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Molecular and genetic analyses of the PP2C-ABA receptor interaction in the abscisic acid signaling pathway

Dissertation submitted in partial fulfillment of the requirements for obtaining the degree of Doctor (PhD) in Biotechnology

By

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CERTIFICA:

Que la presente memoria titulada "Molecular and genetic analyses of the PP2C-ABA receptor interaction in the abscisic acid signaling pathway", ha sido realizada por Regina Antoni Alandes bajo mi dirección y constituye su Memoria de Tesis para optar al grado de Doctor en Biotecnología.

Fdo: Dr. Pedro L. Rodríguez Egea.

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ABSTRACT

The plant hormone abscisic acid (ABA) plays a crucial role in the control of the stress response and the regulation of plant growth and development. ABA binding to PYR/PYL/RCAR intracellular receptors leads to inhibition of clade A PP2Cs such as ABI1 or HAB1, causing the activation of the ABA signaling pathway. To obtain further insights into ABA signaling we have focused on the characterization of members of these two families of proteins. We have generated a mutated version of *HAB1* carrying a mutation in the tryptophan-385, which is a key residue for the interaction with the receptor's gating loops and ABA molecule. As a result, hab1^{W385A} was found to be refractory to inhibition by the PYR/PYL/RCAR proteins. Thus, using *in vitro* kinase assays we found that hab1^{W385A} was able to dephosphorylate OST1 even in the presence of ABA and the receptors. *hab1^{W385A}* and *hab1^{G246D}* can be classified as hypermorphic dominant mutations. hab1^{G246D} shows impaired phosphatase activity, whereas the new dominant allele shows wild type activity. Transgenic Arabidopsis lines overexpressing *hab1^{W385A}* showed strong dominant ABA insensitivity. We also analyzed the role of the clade A PP2Cs belonging to the PP2CA branch in the ABA signaling pathway. The generation of the double mutant *pp2ca-1hai1-1*, which shows enhanced ABA sensitivity compared to wild type and the single mutants, revealed that HAI1 is a negative regulator of ABA signaling pathway. Subcellular localization experiments showed that both HAI1 and PP2CA were localized to the nucleus, but also in cytosol and microsomes. Three members of the PP2CA branch, *i.e.*: PP2CA, AHG1 and HAI1, showed selective inhibition by the different PYR/PYL/RCARs. These results suggest that the PYR/PYL/RCAR receptors can discriminate between members of clade A phosphatases. *pyl8* is the only single mutant that shows reduced sensitivity to ABA in root growth assays. GUS reporter analyses showed that PYL8 was present in stele cells, epidermis, columella and lateral root cap and quantification of GUS activity in root showed that *PYL8* is one of the receptors with higher expression levels in this organ. The root tip plays a crucial role for hydrotropism and ABA is a phytohormone involved in this response. The study of the hydrotropic response of combined mutants of both PP2Cs and PYR/PYL/RCARs revealed that the ABA core pathway regulates root hydrotropism. Thus, while the sextuple mutant *pyr/pyl112458* showed reduced root curvature under a moisture gradient, the quadruple mutant of the PP2Cs (*Qabi2-2*) showed a stronger curvature under these conditions, getting away from areas with low water potential better than the wild type. Finally, the last section of this work was focused on exploring new chemical tools to increase drought resistance. We have performed a chemical genetic approach directed to isolate new ABA agonists.

Based on structural data of ABA receptors, 500 compounds were selected and assayed in *Arabidopsis*. From these, the compound called 2C06 inhibited root growth in wild type more than in *pyr/pyl/rcar* ABA insensitive mutants and it showed promising *in vitro* results to inhibit PP2Cs and interact with them in Y2H assays.

RESUMEN

La fitohormona ácido abscísico (ABA) juega un papel crucial en el control de la respuesta a estrés y en la regulación del crecimiento y desarrollo de la planta. La unión del ABA a los receptores intracelulares PYR/PYL/RCAR conlleva la inhibición de las PP2Cs del clado A tales como ABI1 o HAB1, causando la activación de la ruta de señalización del ABA. Para obtener más información en la señalización del ABA nos hemos centrado en la caracterización de miembros de estas dos familias proteicas. Hemos generado una versión mutada de *HAB1* que contiene una mutación en el Trp-385, residuo clave para la interacción con los receptores y con la molécula de ABA. Como resultado, hab1^{W385A} se mostró refractaria a la inhibición por los receptores PYR/PYL/RCAR. Así, en ensayos de actividad quinasa *in vitro* encontramos que hab1^{W385A} era capaz de desfosforilar a OST1 incluso en presencia de ABA y de los receptores. *hab1*^{W385A} y *hab1*^{G246D} pueden ser clasificadas como mutaciones dominantes hipermórficas. Mientras que hab1^{G246D} posee una actividad fosfatasa reducida, el nuevo alelo dominante muestra una actividad idéntica al genotipo salvaje. Líneas transgénicas de Arabidopsis sobreexpresando *hab1*^{W385A} mostraron una fuerte insensibilidad al ABA. También hemos analizado el papel de las PP2Cs del clado A pertenecientes a la rama representada por PP2CA. La generación de un mutante doble *pp2ca-1hai1-1,* que muestra mayor sensibilidad a la hormona en comparación con el genotipo salvaje y con los mutantes sencillos, reveló que HAI1 es un regulador negativo de la ruta de señalización del ABA. El análisis de la localización subcelular mostró que tanto HAI1 como PP2CA se localizan en el núcleo, aunque también están presentes en el citosol y en la fracción microsomal. Tres miembros de la rama de PP2CA *i.e.:* PP2CA, AHG1 y HAI1, mostraron una inhibición selectiva por los receptores PYR/PYL/RCAR. Estos resultados sugieren que estos receptores pueden discriminar entre miembros del clado A de las PP2Cs. *pyl8* es el único mutante sencillo que muestra sensibilidad reducida al ABA en ensayos de crecimiento de raíz. Análisis usando el gen reportero GUS mostraron que PYL8 estaba presente en la estela, en la epidermis y en la caliptra, y la cuantificación de la actividad beta-glucuronidasa en raíz mostró que *PYL8* es uno de los receptores con mayor nivel de expresión. La caliptra juega un papel crucial en la respuesta hidrotrópica. El estudio de esta respuesta en mutantes múltiples de las PP2Cs y de los PYR/PYL/RCAR reforzó la idea de que el ABA regula este proceso. Así, mientras el mutante séxtuple *pyr/pyl112458* presentó una curvatura menor al aplicársele un gradiente de humedad, el mutante cuádruple de las PP2Cs (*Qabi2-2*) mostró una curvatura más pronunciada en estas condiciones, evitando las zonas con menor potencial hídrico. Finalmente, en la última parte de este trabajo se utilizaron abordajes genético-químicos para aumentar la resistencia a la sequía. Hemos llevado a cabo un rastreo con compuestos químicos para aislar nuevos

agonistas del ABA. Basado en datos estructurales de los receptores, se seleccionaron 500 compuestos que fueron ensayados en Arabidopsis. De estos, el compuesto 2C06 inhibió el crecimiento de raíz en plantas salvajes más que en mutantes *pyr/pyl/rcar* insensibles a ABA y produjo resultados prometedores *in vitro* al inhibir a las PP2Cs e interaccionar con éstas en ensayos de doble híbrido.

RESUM

La fitohormona àcid abscísic (ABA) juga un paper crucial en el control de la resposta a estrés i en la regulació del creixement i desenvolupament de la planta. La unió de l'ABA als receptors intracel·lulars PYR/PYL/RCAR comporta la inhibició de les PP2Cs del clade A com ara ABI1 o HAB1, causant l'activació de la ruta de senyalització de l'ABA. Per a obtindre més informació en la senyalització de l'ABA ens hem centrat en la caracterització de membres d'estes dos famílies proteiques. Hem generat una versió mutada de *HAB1* que conté una mutació en el Trp-385, residu clau per a la interacció amb els receptors i amb la molècula d'ABA. Com a resultat, hab1^{W385A} es va mostrar refractària a la inhibició pels receptors PYR/PYL/RCAR. Així, en assajos d'activitat quinasa *in vitro* trobem que hab1^{W385A} era capaç de desfosforilar a OST1 inclús en presència d'ABA i dels receptors. *hab1*^{W385A} i *hab1*^{G246D} poden ser classificades com a mutacions dominants hipermòrfiques. Mentre que hab1^{G246D} posseeix una activitat fosfatasa reduïda, el nou al·lel dominant mostra una activitat idèntica al genotip salvatge. Línies transgèniques d'Arabidopsis sobreexpressant *hab1*^{W385A} van mostrar una forta insensibilitat a l'ABA. També hem analitzat el paper de les PP2Cs del clade A pertanyents a la branca representada per PP2CA. La generació d'un mutant doble *pp2ca-1hai1-1*, que mostra major sensibilitat a l'hormona en comparació amb el genotip salvatge i amb els mutants senzills, va revelar que HAI1 és un regulador negatiu de la ruta de senyalització de l'ABA. L'anàlisi de la localització subcel·lular va mostrar que tant HAI1 com PP2CA es localitzen al nucli, encara que també estan presents al citosol i a la fracció microsomal. Tres membres de la branca de PP2CA *i.e.*: PP2CA, AHG1 i HAI1, van mostrar una inhibició selectiva pels receptors PYR/PYL/RCAR. Estos resultats suggereixen que estos receptors poden discriminar entre membres del clade A de les PP2Cs. *pyl8* és l'únic mutant senzill que mostra sensibilitat reduïda a l'ABA en assajos de creixement d'arrel. Anàlisi usant el gen reporter GUS van mostrar que PYL8 estava present a l'estela, a l'epidermis i a la caliptra, i la quantificació de l'activitat beta-glucuronidasa a l'arrel va mostrar que *PYL8* és un dels receptors amb major nivell d'expressió. La caliptra juga un paper crucial en la resposta hidrotròpica. L'estudi d'esta resposta en mutants múltiples de les PP2Cs i dels PYR/PYL/RCAR va reforçar la idea que l'ABA regula aquest procés. Així, mentre el mutant sèxtuple *pyr/pyl112458* va presentar una curvatura menor a l'aplicar-se-li un gradient d'humitat, el mutant quàdruple de les PP2Cs (*Qabi2-2*) va mostrar una curvatura més pronunciada en estes condicions, evitant les zones amb menor potencial hídric. Finalment, en l'última part d'aquest treball es van utilitzar abordatges genètic i químics per a augmentar la resistència a la sequera. Hem dut a terme un rastreig amb compostos químics per a aïllar nous agonistes de l'ABA. Basat en dades

estructurals dels receptors, es van seleccionar 500 compostos que van ser assajats en Arabidopsis. D'estos, el compost 2C06 va inhibir el creixement de l'arrel en plantes salvatges més que en mutants *pyr/pyl/rcar* insensibles a ABA i va produir resultats prometedors *in vitro* a l'inhibir a les PP2Cs i interaccionar amb estes en assajos de doble híbrid.

