1.1 from protoplasts co-expressing SnRK1.1 (untagged) with ABI1-HA, but not with control DNA.

Figure 2. Clade A PP2Cs inhibit SnRK1.1 by dephosphorylation. Immunoprecipitated SnRK1.1-HA is dephosphorylated **(A)** and inactivated **(B)** *in vitro* by PP2CA. **(A)** HA-immunoblot following Phos-tag-SDS-PAGE (Kinoshita et al., 2009). **(B)**

916 Autoradiograms showing that SnRK1.1 activity on itself and ABF2 (lane 1) is lost
917 following PP2CA-treatment (lane 2), but rescued by PYL4 and ABA (lane 4). PP2CA
918 dephosphorylates T175 in recombinant SnRK1.1, phosphorylated or not with SnAK2,
919 **(C)** and in immunoprecipitated SnRK1.1 (*n*=3) **(D)** *in vitro*. Numbers below
920 autoradiograms and immunoblots denote band intensities relative to SnRK1.1 control
921 (=1). At least three independent experiments were performed in **(A-C)** with similar
922 results. Co-expression of clade A PP2Cs **(E)** but not of clade E PP2C6-6 **(F)** with
923 SnRK1.1 in protoplasts results into its dephosphorylation of T175. PP2Cs and SnRK1.1
924 bear HA- and GFP-tags, respectively. SnRK1.1(T175) phosphorylation was detected by
925 immunodetection with anti-phospho-AMPK(T172) antibodies from a subset of the
926 samples used in Figures 3A (**E,** *n*=6) and 3B (**F**, *n*=4) and only relevant samples of the
927 same blot are shown. Error bars=SEM; *p*-values, two-tailed paired *t*-test **(D, F)** and one-
928 way ANOVA with Tukey test **(E)** on the non-normalized ratio of SnRK1.1(T175)
929 phosphorylation relative to total SnRK1.1.
930
931
932 **Figure 3.** Clade A PP2Cs repress SnRK1 signaling. **(A)** SnRK1.1 activity, measured as
933 the induction of the *DIN6::LUC* reporter in protoplasts is severely reduced by clade A
934 PP2Cs ABI1 and PP2CA, but to a much lesser extent by the corresponding catalytically
935 inactive mutants ABI1_D177A and PP2CA_D142A (*n*=9). Numbers above columns
936 designate the percentage of SnRK1.1 inhibition as compared to 100% activity in the
937 absence of PP2Cs. **(B)** An unrelated clade E PP2C6-6 does not impinge on SnRK1.1
938 activity (*n*=9). **(C)** Reduced SnRK1 inactivation in double and quadruple *pp2c* knockout
939 mutants Q*hai1-1* and Q*abi2-2*. Relative gene expression of SnRK1.1 marker genes
940 (*DIN6*, *AXP*) in control (light, *L*), activating (dark, *D*), and inactivating (glucose
941 treatment following darkness, *DG*) conditions (*n*=4). *p*-values, one-way ANOVA with
942 Tukey **(A, B)** and two-way ANOVA with Sidak test **(C)**. Error bars=SEM.
943
944 **Figure 4.** Altered glucose response in *pp2c* knockout mutants and PP2C
945 overexpressors. Glucose-hypersensitivity of SnRK1.1 overexpressors (*35S::SnRK1.1;*
946 4-6% glc), double (*abi1-2 pp2ca-1*; 6% glc) and quadruple *pp2c* knockout mutants
947 (Q*hai1-1* and Q*abi2-2;* 4% glc*)*, and glucose-insensitivity of PP2CA overexpressors

948 (*35S::PP2CA;* 6% glc) in early seedling development. *Glc*, glucose; *Sor*, sorbitol
949 osmotic control; *MS*, control media without glucose or sorbitol. Scale bar=1 cm.
950

951 **Figure 5.** ABA promotes SnRK1 signaling. **(A)** PP2C repression of SnRK1 signaling in
952 protoplasts is blocked by co-expression of the PYL4 receptor in the presence of ABA
953 (*n*=3). **(B)** Induction of SnRK1 target genes by ABA (*n*=10) and energy stress
954 (darkness; *n*=12). **(C)** Reduced induction of SnRK1 target genes by ABA in
955 *35S::PP2CA* plants (*n*=3). **(D)** SnRK1 activation and ABA treatment induce largely
956 overlapping transcriptional responses. Percentage of upregulated or downregulated
957 SnRK1.1 targets similarly regulated by ABA. **(E)** ABA enhances SnRK1 activation by
958 darkness and diminishes its glucose-triggered inactivation. SnRK1 target gene
959 expression in light (*L*) and dark with (*DA*) or without ABA (*D*). Following dark
960 activation, SnRK1 repression triggered by glucose was examined with (*DGA*) or
961 without (*DG*) ABA pre-treatment (*n*=4). Error bars=SEM. *p*-values, two-way ANOVA
962 with Fisher´s LSD test. *DIN6*, *SEN5*, *AXP*, SnRK1 target genes.
963

964 **Figure 6.** SnRK1 regulation by energy signals and ABA through clade A PP2Cs.
965 SnRK1 is activated by the energy deficiency triggered by stress and is inactivated by
966 PP2Cs once normal energy levels are restored. PP2Cs repress also SnRK2s and ABA
967 signaling, but are inhibited by PYL receptors upon ABA binding. *Via* its effect on
968 PP2Cs, the ABA-PYL complex induces SnRK1 signaling, potentiating the effect of
969 energy-stress, diminishing the effect of sugar on SnRK1 repression, and complementing
970 the ABA response. SnAK, SnRK1 Activating Kinases.

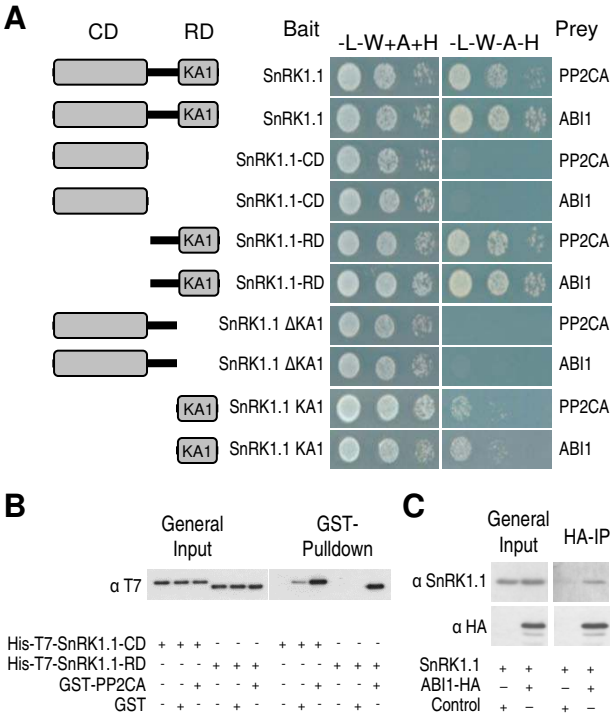


Figure 1. ABI1 and PP2CA interact with SnRK1.1 *in vitro* and *in vivo*. **(A)** SnRK1.1 interacts with ABI1 and PP2CA in yeast-two-hybrid assays. CD, RD, and KA1, catalytic-, regulatory-, and Kinase Associated1-domain, respectively. **(B)** *In vitro* interaction between GST-PP2CA and His-T7-SnRK1.1 detected by GST-pull-down and T7-immunodetection of SnRK1.1 preys. **(C)** HA-immunoprecipitation pulls down SnRK1.1 from protoplasts co-expressing SnRK1.1 (untagged) with ABI1-HA, but not with control DNA.