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1. INTRODUCTION

1. INTRODUCTION

1.1 DISCOVERY AND ROLE OF ABA IN PLANT PHYSIOLOGY.

Abscisic acid is a phytohormone playing critical functions for plant survival and development. The first evidences for a role of this molecule in plants was found in experiments focused on finding growth regulators from plant extracts, but it was not until the 1960s when ABA was isolated and related to his regulatory action. Phillips and Wareing (1958) detected an inhibitory effect on the material extracted from leaves of sycamore whose activity varied with the photoperiod. Later on, the same group proposed the term "dormin" for a substance extracted from birch leaves that induced the formation of resting buds in seedlings (Eagles and Wareing, 1963). Ohkuma *et al*. (1963) succeeded in isolating the crystalline form of the ""abscisin II", a molecule causing an acceleration of abscission in the petiole of cotton seedlings. Comparison of the properties of abscisin II and dormin led to the conclusion that both were the same molecule. Abscisic acid was first synthesized by Cornforth *et al*. (1967). After that, the proposed structure of the compound was confirmed.

1.1.1 THE ROLE OF ABA IN ABIOTIC STRESS

Since the discovery of the ABA other functions apart from dormancy and abscission have been described. The role of ABA in abiotic stress has been well characterized, especially in the response to water, cold and osmotic stress. A common trait of these processes is a low water availability that can be quantified by the water potential (Ψ). Under low water potential plants activate responses in order to maintain the equilibrium of water uptake from the soil (Verslues *et al*., 2006). Figure 1.1 schematically represents the different responses activated under low water potentials depending on the duration of the stress conditions.

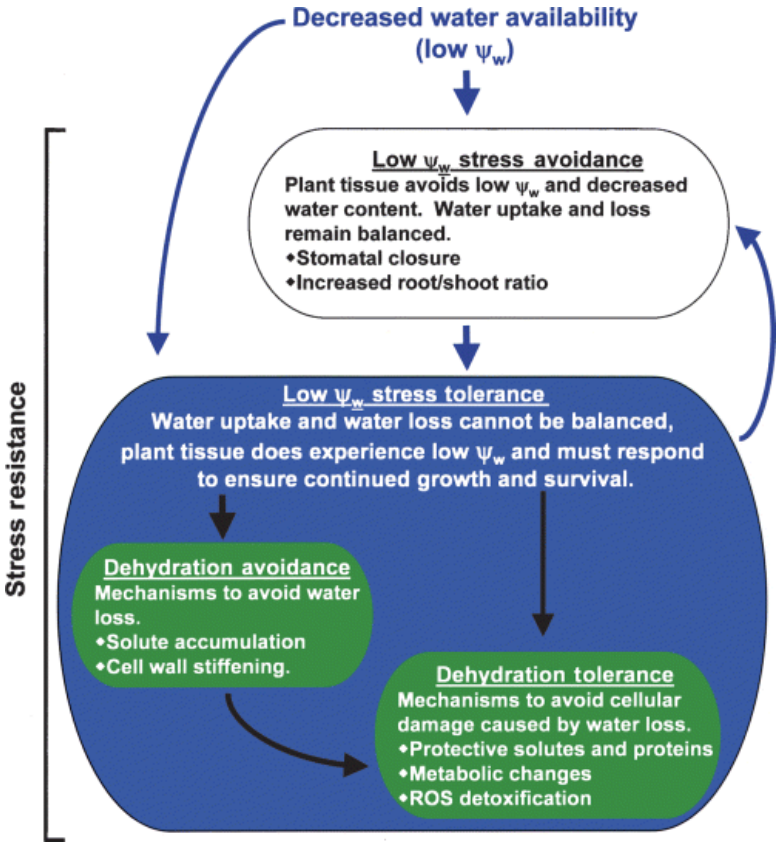


Figure 1.1. **Conceptual diagram of the plant responses triggered by decreased water availability**. In initial stages, the plants tend to avoid low water potentials by preventing water loss. Later, when the stress is prolonged in time, plants employ strategies directed to tolerate these conditions. Between these strategies it can be found the osmotic adjustment by solute accumulation or the synthesis of compounds directed to preserve the cellular components from the damage caused by low water availability. From Verslues *et al*. (2006).

The first response of plants against less water availability is to avoid low water potentials. This is achieved by reducing water loss and increasing water uptake. Therefore, under water stress, one of the initial responses of plants is promoting stomatal closure. Efflux and influx of ions will trigger changes in the polarization state on the plasma membrane causing variations in the turgor that will be translated into the opening or closing of the stomatal pore. Thus, membrane channels have a crucial role in the regulation of this process. The first evidence relating ABA to the stomatal response appeared when mutants involved in ABA signaling or biosynthesis showed increased or decreased transpiration rates; for example the higher transpiration rates of the ABA insensitive mutants *abi1-1* or *abi2-1* (Leung *et al*., 1997). Since then, the identification of proteins regulating stomatal aperture as ion channels or proteins that modulate them has highlighted the role of ABA in this response (Lee *et*

al., 2009; Vahisalu *et al*., 2008). Figure 1.2 shows the elements in charge of regulating the osmotic potential of guard cells and that determine the aperture of the stomatal pore.

In a long term response to low water availability plants modify their growth by increasing the root/shoot ratio and increase cuticle thickness as a way to reduce water loss and increase water uptake from roots. Some evidence reveals that ABA maintains root growth and inhibits shoot growth. Mutants deficient in ABA biosynthesis or wild type plants treated with fluridone (an inhibitor of ABA biosynthesis) showed impaired root growth compared to untreated wild type plants and ABA restored normal root growth. (Saab *et al*., 1990; Sharp *et al*., 2000). However these strategies are directed to bypass a temporary situation and can not be maintained for a long time. In fact, keeping stomata closed will reduce photosynthesis efficiency and promoting increased root/shoot ratio is in detriment of aerial tissues development.

When the transpiration rate decreases, plants activate responses directed to decrease water potential in order to avoid water loss and ensure water uptake from the soil. The accumulation of osmo-compatible solutes (Verslues *et al*., 2006) can decrease water potentials. These molecules are highly soluble compounds that carry no net charge at physiological pH and are nontoxic at high concentrations. The compounds that accumulate most commonly are trehalose, proline and glycine betaine and one of the factors inducing synthesis is ABA (Ishitani *et al*., 1995; Strizhov *et al*., 1997). Increase in the net rate of osmoticum deposition represents an adaptive response that can contribute to growth maintenance under water shortage. For instance, proline deposition in the apex of the root increases dramatically in water-stressed roots, and contributes up to 50% of the osmotic adjustment (Voetberg and Sharp, 1991). But under water stress, osmotic adjustment may not be enough to maintain low values of water potential. Another strategy for maintaining low water potentials is to modify the cell wall yielding properties. Sharp *et al*. (2004) described that primary maize roots were able to continue to elongate under low water potentials. Although an important osmotic adjustment was observed, the decrease in osmotic potential was insufficient to compensate for the decrease in water potential, suggesting that turgor pressure was also reduced. These results suggested that cell wall yielding properties had increased in growing roots at low water potentials, such that cells could maintain their elongation rate even at low turgor pressure.

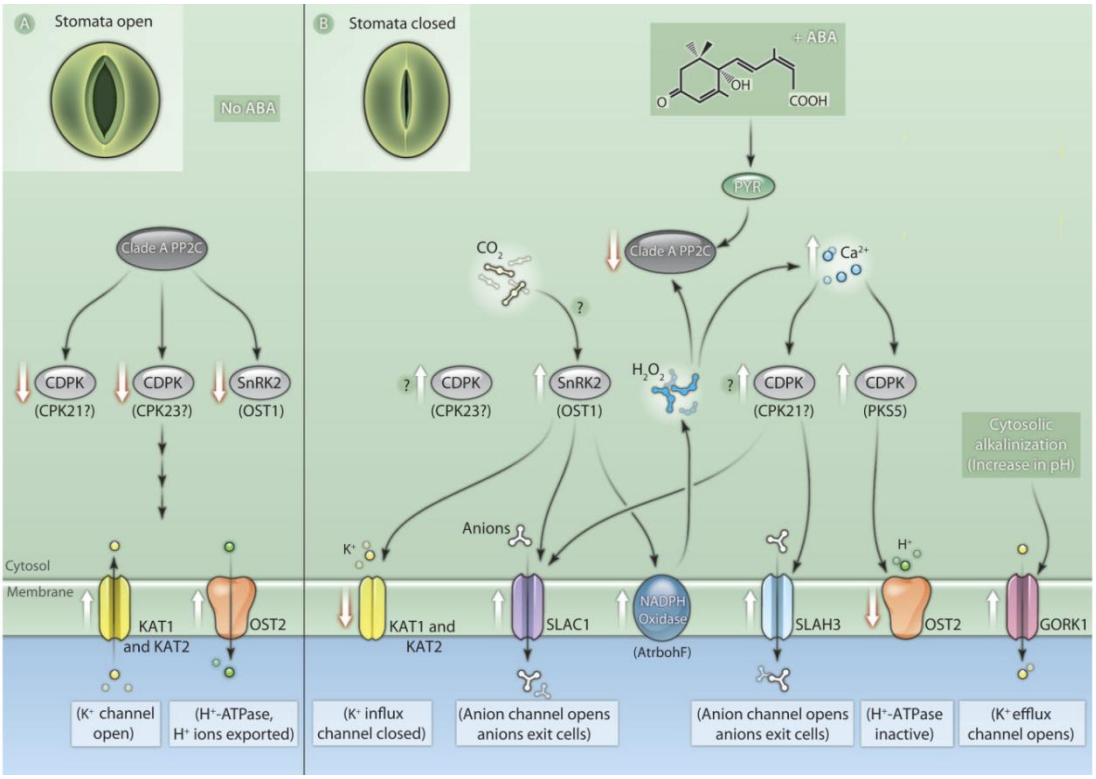


Figure 1.2. Current model of the ABA signaling pathway in the guard cell. (A) After the activation of the H^+-ATPase in the plasma membrane the electrical potential across the membrane increases, with the inside becoming more negative. Thus, the inward-rectifying channels KAT1 and KAT2 (which inactivation is blocked by the PP2Cs in the absence of ABA) trigger K^+ influx and promote stomatal opening. **(B)** An increase of the ABA concentration triggers the inactivation of the PP2Cs by the PYR/PYL/RCAR proteins, thereby liberating the kinases to phosphorylate the downstream targets. The efflux of water from guard cells is carried out by a despolarization of the plasma membrane. OST1 phosphorylates and inhibits the inward-rectifying K^+ channels to prevent the entry of K^+ into the guard cell necessary for stomatal opening. Besides, the NADPH oxidase AtrbohF is activated by OST1 to generate the second messenger H_2O_2, which is linked to Ca^{2+} release. At the same time the inactivation of the H^+-ATPase and the activation of anion channels such as SLAC1 and SLAH3 result in a cytosolic alkalinization and activation of the outward K^+ channel GORK necessary to depolarize the plasma membrane .The combination of all these events produces water efflux from guard cells and ultimately the closure of the stomatal pore. Figure from Joshi-Saha *et al*. (2011).