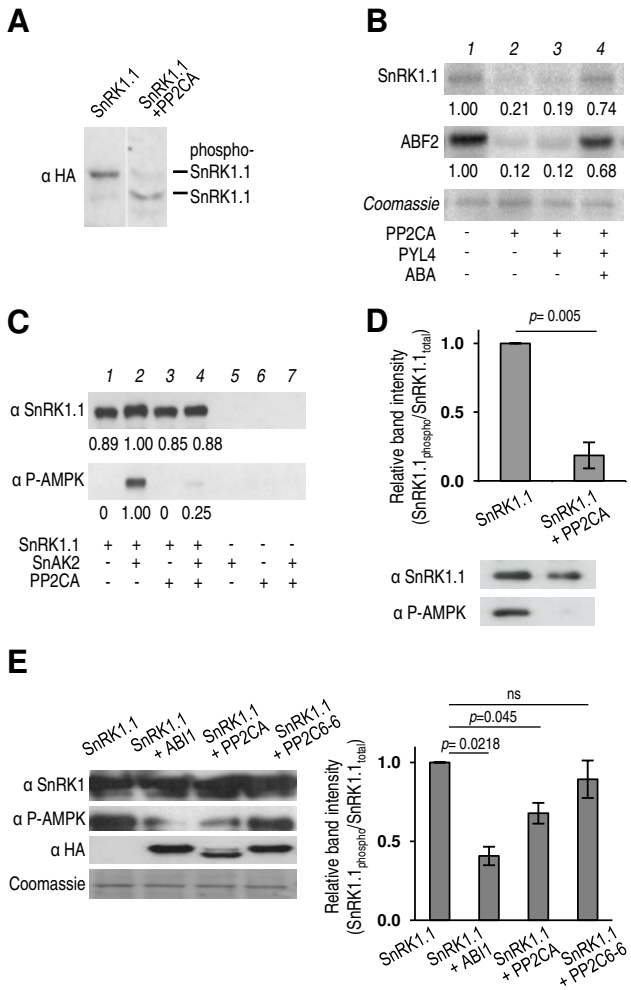


Figure 2. ABI1 and PP2CA inhibit SnRK1.1 by dephosphorylation. Immunoprecipitated SnRK1.1-HA is dephosphorylated **(A)** and inactivated **(B)** *in vitro* by PP2CA. **(A)** HA-immunoblot following Phos-tag-SDS-PAGE (Kinoshita et al., 2009). **(B)** Autoradiograms showing that SnRK1.1 activity on itself and ABF2 (lane 1) is lost following PP2CA-treatment (lane 2), but rescued by PYL4 and ABA (lane 4). PP2CA dephosphorylates T175 in recombinant SnRK1.1, phosphorylated or not with SnAK2 **(C)**, and in immunoprecipitated SnRK1.1 (*n*=3) **(D)** *in vitro*. Numbers below autoradiograms and immunoblots denote band intensities relative to SnRK1.1 control (=1). At least three independent experiments were performed in **(A-C)** with similar results. **(E)** Co-expression in protoplasts of SnRK1.1 with clade A PP2Cs ABI1 and PP2CA but not with clade E PP2C6-6 results into SnRK1.1(T175) dephosphorylation. PP2Cs and SnRK1.1 bear HA- and GFP-tags, respectively. SnRK1.1(T175) phosphorylation was detected by immunodetection with anti-phospho-AMPK(T172) antibodies (*n*=6). Error bars=SEM; *p*-values, two-tailed paired *t*-test **(D)** and one-way ANOVA with Tukey test **(E)** on the non-normalized ratio of SnRK1.1(T175) phosphorylation relative to total SnRK1.1.

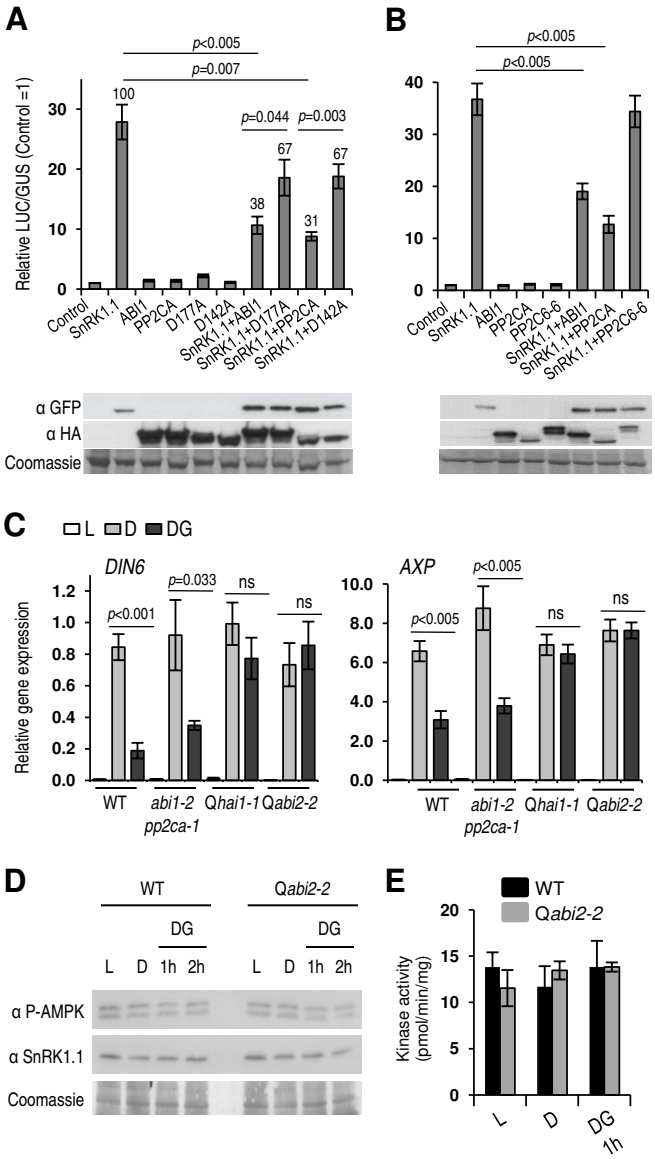
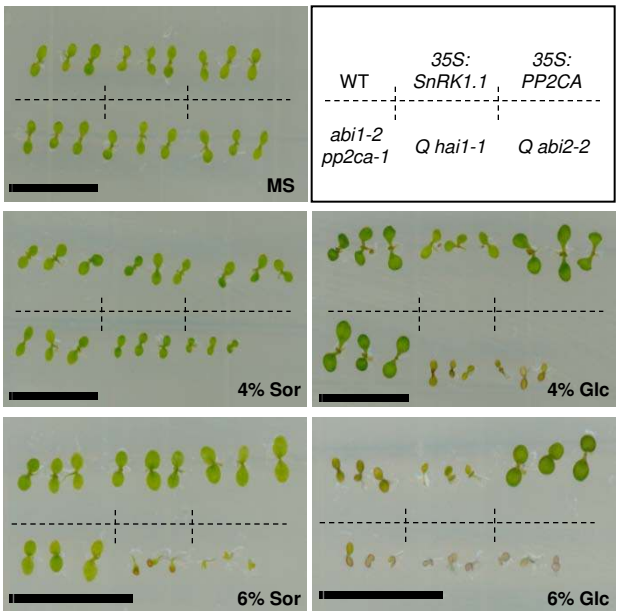


Figure 3. ABI1 and PP2CA repress SnRK1 signaling. **(A)** SnRK1.1 activity, measured as the induction of the *DIN6:LUC* reporter in protoplasts is severely reduced by clade A PP2Cs ABI1 and PP2CA, but to a much lesser extent by the corresponding catalytically inactive mutants ABI1_D177A and PP2CA_D142A (n=9). Numbers above columns designate the percentage of SnRK1.1 inhibition as compared to 100% activity in the absence of PP2Cs. **(B)** An unrelated clade E PP2C6-6 does not impinge on SnRK1.1 activity (n=8). **(C)** Reduced SnRK1 inactivation in double and quadruple *pp2c* knockout mutants Q*hai1-1* and Q*abi2-2*. Relative gene expression of SnRK1.1 marker genes (*DIN6*, *AXP*) in control (light, *L*), activating (dark, *D*), and inactivating (glucose treatment following darkness, *DG*) conditions (n=4). p-values, one-way ANOVA with Tukey **(A, B)** and two-way ANOVA with Sidak test **(C)**. Error bars=SEM. Analyses of SnRK1(T175) phosphorylation **(D)** and SnRK1 activity **(E)** from total cellular extracts reveal no differences in various conditions and between WT and Q*abi2-2* mutant plants. **(D)** SnRK1.1(T175) phosphorylation was detected by immunodetection with anti-phospho-AMPK(T172) antibodies**. (E)** SnRK1 activity was measured using SnRK1 immunoprecipitated from WT or *Qabi2-2* leaves using the AMARA peptide assay. Values represent means±SD (n=2).