Under prolonged stress conditions plants activate mechanisms directed to tolerate this unfavorable situation by preventing the damage caused by dehydration. Accumulation of proteins in charge of maintaining protein and membrane structure is known to cause dehydration tolerance. Some LEA proteins (Late Embryogenesis Abundant) accumulate under water deficit conditions such as seed desiccation or abiotic stress. However, despite the recognized role in abiotic stress, the high degree of unordered structure of these proteins in solution and the big heterogeneity of the family have made

difficult to describe their biological functions and activities (Battaglia *et al*., 2008). A role in preventing protein aggregation or in helping protein folding when water content decreases has been proposed for some of them. For example the overexpression of *HVA1*, a LEA protein from barley rapidly induced in young rice seedlings by ABA and by several stress condition, results in higher growth rates, delayed appearance of damage symptoms and better recovery after being under both water-deficit and salt-stress conditions (Xu *et al*., 1996). A protective effect on protein stability was demonstrated when two LEA proteins prevented irreversible aggregation of two enzymes sensitive to water deficit like the citrate synthase (CS) and the lactate dehydrogenase (LDH) under water-stress conditions (Goyal *et al*., 2005). Protein and membrane stability is also maintained by compatible osmolytes. Osmoprotectants participate into avoid cellular damage maintaining membranes and proteins integrity. For instance, glycine betaine and trehalose act as osmoprotectants by stabilizing quaternary structures of proteins and highly ordered states of membranes (Chinnusamy *et al*., 2005).

When plants are exposed to stress conditions the production of Reactive Oxygen Species (ROS) increases. These molecules cause damage to proteins, DNA and lipids but at the same time act as signaling molecules that trigger further responses directed to control cell homeostasis. ROS also act as second messengers in some signaling cascades (Miller *et al*., 2010). As an example, ROS are involved in the stomatal closure triggered by ABA. In the ABA-induced stomatal closure an increase of the cytoplasmic Ca^{+2} levels promotes changes in the regulation of several channels. The orchestrated activation or inactivation of these channels leads to ion efflux and water loss of guard cells, resulting in loss of wall cell turgor and closing of the stomatal pore (Schroeder *et al*., 2001). ROS levels are enhanced by abscisic acid in Arabidopsis guard cells. For instance, ABA increases H_2O_2 levels in *Vicia faba* guard cells (Zhang *et al*., 2002). It has been reported that ABA-induced ROS production triggers the activation of Ca^{+2} permeable channels in the plasma membrane of guard cells (Pei *et al*., 2000). Consistent with these results the *ost1* mutant shows a strong phenotype in water loss experiments and, interestingly, the ABA-induced ROS production is disrupted (Mustilli *et al*., 2002). Two NADPH oxidase catalytic subunit genes in Arabidopsis (*AtrbohD* and *AtrbohF*) have been found to be responsible of the ABA-triggered ROS production in guard cells since the mutants are impaired in ABA-induced stomatal closure, ABA promotion of ROS production, ABA-induced cytosolic Ca^{2+} increases, and ABA activation of the plasma membrane Ca^{2+}-permeable channels (Kwak *et al*., 2003). In addition, two MAPK proteins, MPK9 and MPK12, which are preferentially expressed in guard cells, function downstream of ROS to regulate guard cell ABA signaling positively. The *mpk9mpk12* double mutant showed an enhanced transpirational water loss and ABA and H_2O_2-insensitive stomatal response (Jammes *et al*., 2009).

Finally, a very important ABA-induced adaptive response is the change in the gene expression pattern. In fact, microarray data show that nearly 10% of the protein coding-genes in Arabidopsis are likely to be regulated by ABA (Nemhauser *et al*., 2006). Among the genes transcriptionally up-regulated by ABA can be found gene products involved in stress tolerance, transcription factors, protein kinases and phosphatases, transporters, enzymes involved in the synthesis of osmoprotectants,etc., whereas genes down-regulated include those involved in growth and development (Fujita *et al*., 2011). The bZIP-type (basic-domain leucine zipper) ABRE-binding (AREB) proteins or ABRE-binding factors (ABFs) are the main transcription factors regulating ABA-dependent gene expression under osmotic stress conditions. They recognize ABA-responsive elements (ABRE) in the promoters of ABA-regulated genes. This family of TFs can be separated in two groups according to the expression pattern and to the function they play. The ABI5/AtDPBF group is mainly involved in processes regulating seed processes and the AREB/ABF group is involved in abiotic stress conditions in vegetative tissues. But this classification can not be followed strictly as *ABI5* is expressed in tissues of adult plants and plants which overexpress *ABI5* are hypersensitive to abscisic acid for instance, in the ABA-dependent inhibition of root growth (Brocard *et al*., 2002). Other families of transcription factors like AP2/ERF, R2R3-MYB, NACs, HD-Zip, BHLH, C2H2, AFLB3 and WRKYs are also involved in the ABA regulation of the gene expression (Fujita *et al*., 2011). Additional mechanisms can modulate transcriptional regulation by ABA. SWI3B, a subunit of SWI/SNF chromatin-remodeling complexes, has been shown to interact *in vivo* with HAB1. ChIP (chromatin immunoprecipitation) experiments revealed the presence of HAB1 in the vicinity of the ABA-responsive and ABA treatment eliminated the localization of this PP2C in the proximity of ABA-responsive promoters (Saez *et al*., 2008). Secondary messengers like Phosphatidic acid (PA), Inositol triphosphate (IP3), Ca^{+2} or ROS can modulate ABA gene expression. For instance, the presence of Ca^{+2} is necessary for the ABA-dependent expression of *RAB18* in the presence of ABA (Ghelis *et al*., 2000).

1.1.2 THE ROLE OF ABA IN BIOTIC STRESS

The main phytohormones regulating biotic stress are jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Moreover, several data evidence a role of ABA in the regulation of the biotic stress response. ABA role in plant defense is complex and differs depending on the type of plant-pathogen interaction.

ABA acts as a negative regulator of plant defense activation caused by biotrophic and necrotrophic pathogens in order to prevent activation of resistance responses in unnecessary situations.

For instance, in the ABA-deficient mutant *aao3* increased resistance was related to low concentrations of ABA and high levels of SA (de Torres-Zabala *et al*., 2007); and the ABA-deficient tomato mutant *sitiens* showed enhanced resistance to *Botrytis cinerea* and increased expression of the SA biosynthesis gene PAL (Audenaert *et al*., 2002). Conversely in the pre-invasive resistance responses ABA plays a crucial role preventing the entry of pathogens through stomatal closure. In fact, regulation of guard cell aperture under biotic stress depends on multiple factors like SA-mediated response but also on elements that regulate stomatal closure under abiotic stress such as ABA biosynthesis, nitric oxide production or the kinase OST1 (Melotto *et al*., 2006). In this particular process either ABA or SA deficient mutants failed to close stomata under *Pseudomonas siringae* application. The positive role of this hormone has been described for others pathogens such as *A.brassiciola* and *P.cucumeria* where ABA application decreased plant susceptibility. In addition, the protective role of ABA against pathogens has been related to the induction of callose deposition and the production of reactive oxygen intermediates (Bari and Jones, 2009). However the molecular mechanisms by which ABA regulates plant defense are still unknown. Further experiments are needed to describe how ABA acts differently against different pathogens and which are the mechanisms that activate these responses.

1.1.3 THE ROLE OF ABA IN PLANT GROWTH AND DEVELOPMENT

In plants, seeds are the propagation structure capable of ensuring the survival of the embryo from its formation process until germination. This complex step of development is controlled by developmental and environmental factors, which means that a large number of genes are needed to obtain fine-tuning regulation. To ensure the success of the next generation, plants have developed several strategies such as regulation of seed development, desiccation tolerance, regulation of germination, in which regulation ABA plays a critical role. Two peaks of ABA accumulation occur during seed development in Arabidopsis. The first one takes place 9 DAF (Days After Flowering), just before the maturation stage, ABA is synthesized in maternal tissues and it helps to prevent premature germination, to regulate embryo growth, seed pigmentation, seed productivity, etc. The second peak occurs at 15-16 DAF, ABA is derived from embryo tissues and is essential to induce dormancy and desiccation tolerance (Frey *et al*., 2004; Karssen *et al*., 1983; Raz *et al*., 2001). Dormancy is a trait that prevents seeds germinating just after completing development. This allows seeds to germinate in favorable conditions increasing thus the likelihood of survival (Gubler *et al*., 2005). The application of norfluorazon, an inhibitor of ABA biosynthesis, triggers germination, showing the role of de novo production of ABA in the induction of dormancy (Debeaujon and Koornneef, 2000). Environmental

conditions such as after-ripening, stratification, dark and smoke lead into a release of dormancy and this correlates with a decrease of the ABA content in the seed. Catabolism of ABA in seeds has shown to be a key step in dormancy release. ABA conversion to its inactive forms phaseic acid or dihydrophaseic acid is observed for instance in after-ripened Arabidopsis seeds 24h after imbibition (Kushiro *et al*., 2004). But ABA is not the only hormone involved in this process. In fact, the balance between ABA and GA content is the factor that determines germination. When ABA catabolism increases, de novo synthesis is suppressed and the GA concentration rises; different mechanisms are activated and finally seed germination is carried out (Finch-Savage and Leubner-Metzger, 2006). But as with other processes in plants, germination is regulated by several factors including other hormones like ethylene or jasmonic acid (Linkies and Leubner-Metzger, 2012).

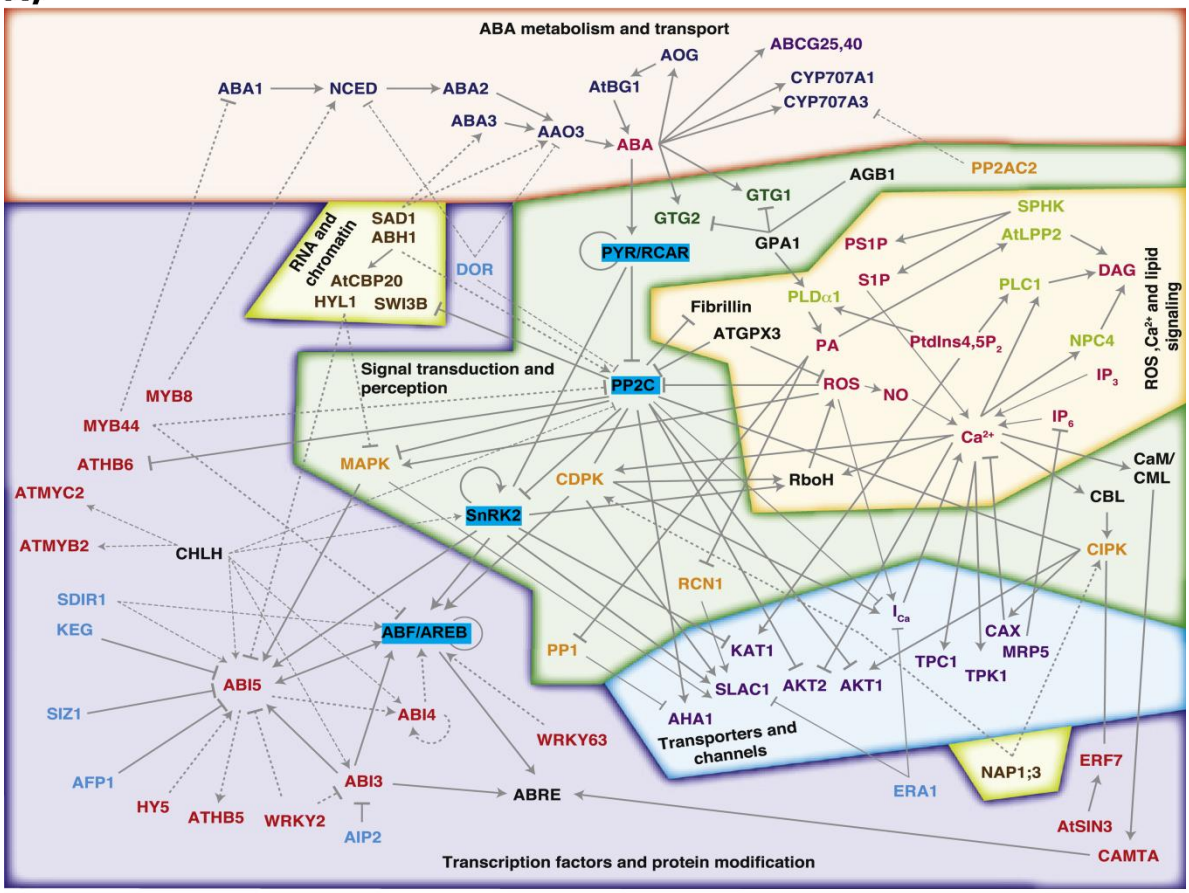
A function into promoting plant growth has been suggested for ABA in the absence of stress. Severe ABA deficient mutants show lower size and smaller leaves even under well watered conditions and ABA application attenuates this phenotype (Sharp *et al*., 2000; Gonzalez-Guzman, 2012). For instance, the *aba1* and *aba2* mutants show growth retardation in most of the tissues, even under high humidity conditions (Barrero *et al*., 2005; Cheng *et al*., 2002). The *aba1* mutant shows a significant reduction in size as well as a disorganized mesophyll with smaller cells in comparison with the wild type. Interestingly, application of low concentration of ABA resulted in a partial restoration of the normal size of the leaf, an increase in dry weight and an increase of the size of the mesophyll cells both in the mutant as in the wild type. Thus, ABA plays a dual function in plant tissues, while in the presence of stress, when ABA levels are high, ABA represses growth; in the absence of stress, small concentrations are essential for a normal development. And this function not only refers to the volume of the cell or the organ size. For instance, the *aba1* mutant is impaired in organogenesis showing abnormalities in the shape and architecture of the leaves (Barrero *et al*., 2005). Recently the isolation of the PYR/PYL/RCAR ABA receptors and the generation of a sextuple mutant with impaired growth and seed yield have reinforced the idea of a positive role of ABA in plant growth (Gonzalez-Guzman *et al*., 2012).

1.2 CORE ELEMENTS OF THE ABA SIGNALING PATHWAY

Since the discovery of ABA several proteins have been isolated as modulators of the ABA response. ABA signaling components form a sophisticated network that integrates and transduces ABA-mediated responses (Figure 1.3, A). Due to this complexity the work here presented is focused

mainly in the core elements of the ABA signaling pathway (Figure 1.3, B). Additionally, mutants affected in the core ABA signaling pathway show the strongest phenotype in ABA response. The first proteins isolated belonging to this core were the clade A of the Serine/threonine phosphatases (PP2Cs). *abi1-1* and *abi2-1*, dominant mutations in the *ABI1* and *ABI2* genes respectively, were isolated by Koornneef *et al*. (1984) in a screen using a ethylmethanesulfonate-mutagenized population of Arabidopsis seeds and focused on the isolation on mutants able to germinate in 10μM ABA. Afterwards both proteins were cloned by positional cloning (Leung *et al*., 1994; Leung *et al*., 1997; Meyer *et al*., 1994; Rodriguez *et al*., 1998). Since *abi1-1* and *abi2-1* are dominant mutations it was not possible to conclude whether they were positive or negative regulators of the ABA signaling pathway. Subsequently, the overexpression of *ABI1* in maize protoplasts (Sheen, 1998) or the generation of intragenic revertants of the *abi1-1* and *abi2-1* mutants (Gosti *et al*., 1999; Merlot *et al*., 2001) suggested a negative role of these proteins in the ABA cascade. Finally, the direct confirmation of this hypothesis came with the characterization of loss of function mutants in different clade A PP2Cs. A null mutant of another PP2C, HAB1, was the first null mutant characterized of this group of proteins (Saez *et al*., 2004). Subsequently, other mutants of other PP2Cs as well as combined mutants have been characterized. It allowed to assign to these proteins a negative role in the regulation of the ABA pathway (Kuhn *et al*., 2006; Nishimura *et al*., 2007; Rubio *et al*., 2009; Saez *et al*., 2006; Yoshida *et al*., 2006b). All these mutants present hypersensitivity to ABA in all or some of the common plant responses affected by ABA such as germination, root and shoot growth inhibition, transpiration water loss and expression of stress responsive genes regulated by ABA. The generation of two triple mutants with extreme ABA sensitivity and impaired in growth revealed an essential role of clade A PP2Cs in the ABA pathway as well as confirmed the role of ABA in plant growth (Rubio *et al*., 2009). The nature of the dominant gain-of-function phenotype caused by a missense mutation in the *abi1-2* and *abi2-1* mutants remained a mystery for 25 years. Finally, the discovery of the PYR/PYL/RCAR receptors provided an elegant molecular explanation to understand the insensitive phenotype of these mutants. These mutations negatively affect the interaction between these proteins and make PP2Cs refractory to the inhibition by the PYR/PYL/RCAR proteins (Dupeux *et al*., 2011a; Park *et al*., 2009; Santiago *et al*., 2012).

A)


ABA metabolism and transport
ABA1
NCED
ABA2
ABA3
AAO3
ABA
AtBG1
AOG
ABCG25,40
CYP707A1
CYP707A3
PP2AC2
AGB1
GTG1
GTG2
GPA1
PYR/RCAR
SPHK
AtLPP2
PS1P
S1P
DAG
PLC1
PLDα1
PA
PtdIns4,5P2
NPC4
IP3
IP6
ROS, Ca2+ and lipid signaling
Fibrillin
ATGPX3
RNA and chromatin
SAD1
ABH1
AtCBP20
HYL1
SWI3B
DOR
Signal transduction and perception
PP2C
ROS
NO
Ca2+
CaM/CML
CBL
CIPK
MAPK
CDPK
RboH
SnRK2
MYB8
MYB44
ATHB6
ATMYC2
ATMYB2
CHLH
SDIR1
KEG
ABF/AREB
ABI5
ABI4
ABI3
SIZ1
AFP1
HY5
ATHB5
WRKY2
AIP2
WRKY63
ABRE
RCN1
PP1
ICa
KAT1
SLAC1
AKT2
AKT1
CAX
MRP5
TPC1
TPK1
AHA1
Transporters and channels
NAP1;3
ERF7
ERA1
AtSIN3
CAMTA
Transcription factors and protein modification

B)