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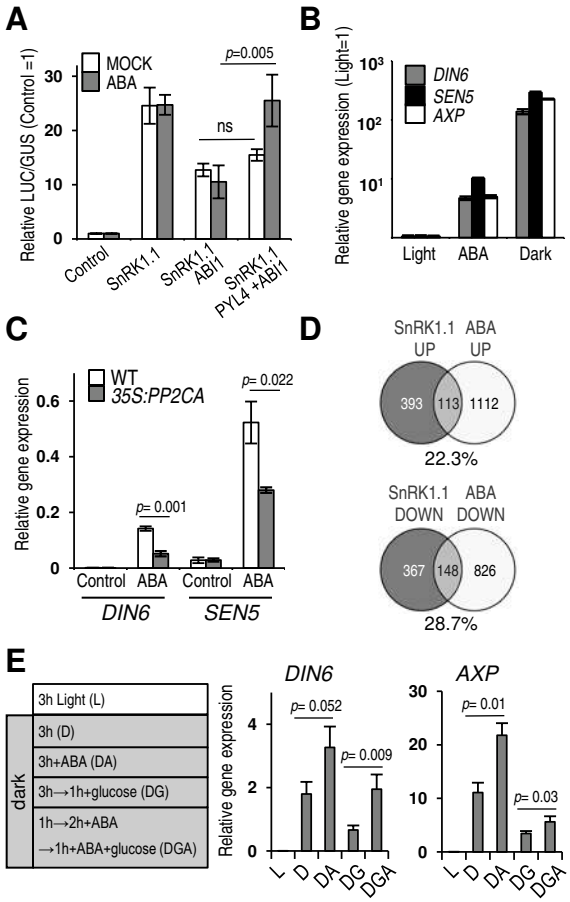


Figure 5. ABA promotes SnRK1 signaling. **(A)** PP2C repression of SnRK1 signaling in protoplasts is blocked by co-expression of the PYL4 receptor in the presence of ABA (n=3). **(B)** Induction of SnRK1 target genes by ABA (n=10) and energy stress (darkness; n=12). **(C)** Reduced induction of SnRK1 target genes by ABA in *35S:PP2CA* plants (n=3). **(D)** SnRK1 activation and ABA treatment induce largely overlapping transcriptional responses. Percentage of upregulated or downregulated SnRK1.1 targets similarly regulated by ABA. **(E)** ABA enhances SnRK1 activation by darkness and diminishes its glucose-triggered inactivation. SnRK1 target gene expression in light (*L*) and dark with (*DA*) or without ABA (*D*). Following dark activation, SnRK1 repression triggered by glucose was examined with (*DGA*) or without (*DG*) ABA pre-treatment (n=4). Error bars=SEM. p-values, two-way ANOVA with Fisher´s LSD test. *DIN6*, *SEN5*, *AXP*, SnRK1 target genes.

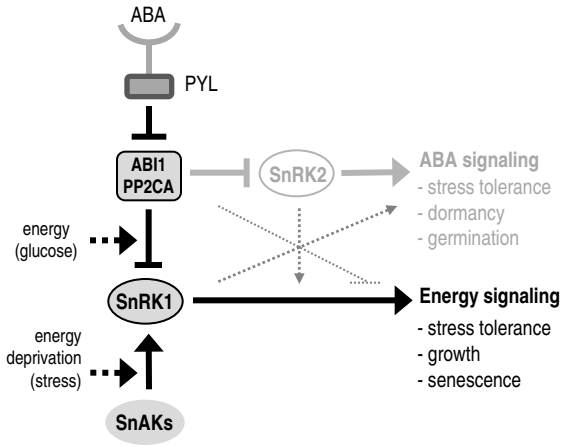


Figure 6. SnRK1 regulation by energy signals and ABA through PP2Cs. SnRK1 is activated by the energy deficiency triggered by stress and is inactivated by ABI1, PP2CA and other PP2Cs once normal energy levels are restored. PP2Cs repress also SnRK2s and ABA signaling, but are inhibited by PYL receptors upon ABA binding. *Via* its effect on PP2Cs, the ABA-PYL complex induces SnRK1 signaling, potentiating the effect of energy-stress, diminishing the effect of sugar on SnRK1 repression, and complementing the ABA response. The SnRK1 and ABA pathways are likely to crosstalk also at other levels (dotted lines). SnAK, SnRK1 Activating Kinases.

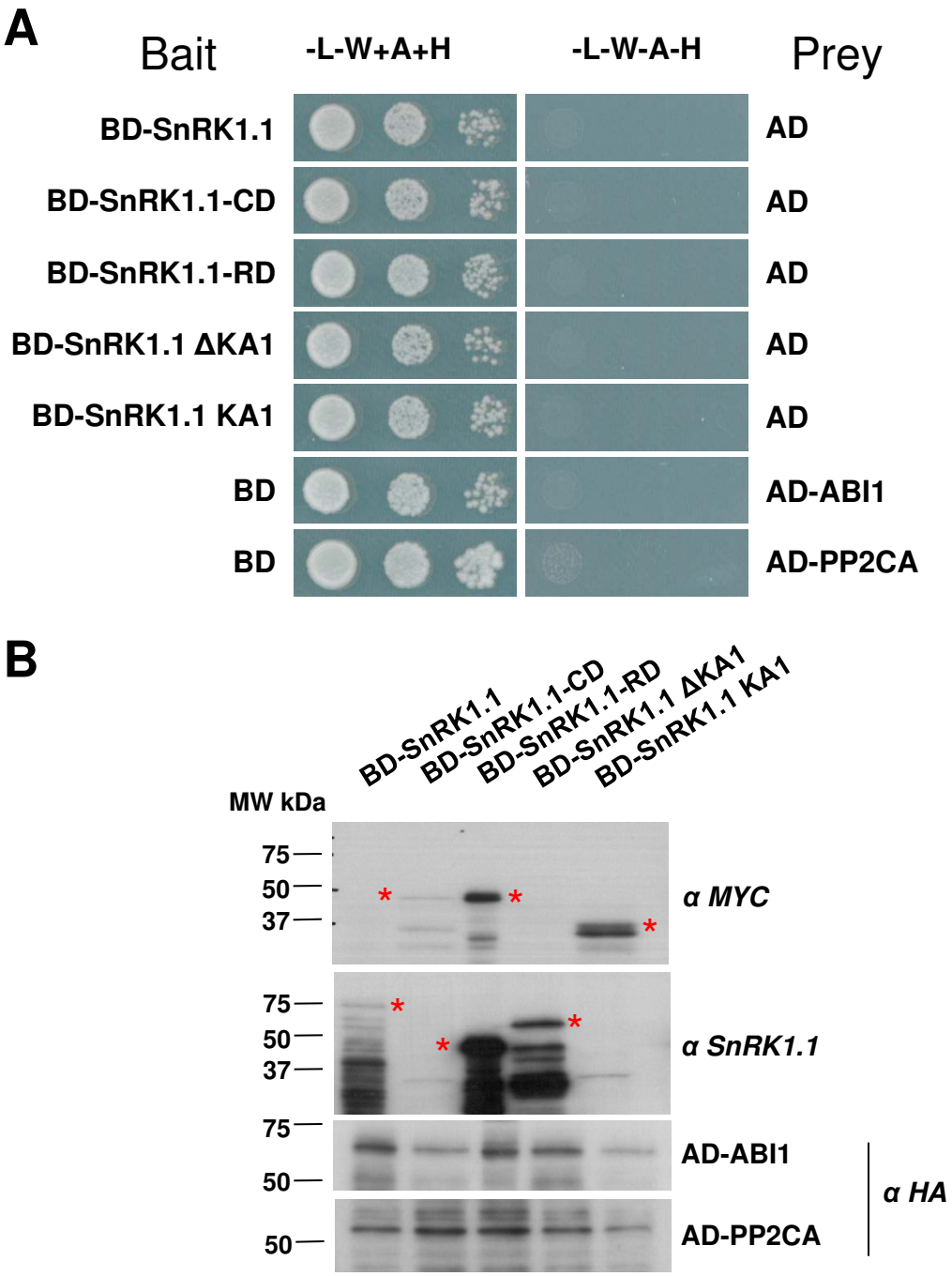
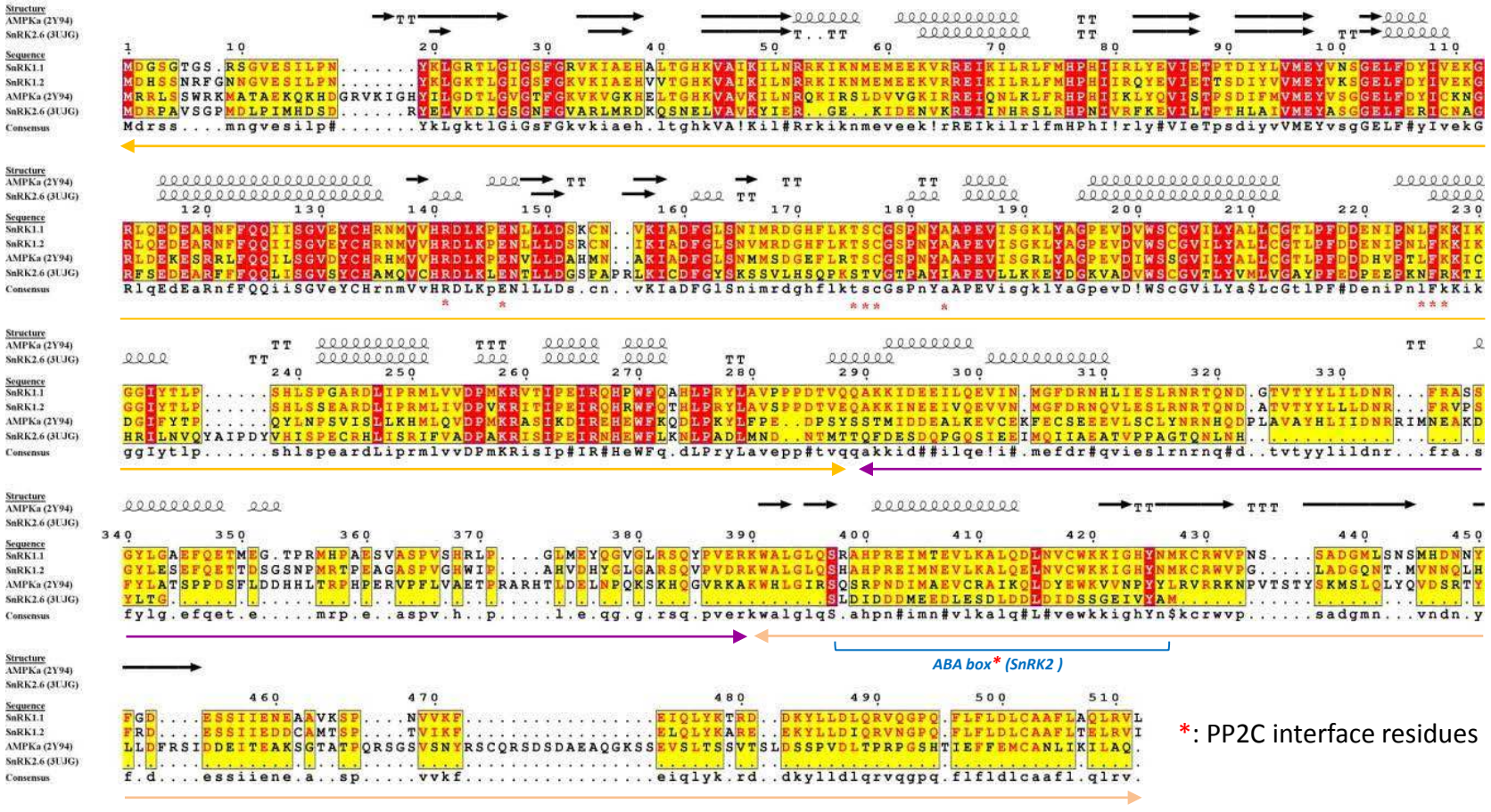
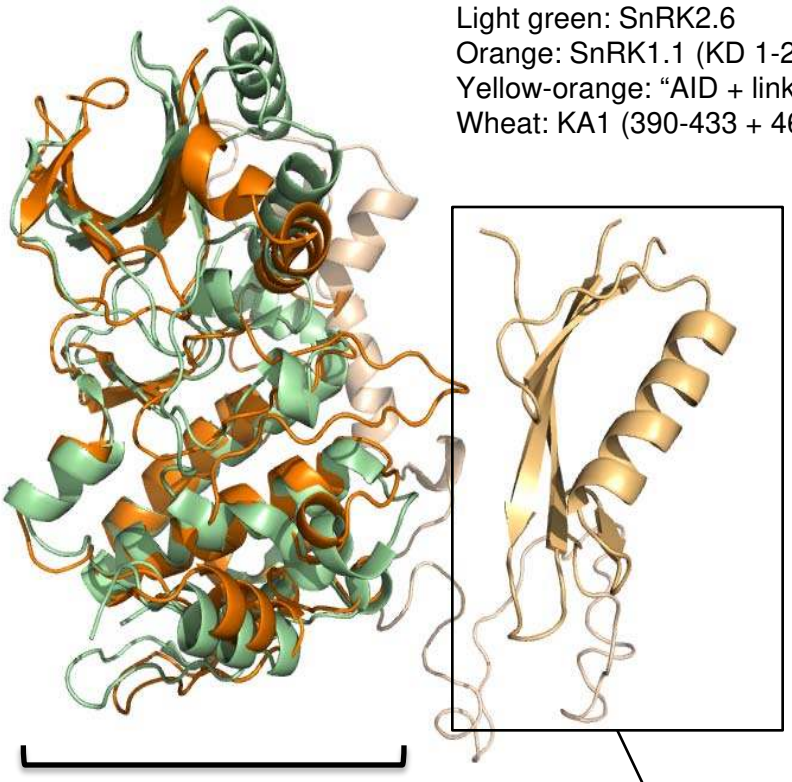