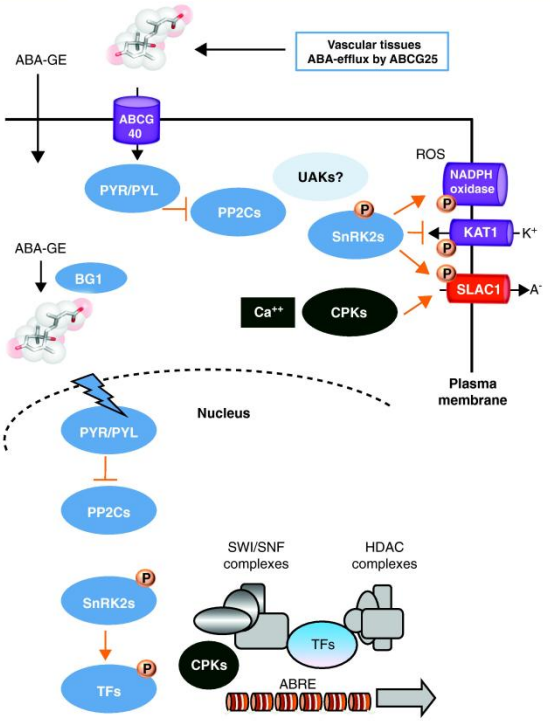

Vascular tissues
ABA-efflux by ABCG25
ABA-GE
ABCG 40
PYR/PYL
PP2Cs
UAKs?
ROS
NADPH oxidase
SnRK2s
KAT1
K+
SLAC1
A-
ABA-GE
BG1
Ca++
CPKs
Plasma membrane
Nucleus
PYR/PYL
PP2Cs
SWI/SNF complexes
HDAC complexes
SnRK2s
TFs
CPKs
TFs
ABRE

Figure 1.3. **A)** ABA signaling network divided into six main functional categories: ABA metabolism and transport (red); perception and signal transduction (dark green); ROS, Ca2+ and lipid signaling (orange); transporters and channels (blue); transcription factors and protein modification (purple); and RNA processing and chromatin remodeling (light green). ABA signaling nodes are given by their protein or molecule names and colored according to their role in ABA metabolism. Connections represent positive (arrow) and negative (block) regulation or currently unknown (line). Regulations are direct (bold line), indirect (faint line) or transcriptional (dashed line). Core elements of the ABA signaling pathway are represented as blue rectangles. The H subunit of the Mg-chelatase (CHLH) and the putative G protein-coupled receptors (GPCRs) GTG1 and GTG2 have been proposed as ABA receptors (Pandey *et al*., 2009; Zhang *et al*., 2002). However, further data have questioned this role. ABA binding experiments as well as the response to ABA of the loss of function mutants have been questioned due to the inability to reproduce the same results by other groups (Jaffé *et al*., 2012; Muller and Hansson, 2009; Risk *et al*., 2009; Tsuzuki *et al*., 2011). Modified from Hauser *et al*. (2011). **B)** Simplified model of the ABA pathway that integrates ABA transport and signaling. From Antoni *et al*. (2011).

The molecular features of the interaction between the elements of the ABA signaling pathway are explained in later sections. PP2C phosphatases regulate negatively a group of SNF1-related protein kinases of the group 2 (SnRK2s); these kinases are positive regulators of the ABA signaling pathway and between their targets include transcription factors that regulate ABA responsive genes, like ABRE-binding factors (ABFs), or anion channels involved in stomatal closure such as SLAC1. The first SnRK2 related to ABA was OST1 and was isolated through a genetic screen for Arabidopsis mutants with altered stomatal responses to drought (Mustilli *et al*., 2002). Later, two other kinases were described as regulators of the ABA response in vegetative tissue (Fujii *et al*., 2007). Finally, the discovery of the PYR/PYL/RCAR receptors allowed the description of a schematic picture of the ABA signaling pathway from the binding of the hormone to the triggering of the different ABA responses (Park *et al*., 2009; Ma *et al*., 2009).

1.2.1 PROTEIN SERINE/ THREONINE PHOSPHATASES 2C (PP2C)

Phosphorylation and dephosphorylation are important mechanisms responsible for regulating a lot of biological processes; in fact, around the 30% of the intracellular proteins are likely to be phosphorylated. The remarkable number of kinases and phosphatases present in all organisms highlights this fact. The Arabidopsis genome encodes 1,085 typical protein kinases (Hrabak *et al*., 2003) and 112 phosphatase catalytic subunit sequences (Schweighofer *et al*., 2004).

Based on the substrate specificity, protein phosphatases can be divided in 2 major groups, Protein Tyrosine Phosphatases (PTPs) and Protein Serine/Threonine Phosphatases. This last group includes the Phosphoprotein Phosphatases (PPP) represented by the PP1, PP2A and PP2B families (no

representative of the PP2B has been found in plants) and by the Metal ion-dependent Protein Phosphatases (PPM) represented by the PP2Cs and the pyruvate dehydrogenase phosphatases. Despite the sequence divergence between the PPP and PPM families, the structural information obtained from the human PP2C protein phosphatase (Das *et al*., 1996), the plant PP2Cs (Dupeux *et al*., 2011a; Miyazono *et al*., 2009) and some PPP protein phosphatases (Goldberg *et al*., 1995; Kissinger *et al*., 1995; Xu *et al*., 2006) underlines a similar ternary structure of their catalytic domain. There are several important differences between PPP and PPM families. The PP2Cs are monomeric enzymes that require Mg^{+2} or Mn^{+2} for their activity. These divalent cations play a key role through the activation of a water molecule in the dephosphorylation reaction. While the PPP family forms holoenzymes with their regulatory subunits, the PPM family contains instead additional domains and conserved sequence motifs that may help to determine substrate specificity (Luan, 2003; Shi, 2009). All the PP2Cs contain a core catalytic domain formed by 11 subdomains (Schweighofer *et al*., 2004). Additionally, they contain the N-terminal and C-terminal extensions which confer specific traits to each protein related to subcellular localization, activity regulation, interaction with other proteins, etc. For instance, in most of the clade B of the PP2C family can be found a kinase interaction motif (KIM) in the N-terminal region. A case of subcelular localization regulation is HAI1, where a bipartite nuclear localization signal located in the N-terminus determines protein localization (Antoni *et al*., 2012).

Phosphorylation plays a key role in the regulation of the ABA pathway in plants. Some evidence relates the PP2A family of phosphatases to this phytohormone. Mutants of RCN1, a PP2A regulatory subunit, are ABA insensitive in some characteristic ABA regulated responses such as seed germination, guard cell responses and gene expression (Kwak *et al*., 2002). In addition Pernas *et al*. (2007) have characterized a null mutant of the PP2A catalytic subunit (PP2Ac) that shows hypersensitivity to ABA in different processes regulated by this hormone. However the clade A of the PP2C family is the most important group of phosphatases related to the ABA pathway. These proteins are negative regulators of the cascade and are part of the key elements responsible of the transduction pathway. While the PPP family shows a high degree of homology at the structural level between animals and plants, the PP2C family possesses very particular characteristics with respect to its homologues in other organisms. It's represented by a larger group of proteins, suggesting that its role in plants is more crucial and diverse. These proteins show little sequence homology compared to their homologues in animals and most of them posses the catalytic domain in the C-terminus and an unique N-terminal extension (Luan, 2003). The PP2C family in plants is represented by 76 putative proteins, many of them without known function, divided in ten clades from A to J (Schweighofer *et al*., 2004).

Phosphatases of the clade B have a MAP kinase interaction motif (KIM); this motif is conserved in yeast and mammals and is supposed to be responsible of the specific interaction between these phosphatases and the mitogen activated protein kinases (MAPKs). MAPKs are responsible for mediating one of the most important and conserved signal transduction cascades in eukaryotes and mediate biotic and abiotic stress responses. There is evidence relating clade B of PP2Cs to MAPK kinases. MP2C, is an alfalfa PP2C that negatively regulates the stress-induced MAPK SIMK (Meskiene *et al*., 2003). There are data supporting that Arabidopsis clade B of PP2Cs also carry out the same function. For instance, AP2C1 interacts and negatively regulates MPK4 and MPK6 in processes regulated by these AMPs such as the wounding response, JA and ET levels, and biotic stress (Schweighofer *et al*., 2007). Moreover, the null mutant of PP2C5 shows increased stomatal aperture, partial ABA-insensitive phenotype in seed germination and less induction of the expression of ABA-induced genes and interacts in Arabidopsis protoplasts with MPK3, MPK4, and MPK6 (Brock *et al*., 2010).

In the clade C of PP2C can be found phosphatases involved in cell differentiation. Specifically, POL and PLL1 take part in the clavata pathway responsible of regulating the meristem size. POL and PLL1 positively regulates WUSCHEL and lead into the maintenance of the root and shoot apical meristems (Gagne and Clark, 2007; Gagne and Clark, 2010).

Another PP2C unrelated to any other group and with known function is KAPP. This protein shows at the N-terminal extension a type I signal anchor (SAI) that makes the protein to be membrane-anchored with cytoplasmatic orientation followed by a kinase interacting domain (KI). The PP2C catalytic domain is located in the C-terminus. KAPP was isolated by interacting through the KI domain with RLK5, a receptor like kinase with unknown function (Stone *et al*., 1994). Later, other RLKs were found to interact with KAPP. This is the case for CLAVATA1, a RLK responsible of maintaining the size of shoot and inflorescence meristems in Arabidopsis. The analyses suggest that KAPP is a negative regulator of the pathway as low KAPP mRNA levels correlate with suppression of the *clv1* phenotype. The ubiquitous expression pattern and the interaction with several RLKs suggest an important role for this protein in the RLK pathways (Shiu and Bleecker, 2001).

Finally, the clade A of PP2C protein phosphatases is formed by nine proteins related mostly to the ABA pathway. Two branches can be distinguished based on amino acid sequence alignments (Schweighofer *et al*., 2004) (Figure 1.4). The branch formed by ABI1, ABI2, HAB1 and HAB2 has been historically better characterized than the branch represented by PP2CA/AHG3 and AHG1. The first PP2Cs of this clade were isolated in a screen focused on the isolation of ABA insensitive mutants (Koornneef *et al*., 1984). *abi1-1* and *abi1-2* are dominant mutations of *ABI1* and *ABI2*, respectively,

that show insensitivity to ABA in several processes such as germination, stomatal regulation, gene expression and root growth (Leung *et al*., 1994; Leung *et al*., 1997; Meyer *et al*., 1994; Rodríguez *et al*., 1998) . Since *abi1-1* and *abi1-2* are dominant mutations it was not possible to conclude whether they were positive or negative regulators. These mutations are caused by the substitution of a Gly for an Asp in a conserved motif of the PP2C catalytic site, causing a decrease in the phosphatase activity, and at the same time showing insensitive responses to ABA. The characterization of intragenic revertants and especially the obtaining of the first loss-of-function mutants confirmed the negative role in the ABA signaling pathway (Gosti *et al*., 1999; Merlot *et al*., 2001; Saez *et al*., 2004). Initially, a negative dominant effect of the *abi1-1* and *abi1-2* mutations was proposed to explain the phenotypes. However, since loss-of-function alleles show ABA-hypersensitivity phenotype, such a hypothesis was unlikely. The discovery of the interaction between PP2Cs and the ABA receptors, which is severely reduced in the *abi1-1* and *abi1-2* proteins, shed definitive light on the nature of these mutations. Structural data have shown that these mutations cause a decrease of the PP2C activity because they affect a residue located in the active site. Particularly, this Gly residue establishes a hydrogen bond with a residue in the PYR/PYL/RCAR proteins (Dupeux *et al*., 2011a; Hao *et al*., 2011; Melcher *et al*., 2009; Miyazono *et al*., 2009; Yin *et al*., 2009). However, in the presence of ABA, the corresponding mutant proteins are refractory to inhibition by ABA, whereas wild type PP2Cs are inhibited. Therefore, in the presence of ABA, *abi1-1* and *abi1-2* PP2Cs show more activity than the wild type and they represent hypermorphic mutations (Dupeux *et al*., 2011a; Park *et al*., 2009; Robert *et al*., 2006; Santiago *et al*., 2012). After the isolation of these phosphatases many efforts have been conducted to describe their function in the different responses of the ABA pathway. Particularly, ABI1, ABI2 and HAB1 have been used as models to describe the ABA signaling together with the new family of cytosolic ABA receptors, the PYR/PYL/RCAR proteins, and the SnRK2s. Thus, structural and biochemical data have allowed the description of the ABA cascade in a very detailed way (Fujii *et al*., 2009; Ng *et al*., 2011; Soon *et al*., 2012).

Besides drought, these phosphatases are also involved in other abiotic stresses. The serine/threonine protein kinase SOS2 activates the plasma membrane Na^+/H^+ antiporter encoded by the *SOS1*. These two proteins play an important role in salt stress tolerance (Qiu *et al*., 2002). ABI2 interacts with SOS2 through the PPI motif in the kinase and, in addition, *abi1-1* and *abi2-1* null mutants show insensitivity to salt stress (Ohta *et al*., 2003). *abi1-1* mutant is also impaired in development of freezing tolerance and PP2CA/AHG3 antisense plants show accelerated cold acclimatation (Mantyla *et al*., 1995; Tahtiharju and Palva, 2001); this is in agreement with the fact that ABA treatment triggers freezing tolerance. All these data highlight the role of PP2Cs in the regulation

of cold acclimatation by ABA (Mantyla *et al*., 1995; Tahtiharju and Palva, 2001). Finally, A role in the regulation of K^+ channels of PP2CA and HAI2/AIP1 is explained below.

HAB1 and *HAB2* show 75% sequence similarity between themselves. *hab1-1* was the first null mutant characterized of a PP2C (Saez *et al*., 2004). The hypersensitive phenotype and the characterization of constitutive overexpression lines confirmed the suggested role of these PP2Cs as negative regulators. The existence of functional redundancy between this group of phosphatases was confirmed when the *hab1-1abi1-2* double mutant showed a more severe sensitivity to ABA than the single mutants (Saez *et al*., 2006). The mutation $hab1^{G246D}$ (equivalent to that responsible of the phenotypes in the *abi1-1* and in the *abi2-1* mutants) presented a phenotype similar to *abi1-1* and *abi2-1* and reinforced the idea that these proteins have the same way of interacting with their substrates (Robert *et al*., 2006). In addition, the interaction of SWI3b, a subunit of SWI/SNF chromatin remodeling complexes, and HAB1 provided the first evidence of direct transcriptional regulation by a PP2C (Saez *et al*., 2008).

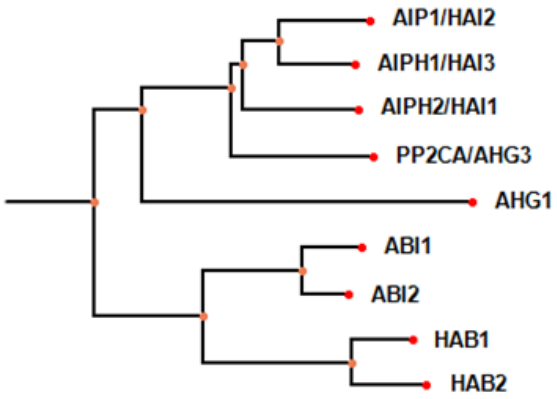


Figure 1.4. Cladogram of the clade A PP2Cs. The amino acid similarity tree based on the catalytic site sequences shows the existence of two subbranches.

While AHG1 and PP2CA/AHG3 mutants have been characterized and some of their principal roles in the ABA response are known, other three phosphatases belonging to this branch (AIP1/HAI2, AIPH1/HAI3 and AIPH2/HAI1) have been less studied. All three have been described as highly ABA induced (HAI) as microarray data showed high inductions levels in wild type plants and reduced expression levels in both *snrk2.2snrk2.3snrk2.6* and *areb1/areb2/abf3* triple mutants after treatment with 50 μM ABA . In addition, AIPH2/HAI1 has been characterized as a SnRK2.2 interacting protein

in BiFC (Bimolecular Fluorescence Complementation) analyses. The expression pattern of these proteins suggests that they take part in the ABA response in vegetative tissue (Fujita *et al*., 2009). PP2CA/AHG3 and AHG1 were discovered in a screening for mutants with altered sensitivity to ABA (Kuhn *et al*., 2006; Nishimura *et al*., 2004; Yoshida., 2006b). The knock-out mutants *pp2ca/ahg3* and *ahg1* are very hypersensitive to ABA in germination assays but no clear phenotypes have been found in adult plants. In addition, they show high expression levels in seeds and the double mutant presents an enhanced hypersensitive phenotype to ABA in germination. Whereas AHG1 is expressed specifically in the seed during its formation *PP2CA* is also expressed in vegetative tissue. The overexpression lines of PP2A show ABA-insensitivity in water loss experiments and in germination. All these data suggest a major role in seeds for these two phosphatases but also a role for PP2CA in processes other than germination, which would be masked in the single mutant because of genetic redundancy with other PP2Cs (Kuhn *et al*., 2006; Nishimura *et al*., 2007; Yoshida *et al*., 2006b).

A role in the control of K^+ channels has been found for 2 members of this branch, PP2CA and AIP1/HAI2. K^+ is a macronutrient essential for multiple processes in the plant such as enzyme activity, plant nutrition and osmoregulation. As can be seen in table 1.1 the family of the shaker-like K^+ can be separated in 4 functional subgroups (Wang and Wu, 2012). PP2CA interacts and inhibits AKT2 activity in Xenopus oocytes and in mammalian cells and these two proteins have overlapping expression patterns. Thus, AKT2 regulation by PP2CA might have a physiological role both in guard cells and in phloem vasculature, where both proteins show their highest expression levels (Cherel *et al*., 2002). Another member of the inward-rectifying K^+ channel family, AKT1, has been found to be regulated by the CBL-CIPK pathway. It is highly expressed in root epidermal cells and the null mutant shows impaired growth under limiting concentrations of K^+ (Dennison *et al*., 2001). CBL proteins interact with the CIPK kinases in the presence of Ca^{+2} and these two groups of proteins form different pair combinations regulating different processes. AKT1 is activated by the CBL1/CBL9-CIPK23 and inactivated by AIP1/HAI2 (AKT1 interacting protein) (Lee *et al*., 2007). The finding that a novel PP2C interacts with AKT1 reveals a fine-tuning regulation for this kind of K^+ channels and suggests the involvement of ABA in this process. The isolation of SLAC-1, a slow anion channel involved in stomatal closure, and the fact that its activity is modulated by OST1 and ABI1/PP2CA proved the direct role of clade A PP2Cs in regulation of stomatal aperture (Geiger *et al*., 2009; Lee *et al*., 2009; Vahisalu *et al*., 2008). Recently, the SLAC-1 activation has been reconstituted in oocytes using the core elements of the ABA signaling pathway (Brandt *et al*., 2012).

Name	Functions	Organ(s)/tissue(s)
KAT1	Inward-rectifying K^+ channel, K^+ uptake into guard cells, stomatal regulation	Leaf (guard cells)
KAT2	Inward-rectifying K^+ channel, K^+ uptake into guard cells, stomatal regulation	Leaf (guard cells, phloem)
AKT1	Inward-rectifying K^+ channel, K^+ uptake into root cells	Root (root hairs, epidermis, cortex), leaf (primordia, mesophyll, hydathodes, guard cells)
SPIK	Inward-rectifying K^+ channel, K^+ uptake into pollen tubes, pollen tube development regulation	Pollen (pollen, pollen tubes)
SKOR	Outward-rectifying K^+ channel, K^+ release into xylem, K^+ translocation from roots to shoots	Root (vascular tissues), pollen
GORK	Outward-rectifying K^+ channel, K^+ release from guard cells, stomatal regulation	Root (root hairs, epidermis), leaf (guard cells)
AtKC1	Silent K^+ channel, assembly with Shaker inward K^+ channels and regulation of K^+ uptake into cells	Root (root hairs, epidermis, cortex), leaf (epidermis, hydathodes, trichome)
AKT2	Weakly rectifying K^+ channel, K^+ circulation in phloem	Root (phloem), stem, leaf (phloem, mesophyll, epidermis, guard cells), flower (sepal)

Table 1.1. The members of the Shaker-like K^+ Channel family in Arabidopsis. In Arabidopsis, AKT1 functions in K^+ uptake from the soil. K^+ translocation from root cortex cells into the xylem is mediated by the outward-rectifying channels, such as SKOR and allows K^+ transport between tissues or organs. Weakly rectifying K^+ channels, such as AKT2, load and unload K^+ in phloem tissues. In guard cells KAT1 and KAT2 control the K^+ influx across the plasma membrane during stomatal opening and the outward K^+ channel GORK conducts the K^+ efflux during stomatal closure. In pollen and pollen tube cells, the pollen-specific Shaker channel SPIK mediates K^+ influx regulating pollen tube growth and development. (Based on Wang and Wu, 2012).