Figure S1. Yeast-two-hybrid controls for the SnRK1.1 and PP2C interaction (Fig. 2*A*). **(A)** None of the AD and BD constructs activate the *ADE* and *HIS* reporters. Colony growth was assessed on medium lacking adenine and histidine (-A-H) using serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of saturated cultures. The different SnRK1.1 deletions are shown. CD=catalytic domain, residues 1-293; RD=regulatory domain, residues 294-512; KA1 domain=residues 390-512. AD=GAL4 activation domain, BD=GAL4 binding domain. **(B)** Expression of the indicated constructs in yeast as revealed by immunodetection with anti-HA (for AD-constructs) and anti-c-MYC (for BD-constructs) antibodies. Full-length SnRK1.1 and SnRK1.1 ΔKA1 have low expression levels and are more readily detected with the anti-SnRK1.1 antibody. Note that this antibody is against a peptide in the more proximal part of the RD-region and thus does not detect SnRK1.1-CD nor SnRK1.1 KA1. Red asterisks indicate the band with the expected molecular weight.

A


Structure
AMPKa (2Y94)
SnRK2.6 (3UJG)
Sequence
SnRK1.1
SnRK1.2
AMPKa (2Y94)
SnRK2.6 (3UJG)
Consensus
Kinase Domain (Catalytic Domain)
AID+ linker
KA1 Domain
ABA box* (SnRK2)
*: PP2C interface residues

B



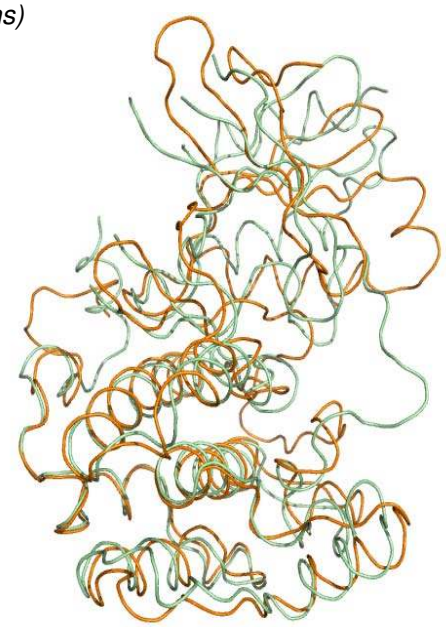
Light green: SnRK2.6
Orange: SnRK1.1 (KD 1-293)
Yellow-orange: "AID + linker" (294-389)
Wheat: KA1 (390-433 + 467-494)

Kinase domain

C

Kinase domain (KD)
Light green: SnRK2.6
Orange: SnRK1.1

RMSD: 1.62Å (on 73% of atoms)



D

KA1 domains:
Wheat: SnRK1.1 (390-512)
Slate blue: MARK3 (1UL7)
Yellow: SnRK3.11 (2HEB, 305-446)
Red: AMPKα (2YA3, 397-549)

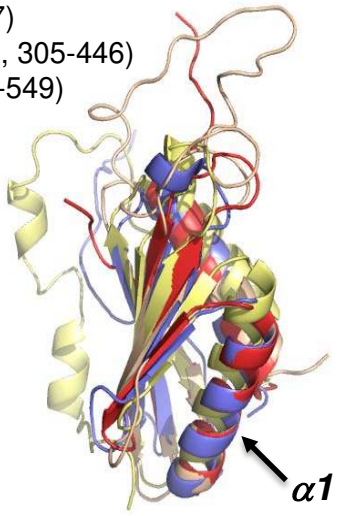


Figure S2. Alignment and structural comparison of SnRK1 and SnRK2. **(A)** Alignment of SnRK1.1 (Q38997), SnRK1.2 (P92958), AMPKα (PDB: 2Y94-A) and SnRK2.6 (PDB: 3UJG-A) was performed with ClustalW and represented with ESPript (Gouet *et al.*, 1999), displaying the known secondary structures on the top. Residues fully conserved in all four sequences are in red and those conserved in three in yellow. Residues marked by a red asterisk are implicated in physical interaction with the HAB1 PP2C phosphatase (3UJG) (Soon *et al.*, 2012). Kinase Domain (KD, catalytic domain, CD; common to the four proteins) is marked by orange arrows and the KA1 domain (only for SnRK1 and AMPK) in marked by blue arrows. "AID + linker" (marked by purple arrows) stands for "Auto-Inhibitory Domain" followed by a linker region by analogy with the AMPKα (Hardie *et al.*, 2012). No function has been assigned to this sub-domain in plants. **(B)** Structural alignment of the SnRK1.1 model [performed from template 2Y94S (Xiao *et al.*, 2011) with Swiss-model (Arnold *et al.*, 2006)] with SnRK2.6 (3UJG-A). Colored as described, cartoon representation. **(C)** Structural alignment of the kinase domain of SnRK1.1 model with SnRK2.6. RMSD of kinase domain alignment is 1.62Å on 73% of aligned atoms, giving confidence on the conservation observed in alignment (see **A**). As almost all the important residues (* in **A**) are in loops, no more can be assessed for these. The other three are located in the αG helix of the kinase domain in its large lobe (subdomain XS) (Hanks & Hunter, 1995). The large lobe alignment of these kinases is good (RMSD=0.81Å on 74% of aligned atoms) giving confidence in these conservation. Colored as described, ribbon representation. **(D)** Validation of the Kinase Associated1 (KA1) domain model of SnRK1.1. KA1 domain from Uniprot database is annotated as shorter (486-512) than our considered model (390-512). Comparison of the actual structures of a SnRK3.11/SOS2 (2HEB) (Sánchez-Barrena *et al.*, 2007), MARK3 (1UL7) (Tochio *et al.*, 2006), the AMPKα "core complex" part (2YA3) (Xiao *et al.*, 2011) with a model of the last 122 residues of SnRK1.1 (part colored blue in **(A)** modeled by Phyre (Kelley & Stenberg, 2009). This part is clearly exhibiting a KA1 fold with a β-sheet (of four β-strands) and two α-helixes on the same side of the β-sheet. Colored as stated, cartoon representation. All images and structural alignment were generated with Pymol (from Delano Scientific). α1 refers to the α-helix part of the phosphatase interacting domain (PPI) (Sánchez-Barrena *et al.*, 2007).

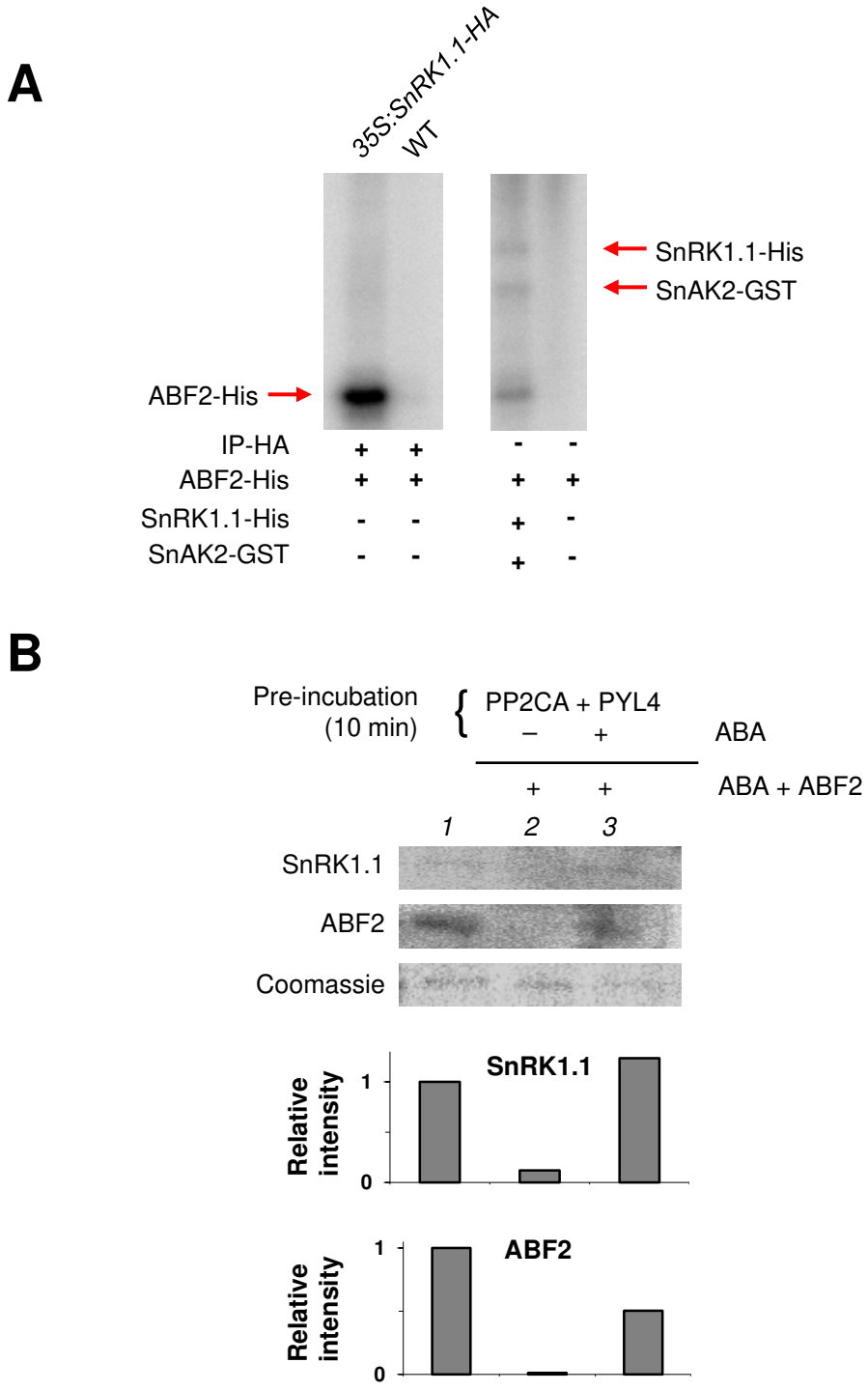


Figure S3. SnRK1.1 is inactivated by recombinant His-PP2CA *in vitro*. **(A)** Control HA-immunoprecipitation from WT plants retrieves no ABF2 phosphorylating activity, showing that the activity measured from *35:SnRK1.1-HA* plants is specific to SnRK1.1. Right panel, positive control showing that recombinant SnRK1-His preactivated with SnAK2-GST phosphorylates ABF2. **(B)** Where indicated SnRK1.1 was pre-incubated, for 10 min, with PP2CA and PYL4 in the absence (lane 2) or presence (lane 3) of ABA, to allow or prevent PP2CA activity, respectively. After this pre-incubation ABA was added to all samples to inactivate PP2CA, the ABF2 substrate was supplied, and the reaction was further incubated for 1h.