Lately, the role in the ABA pathway of other uncharacterized clade A PP2Cs has been analyzed. (Antoni *et al*., 2012; Bhaskara *et al*., 2012; Guo *et al*., 2010; Lim *et al*., 2012). As other clade A PP2Cs, AIP1/HAI2 and AIPH2/HAI1 interact with the PYR/PYL/RCAR receptors, which suggest the same mode of action than other phosphatases characterized so far. Lim *et al*. (2012) and Guo *et al*. (2010) analyzed knock-out mutants of AIP1/HAI2 and AIPH2/HAI1 respectively. Although the authors suggest a positive role in the regulation of the ABA pathway during germination these mutants show very subtle differences compared to the wild type and only one mutant allele has been characterized for each phosphatase. Recently Bhaskara *et al*. (2012) have performed a characterization

of single, double and the triple mutants of AIP1/HAI2, AIPH2/HAI1 and AIPH1/HAI3. The single mutants showed similar germination rates under ABA treatment compared to the wild type, which is in accordance with the results obtained by Antoni *et al*. (2012) for the case of AIPH2/HAI1. However double and triple mutants showed ABA hyposensitivity in germination and hypersensitivity in postgermination responses to ABA. Nevertheless the single mutants showed grater proline and osmoregulatory solute accumulation under low water potentials than other PP2Cs and than the wild type. All these data together suggest a role in ABA responses but a predominant role of these PP2Cs on osmotic adjustment. A possible explanation for the ABA hyposensitivity in germination of the combined mutants could be the effect of the higher accumulation of proline, which has been reported as a molecule involved in the osmotic adjustment under water stress (Voetberg and Sharp, 1991).

1.2.2 ABA RECEPTORS IDENTIFIED UP TO NOW

Several advances in the last years have helped to establish the ABA core signaling pathway. However, ABA perception has been subject of controversy due to both lack of reproducible phenotype in ABA-receptor mutants and ABA-binding by different putative ABA receptors (Christmann and Grill, 2009; Cutler *et al*., 2010; Gao *et al*., 2007; Guo *et al*., 2008; Jaffé *et al*., 2012; Johnston *et al*., 2007; Liu *et al*., 2007; Muller and Hansson, 2009; Pandey *et al*., 2009; Razem *et al*., 2004; Razem *et al*., 2006; Risk *et al*., 2008; Risk *et al*., 2009; Shen *et al*.,2006; Tsuzuki *et al*., 2011; Zhang *et al*., 2002). Finally, the independent isolation in 2009 of the PYR/PYL/RCAR proteins by several research groups (Ma *et al*., 2009; Nishimura *et al*., 2010; Park *et al*., 2009; Santiago *et al*., 2009b) allowed obtaining conclusive genetic evidence and ABA binding by these receptors. Additionally, it was established the connection of members of this pathway, like the PP2Cs or the SnRK2s, with ABA perception.

FCA

FCA is an Arabidopsis protein homologue to ABAP1, a protein isolated as an abscisic acid-binding protein from a barley DNA expression library (Razem *et al*., 2004). Binding experiments using recombinant FCA proteins pointed out a direct interaction with ABA. ABA affected the flowering times in wild type plants but not in the *fca-1* mutants and also changed the mRNA levels of *FLC*, a MADS box transcription factor negatively regulated by FCA that repress the floral transition. The

authors proposed a new role of ABA in the RNA-processing machinery through the direct binding to FCA as no relevant phenotypes has been found for the null mutants in germination or stomatal aperture (Razem *et al*., 2006). Unfortunately, further investigations have failed to reproduce the binding of ABA to FCA and this work has been retracted. Risk *et al*. (2008) remark that there is a small amount of the total protein binding ABA and suggest that the difficulty into removing unbound ABA from samples could have led to wrong interpretations.

CHLH/ABAR

ABAR was isolated from epidermis extracts of broad bean leaves using an affinity-chromatography column with the ABA molecule coupled through its carboxyl group (Zhang *et al*., 2002). This protein encodes the H subunit of the Mg-chelatase (CHLH), a protein complex localized in chloroplasts responsible of the insertion of Mg^{2+} into protoporphyrin IX to form Mg-protoporphyrin IX. The isolation of a missense mutation of this Arabidopsis protein (*gun5)* contributed to assign it a function in plastid-to-nucleus retrograde signaling (Mochizuki *et al*., 2001). The T-DNA insertion mutant *abar-1* is lethal but the characterization of RNAi lines as well as overexpression lines provided evidence for a positive role of ABAR/CHLH in some ABA responses such as germination, early seedling growth or gene expression. RNAi lines showed insensitivity to ABA in germination and stomatal regulation and, by contrast, the ABAR overexpression resulted in dehydration resistance (Shen *et al*., 2006). *rapid transpiration in detached leaves 1* (*rtl1*) is another allele of ABAR/CHLH, which was isolated in a screen for mutants showing enhanced transpiration. Plants of *rtl1* showed insensitivity to ABA-induced stomatal closure but no differences compared to the wild type were found in other responses such as germination or root growth. Binding assays performed by these authors did not detect ABA binding by ABAR/CHLH. In contrast, these authors detected ABA binding by PYR1, an ABA receptor whose binding to ABA has been previously reported (Santiago *et al*., 2009a; Tsuzuki *et al*., 2011). Moreover, Muller and Hansson (2009) have reported a normal behavior of different barley Mg-chelatase large subunit (XanF) mutants in its response to ABA, and they could not detect ABA binding by barley CHLH/XanF. Since the carboxylic group of ABA (which is required for ABA bioactivity) was dispensable for binding by ABAR, it is questionable whether this protein represents a genuine ABA receptor (Cutler *et al*., 2010). All these data reflect different criticisms to accept ABAR/CHLH as an ABA receptor.

G-protein-coupled receptors (GPCRs)

Signaling cascades associated to G proteins are very common in eukaryotes. After ligand binding to the G protein-coupled receptors (GPCRs) conformational changes trigger the change of the Gα subunit to its GTP-bound activated form and the dissociation of the heterotrimeric G complexes; subsequently the different responses are activated. Despite the large number of protein G complexes existing in other organism, in Arabidopsis there is only one Gα subunit (GPA1), one Gβ subunit and two Gγ subunits. The involvement of some of these genes in the ABA response has been probed with the characterization of mutants in Arabidopsis. Mutants of GCR1, a predicted GPCR protein, mutants of one of the two Gγ subunits, AGB1, and mutants of GPA1 are affected in some ABA responses (Pandey *et al*., 2006; Pandey and Assmann, 2004). The ABA phenotypes associated to the GCR1 mutant have suggested that unknown GPCRs in Arabidopsis could participate in the ABA signaling pathway and may act as membrane receptors. In this direction Liu *et al*. (2007) isolated in the Arabidopsis genome the protein GCR2, a putative GPCR protein with a seven transmembrane domain whose loss of function mutants show hyposensitivity to ABA. GCR2 was able to interact with the Gα subunit GPA1 using different approaches and ABA binding was detected with a Kd of 20.1 nM. Despite that there is interaction in BiFC and in coinmunoprecipitation experiments Johnston *et al* (2007) argued that with the SPR (Surface Plasmon Resonance) results it's not possible to conclude an interaction between GPA1 and GCR2. Bioinformatic analyses were not able to confirm a seven transmembrane domain in the protein and the prediction method used was questioned by Johnston *et al*. (2007). In fact, the classification of GCR2 as a GPCR protein has been questioned and it has been proposed to include this protein in the LanC protein superfamily with which shares amino acid sequence similarity (Gao *et al*., 2007; Johnston *et al*., 2007). It has been also questioned the role of GCR2 in the ABA pathway. With the aim of checking the participation of GRC2 in the ABA pathway, analyses with the *gcr2* mutant and two of its homologues in Arabidopsis (GCL1 and GCL2) were performed. There were no significant differences between the single, double or the triple mutants and the wild type in germination experiments in response to ABA or in the expression levels of some ABA-induced genes after ABA treatment (Gao *et al*., 2007; Guo *et al*., 2008). Finally, as for the case of FCA, ABA binding by GCR2 could not be reproduced (Risk *et al*., 2009).

With the aim of isolating new GPCR proteins in plants Pandey *et al*. (2009) performed an screen *in silico* and found two novel GPCR-like proteins with GTP binding and GTPase activity and able to bind ABA, GTG1 and GTG2. While the single mutants did not show any defect in the ABA response, the double mutant presents insensitivity in germination, root growth, stomatal closure and less induction of the expression of ABA-induced genes. As other GPRC, these proteins are localized in

the membrane and interact with GPA1. These proteins show more affinity to ABA in the presence of GDP than in the presence of GTP. This and the fact that the effect of GPA1 in GTGs proteins is to accelerate the binding to GTP and decrease GTPase activity, creates a scenario where, in the presence of GPA1, GTGs would be bound to GTP and would present less affinity for ABA which would block the ABA cascade. This negative regulation of GPA1 is in agreement with the hypersensitive phenotype of the null mutant. However, the fact that the ABA response is not completely abolished in the double mutant, leads to speculation that other mechanisms of perception are still to be discovered. This hypothesis is particularly important in guard cells, where the previous model doesn't match with the insensitivity to ABA showed by the *gpa1* mutant in inhibition of stomatal opening. The study of the relations between GTG1 and GTG2 with unknown and known elements of the ABA pathway such as the PP2Cs or the SnRK2s as well as binding experiments would be required to confirm the function of these proteins in the ABA cascade (Christmann and Grill, 2009; Cutler *et al*., 2010; Pandey *et al*., 2009; Risk *et al*., 2009). Moreover, recently Jaffé *et al*. (2012) have isolated new null mutant alleles of GTG1 and GTG2 and have generated two double mutants. Both double mutants showed similar sensitivity to ABA-mediated inhibition of root growth and germination compared to the wild type, and after ABA treatment, no significant difference was found in ABA transcriptional response between the mutant and the wild type. These results are inconsistent with their role as ABA receptors and based on the phenotypes observed the authors assign a role of these proteins in growth and development of seedlings, in pollen tube growth and plant fertility. Again, as in the case of FCA, GCR2 and CHLH/ABAR, the role of GTG1/GTG2 as ABA receptors is questionable.