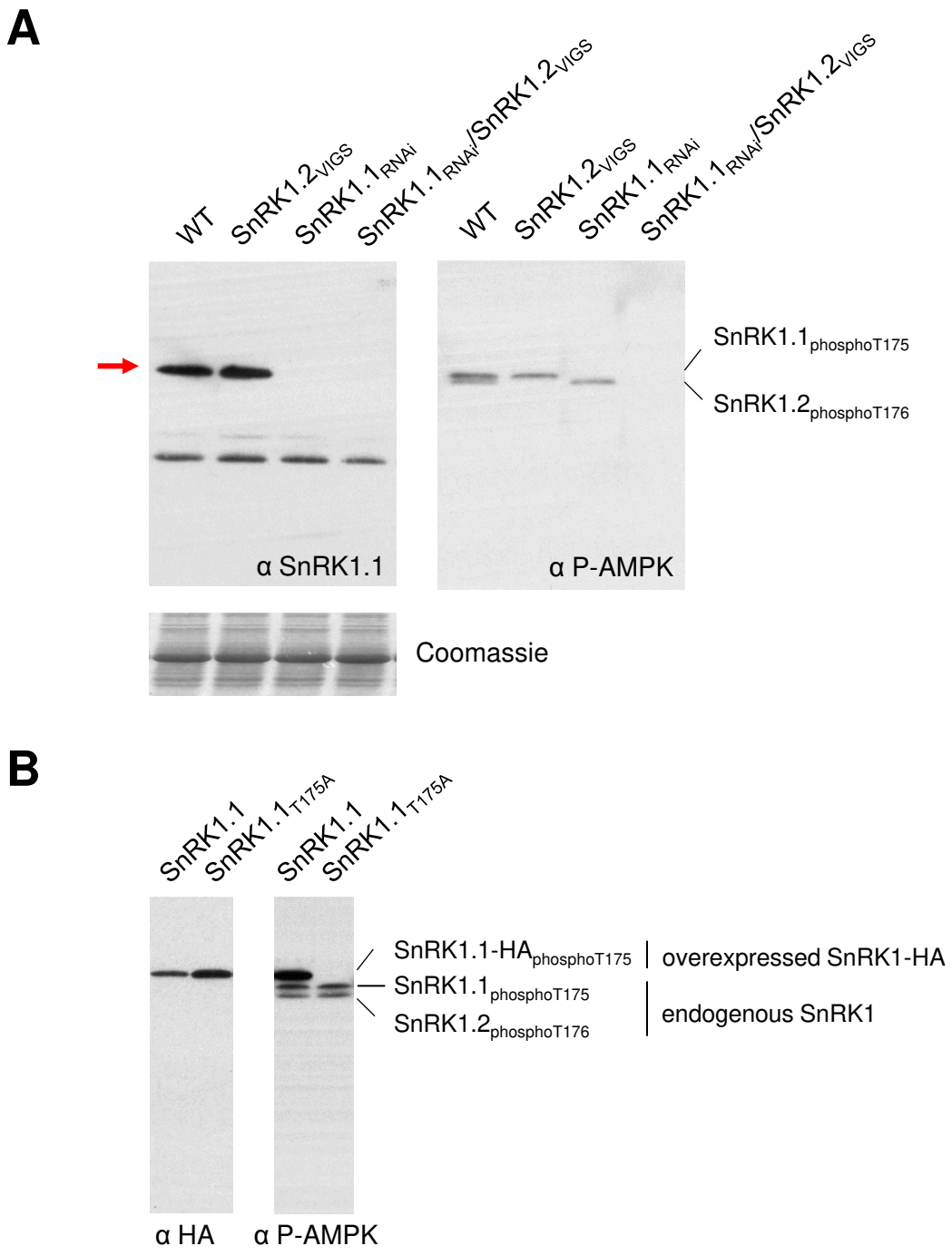


Figure S4. Specific detection of phosphorylated SnRK1. **(A)** The P-AMPK antibody recognizes specifically SnRK1.1 and SnRK1.2 in total protein extracts from *Arabidopsis* leaves. WT and SnRK1.1 RNAi plants were infiltrated with *Agrobacterium* containing viral vectors for a GFP control (WT) or for VIGS of SnRK1.2 and analyzed 3 weeks after, using anti-SnRK1.1 and anti-P-AMPK antibodies (Baena-González et al., 2007). The red arrow indicates the band corresponding to SnRK1.1. **(B)** Mutation of T175 to A abolishes SnRK1.1-HA recognition by the P-AMPK antibody. *Arabidopsis* mesophyll protoplasts were transfected with constructs expressing SnRK1.1-HA or SnRK1.1$_{T175A}$-HA and proteins were detected after SDS-PAGE by immunoblotting with anti-HA or anti-P-AMPK antibodies.

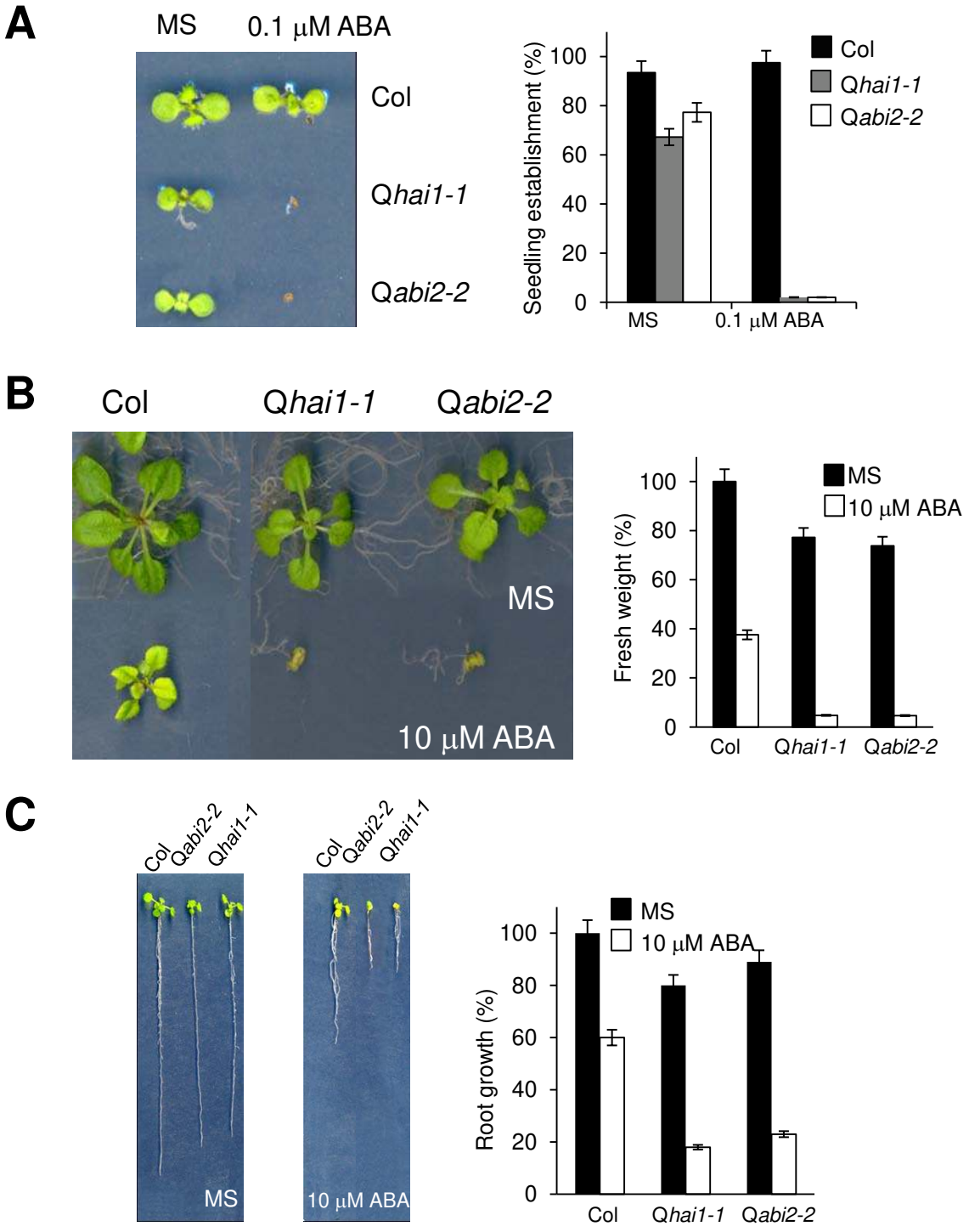


Figure S5. Clade A *pp2c* quadruple mutants are ABA–hypersensitive. **(A)** Enhanced sensitivity to inhibition of seedling establishment by ABA. Seeds were germinated and grown in medium lacking or supplemented with 0.1 μM ABA for 10 days (*n*=100). **(B)** The growth of the *pp2C* mutants is not strongly affected in control MS medium but is impaired in medium containing 10 μM ABA. Photographs were taken 20 days after transferring 5-day-old seedlings from MS medium to plates lacking or containing 10 μM ABA (*n*=15). **(C)** ABA-hypersensitive root growth inhibition of *pp2c* mutants. Photographs were taken 10 days after transferring 4-day-old seedlings to MS plates lacking or supplemented with 10 μM ABA (*n*=15). *Col,* Columbia wild-type*;* Q*hai1-1, hab1-1 abi1-2 pp2ca-1 hai1-1*; Q*abi2-2, hab1-1 abi1-2 pp2ca-1 abi2-2* (Antoni *et al.*, 2013). Values represent means±SEM.

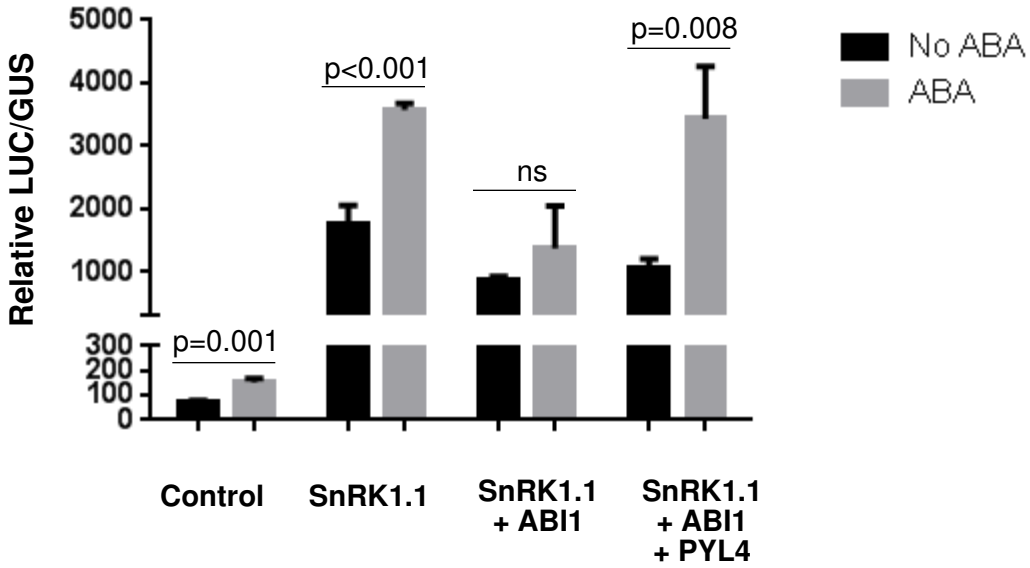


Figure S6. ABA promotes SnRK1 signaling in protoplasts. Cells were transfected with control DNA, or with plasmids expressing SnRK1.1 alone or in combination with ABI1 and the PYL4 receptor. In the absence of overexpressed PYL4, ABA and the endogenous receptors are not sufficient to inhibit overexpressed ABI1. Samples are the same as in Fig. 2A, but instead of normalizing the mock and ABA sets to their corresponding controls, all samples were normalized to the mock control (n=3). Values represent means±SEM. p-values, multiple t-test with Holm-Sidak correction.

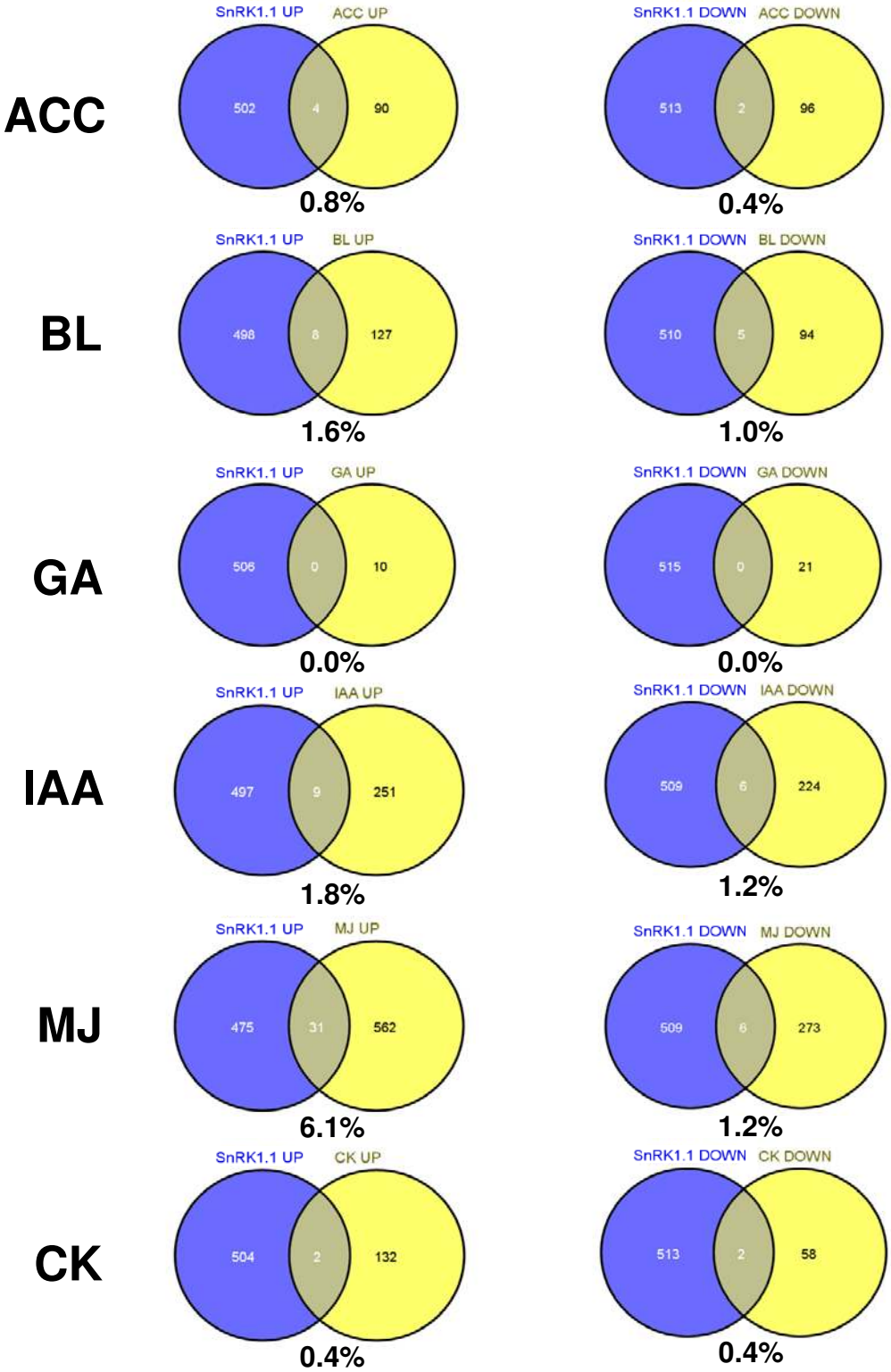


Figure S7 (cont.) Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and indicated hormone treatments (Nemhauser et al., 2006; AtGenExpress). UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets. ACC, 1-aminocyclopropane-1-carboxylic acid (ethylene precursor); BL, brassinolide; GA, gibberellic acid; IAA, indole-3-acetic acid (auxin); MJ, methyl jasmonate; CK, cytokinin

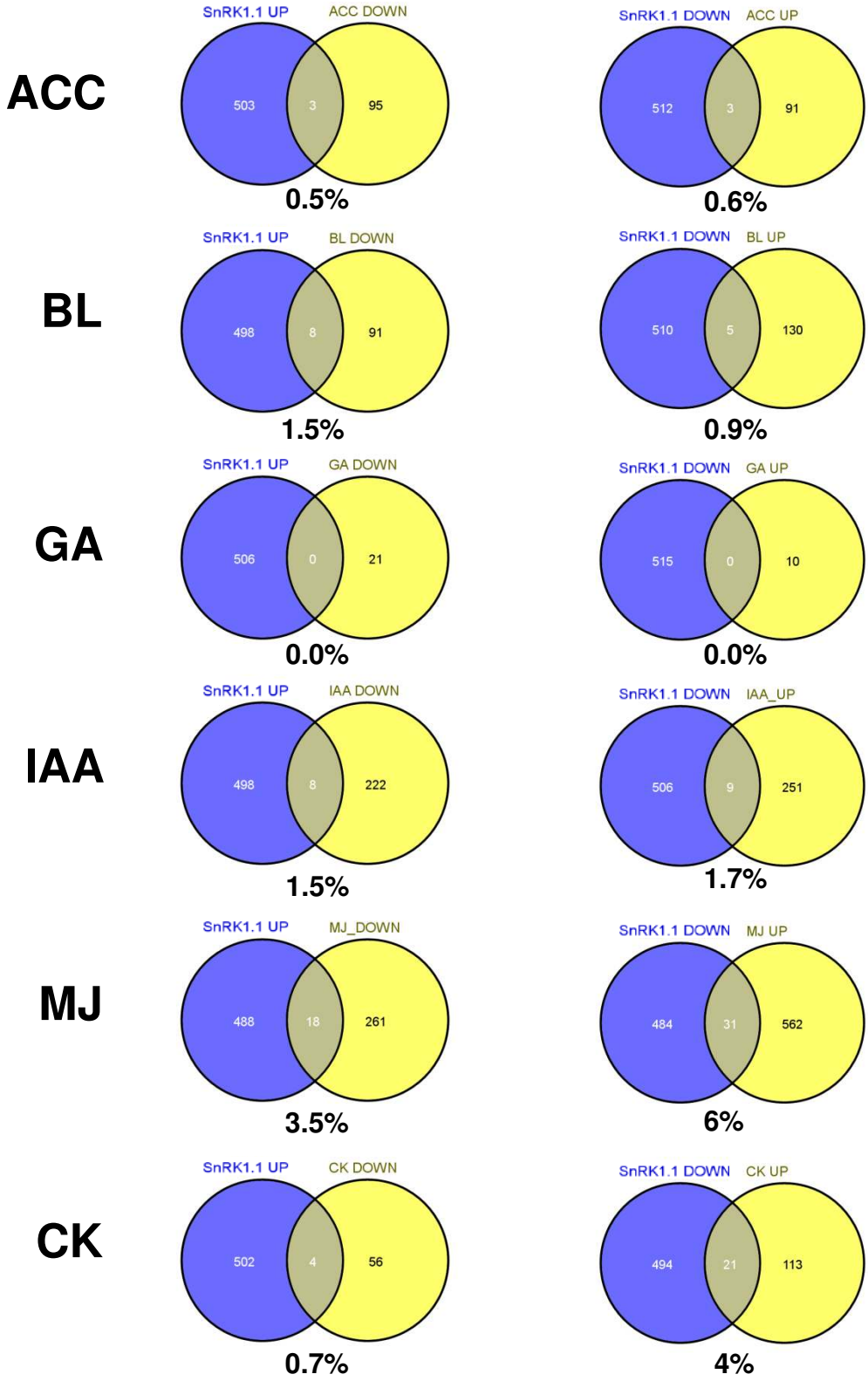


Figure S7 (cont.) Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and indicated hormone treatments (Nemhauser et al., 2006; AtGenExpress). UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets. ACC, 1-aminocyclopropane-1-carboxylic acid (ethylene precursor); BL, brassinolide; GA, gibberellic acid; IAA, indole-3-acetic acid (auxin); MJ, methyl jasmonate; CK, cytokinin

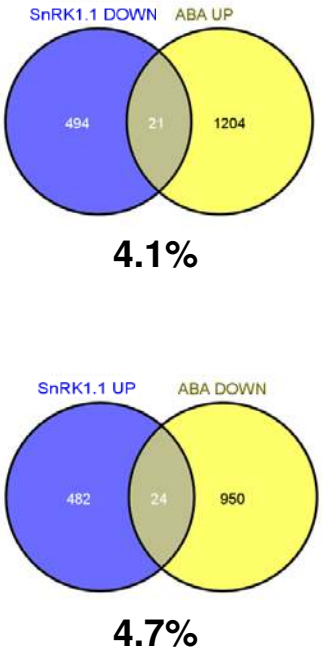


Figure S7. Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and ABA (Nemhauser et al., 2006; AtGenExpress). Overlap between the genes induced by SnRK1.1 and repressed by ABA, and between the genes repressed by SnRK1.1 and induced by ABA. UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets.

Rodrigues_Supplementary Table S1
Cloning primers
Restriction sites introduced by PCR are marked in blue

Name	Primer sequence	Vector	Description	Sites used for the cloning
SnRK1.1 Fw	CGGGATCCATGGATGGATCAGGCACAGG	pHBT95	SnRK1.1 overexpression in protoplasts	BamHI/StuI
SnRK1.1 Rev	AAGGCCTGAGGACTCGGAGCTGAGC	pHBT95		
ABI1 BamHI Fw	TTTGGATCCATGGAGGAAGTATCTCCGGCG	pHBT95	ABI1 overexpression in protoplasts	BamHI/SmaI (insert)
ABI1 SmaI Rev	TTTCCCGGGGTTCAAGGGTTTGCTCTTGAG	pHBT95		BamHI/StuI (vector)
PP2CA Fw	GCGGATCCATGGCTGGGATTTGTTGC	pHBT95	PP2CA overexpression in protoplasts	BamHI/StuI
PP2CA Rev	AAGGCCTAGACGACGCTTGATTATTCCT	pHBT95		
At1g03590 BamHI Fw	CGCGGATCCATGGGAGGTTGTATCTCTAAG	pHBT95	At1g03590 (URP) overexpression in protoplasts	BamHI/StuI
At1g03590 StuI Rev	GAAGGCCTAGTCTTTGGTTCCTCTCCAGG	pHBT95		
PYL4_StuI_Fw	AAGGCCTCTTGCCGTTCACCGTCCTT	pHBT95	PYL4 overexpression in protoplasts	StuI/PstI
PYL4_PstI_Rev	AACTGCAGTCACAGAGACATCTTCTTC	pHBT95		
2CASal2bFW	AAAGTCGACTCATGGCTGGGATTTGTTGCGGT	pGEX-4T1	Production of recombinant PP2CA-GST	SalI/NotI
2CANotI Rev	AAAGCGGCCGCTTAAGACGACGCTTGATTATTC	pGEX-4T1		
SnRK1.1BamHI-F	CGGGATCCGATGGATCAGGCACAGGCAG	pET28a	Production of recombinant His-T7-SnRK1.1CD	BamHI/EcoRI
SnRK1.1I293_EcoRI_RP_STOP	CCGGAATTCTCAAATCTTTTTTGCCTGTTGC	pET28a		
SnRK1.1D294EcoRIFw	CCGGAATTCGACGAGGAGATTCTCCAAGAAG	pET28a	Production of recombinant His-T7-SnRK1.1RD	EcoRI
SnRK1.1EcoRI-R	CGGAATTCTCAGAGGACTCGGAGCTGAG	pET28a		
PP2CA_NdeI_Fw	TTTGTCGACTACATATGGCTGGGATTTGTTGCGGT	pGADT7	Expression GAL4 AD-PP2CA for Y2H	NdeI/SmaI
PP2CA_SmaI_Rev	TTTGTCGACTTACCCGGGAGACGACGCTTGATTATTCC1	pGADT7		
SnRK1.1EcoRIFw	CCGGAATTCATGGATGGATCAGGCACAGGC	pGBKT7	Expression of GAL4 BD-SnRK1.1 full-length for Y2H	EcoRI/BamHI
SnRK1.1NOSTOPBamRev	CGCGGATCCGAGGACTCGGAGCTGAGCAAG	pGBKT7		
SnRK1.1D294EcoRIFw	CCGGAATTCGACGAGGAGATTCTCCAAG	pGBKT7	Expression of GAL4 BD-SnRK1.1 RD for Y2H	EcoRI/BamHI
SnRK1.1NOSTOPBamRev	CGCGGATCCGAGGACTCGGAGCTGAGCAAG	pGBKT7		
BD adapt1	TATGGGATCCATGGAAGCTTTAGGCCTCTGCA	Adaptors to create BamHI and StuI sites in the pGBKT7 MCS		
BD adapt2	GAGGCCTAAAGCTTCCATGGATCCCA	to generate the following BD-KIN delections		
SnRK1.1BamNdeFw	CGGGATCCCATATGGATGGATCAGGCACAGGC	pGBKT7	Expression of GAL4 BD-SnRK1.1 CD for Y2H	BamHI/StuI
SnRK1.1CatDStuRev	TAGGCCTGTCAATCTTTTTTGCCTGTTG	pGBKT7		
SnRK1.1BamNdeFw	CGGGATCCCATATGGATGGATCAGGCACAGGC	pGBKT7	Expression of GAL4 BD-SnRK1.1 ΔKA1 for Y2H	BamHI/StuI
SnRK1.1D390StuRev	TAGGCCTTCTCTCAACAGGGTATTGAG	pGBKT7		
SnRK11 KA1BamH1-Fw	CGGGATCCAAATGGGCTCTTGGACTTCAG	pGBKT7	Expression of GAL4 BD-SnRK1.1 KA1 for Y2H	BamHI/StuI
SnRK1.1D390StuRev	TAGGCCTTCTCTCAACAGGGTATTGAG	pGBKT7		

Mutagenesis primers

ABI1_D177A_Fw	CATTTCTTCGGTGTTTACGCTGGCCATGGCGGTTCTCAGG	To generate a catalytically inactive ABI1
ABI1_D177A_Rev	CCTGAGAACCGCCATGGCCAGCGTAAACACCGAAGAAATG	
PP2CA_D142A_Fw	CATTTCTACGGTGTCTTTGCTGGCCATGGCTGCTCTCATG	To generate a catalytically inactive PP2CA
PP2CA_D142A_Rev	CATGAGAGCAGCCATGGCCAGCAAAGACACCGTAGAAATG	

qPCR primers

EIF4 A	TCATAGATCTGGTCCTTAAACC	amplifying eIF4, house-keeping gene
EIF4 B	GGCAGTCTCTTCGTGCTGAC	
DIN6 A	AACTTGTCGCCAGATCAAGG	amplifying DIN6, SnRK1.1 activated marker gene
DIN6 B	GGAACACGTGCCTCTAGTCC	
SEN5 A	GCGAAACTCTCTCCGACTTC	amplifying SEN5, SnRK1.1 activated marker gene
SEN5 B	CCACAGAACAACCTTTGACG	
AXP A	CTTCGACAAGCCTTCTCACC	amplifying AXP, SnRK1.1 activated marker gene
AXP B	TCGTCGCTGTATAGCCAATC	
RAB18 A	TGGCTTGGGAGGAATGCTTCA	amplifying RAB18, ABA activated marker gene
RAB18 A	CCATCGCTTGAGCTTGACCAGA	
RD29B A	CTTGGCACCACCGTTGGGACTA	amplifying RD29B, ABA activated marker gene
RD29B B	TCAGTTCCCA GAATCTTGAACT	