PYR/PYL/RCAR

In 2009 different groups isolated independently a new family of ABA receptors called PYR (pyrabactin resistance)/PYL (PYR1-like)/RCAR (regulatory components of ABA receptor) (Ma *et al*., 2009; Park *et al*., 2009; Santiago *et al*., 2009b). This family of proteins belongs to the Bet v I-fold superfamily and is formed by 14 members in Arabidopsis. Several structural data have revealed the molecular mechanism by which these proteins bind ABA and activate the ABA signaling pathway trough the inhibition of the PP2Cs (Melcher *et al*., 2009; Miyazono *et al*., 2009; Nishimura *et al*., 2009; Santiago *et al*., 2009a; Soon *et al*., 2012; Yin *et al*., 2009; Yuan *et al*., 2010; Hao *et al*., 2011; Zhang *et al*., 2012). The Bet v I domain is formed by 7 β-sheets and a α-helix that form a cavity closed in the bottom by two small helices. In the top of the cavity two loops called gate and latch adopt an open conformation in the absence of ABA. The entrance of ABA in the cavity entails conformational changes in these gating loops that result in a closed conformation of the receptor that now shows a

protein surface able to interact with the PP2C. Despite the highly conserved sequences, this family of receptors presents differences in oligomeric state. Although structural data are not available for all the PYR/PYL/RCAR proteins other approaches suggest that receptors such as PYR1, PYL1, PYL2 and PYL3 form homodimers and PYL4, PYL5, PYL6, PYL8, PYL9 and PYL10 are monomeric (Dupeux *et al*., 2011b; Hao *et al*., 2011). The oligomeric state of PYL7, PYL11 and PYL12 hasn't been determined yet, and recently Zhang *et al*. (2012) reported a particular trans-dimeric structure for PYL3. The apo-form is a cis-homodimer canonical to those formed by other dimeric receptors, but, in the presence of ABA the two proteins bind to each other in a reverse direction forming a trans-homodimer that precedes dimer dissociation.

Residues involved in ABA binding and dimerization are situated very close and this implies that, for dimeric receptors, ABA binding affects dimer stability and promotes dimer dissociation. In addition, the dimerization zone and the residues involved in the interaction with the PP2Cs are overlapping, which means that these two processes can not occur simultaneously. Therefore, dissociation of the dimers by ABA binding is necessary for the interaction with the PP2Cs. Dimeric receptors show lower affinities for ABA than monomerics, since ΔH for dimer dissociation is positive. The differences in the Kd of these two kinds of receptors are based on the negative contribution of dimerization to the receptor activation process and this was demonstrated when a PYR1 mutant, $PYR1^{H60P}$, that forms less stable dimers showed a value of Kd similar to those of monomeric receptors (Dupeux *et al*., 2011b). However, in the presence of ABA, both dimeric and monomeric receptors form ternary complexes with PP2Cs with similar affinities. Receptor and PP2C establish contacts using the gating loops that cover the ABA-binding pocket. The inhibition of the phosphatase occurs by blocking the access of its active site through the β3-β4 (gate) loop of PYR/PYL/RCAR proteins. Besides, the residue W385 situated in the flap subdomain of the phosphatase inserts between the gating loops and establishes a H-bond with the ketone group of ABA through a water molecule stabilizing the closed conformation of the receptor. This mechanism probably explains the higher ABA binding affinities presented by the ternary complexes (Kd between 30-60nM) compared to individual receptors (Kd between 1-50μM).

Genetic redundancy precluded the isolation of this family of ABA receptors using classical genetic screenings and only through a chemical genetics approach, or protein-protein interaction studies followed by ABA binding assays these receptors were discovered (Ma *et al*., 2009; Park *et al*., 2009; Santiago *et al*., 2009b; Nishimura *et al*., 2010). This family is composed of 14 members and it can be divided in two subgroups, monomeric and dimeric, according to their oligomeric state. Microarray data show low expression levels for *PYL3* and *PYL10-13* whereas *PYL1-9* present

significant expression levels (Kilian *et al*., 2007; Winter *et al*., 2007). GUS reporter gene analyses of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* reveal overlapping expression in some tissues, although some differences could also be observed (Gonzalez-Guzman *et al*., 2012). The comparison of ABA sensitivity in single and combined pyr/pyl/rcar mutants (for instance the quadruple mutant *pyr1pyl1pyl2pyl4)* and more recently in a sextuple mutant (*pyr1pyl1pyl2pyl4pyl5pyl8)* able to germinate on 100µM ABA has confirmed that functional redundancy precludes the detection of strong ABA-insensitive phenotypes in single mutants (Park *et al*., 2009; Gonzalez-Guzman *et al*., 2012). In contrast, the sextuple mutant shows strong ABA insensitivity to ABA-mediated inhibition of germination and growth, regulation of stomatal aperture and ABA responsive gene expression. This genetic evidence proves that these receptors play a major and quantitative role in ABA perception.

1.2.3 SNF1-RELATED PROTEIN KINASES 2 (SNRK2s)

Arabidopsis contains 38 protein kinases that are related to *SNF1* (Sucrose non-fermenting-1) from yeast. The SnRKs form three subgroups based on sequence similarity and domain structure (Hrabak *et al*., 2003). The group of SnRK2s in Arabidopsis is formed by ten kinases three of which are strongly activated by ABA (Figure 1.5). The first report of a SnRK2 involved in ABA signaling was the wheat SnRK2 *PKABA1*, which is induced at the transcript level in ABA-treated embryos (Anderberg and Walker-Simmons, 1992). Orthologous SnRK2s whose transcription or activity is induced by ABA in other plant species have been described in Arabidopsis, faba bean, rice or wheat (Gomez-Cadenas *et al*., 1999; Kobayashi *et al*., 2004; Li and Assmann, 1996). *OST1* (*SnRK2E/SnRK2.6*) was the first Arabidopsis SnRK2 described to be involved in ABA signaling. This protein was firstly isolated in a screen based on thermal imaging of drought-stressed plants. Later on, OST1 was isolated in experiments directed to identify ABA-activated protein kinases in Arabidopsis plant extracts (Mustilli *et al*., 2002; Yoshida *et al*., 2002). The *ost1* (*open stomata 1*) single mutant is impaired in ABA-mediated stomatal closure but is not affected in other ABA responses.

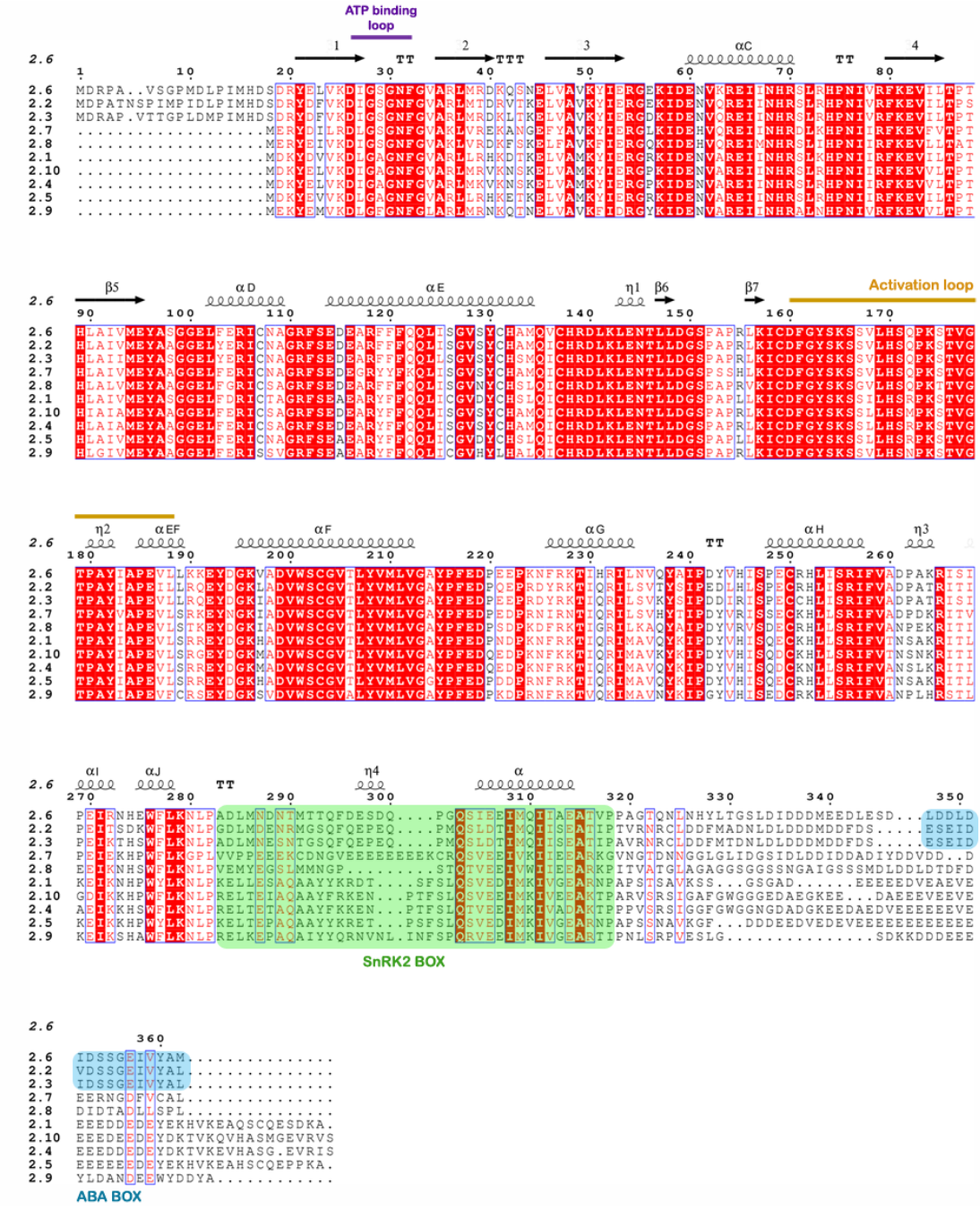


Figure 1.5. Structure-based sequence alignment of the ten SnRK2s of Arabidopsis with the structure of OST1. All ten SnRK2s present at the C-terminus a SnRK-box (also called activation motif or DI) responsible of the activation of the kinases under osmotic stress. This domain is located parallel with the αC, an α-helix whose position is crucial in the adoption of a closed (active) or open conformation (inactive). Mutants lacking the interaction between these two regions abolish the kinase activity and confirm the importance of the SnRK-box to modulate the kinase activity. In addition the three SnRK2s activated by ABA posses a specific region at the C-terminus called ABA box. This region interacts with the PP2C and is responsible of the ABA-dependent activation of these three kinases.

From the ten SnRK2s of Arabidopsis SnRK2.2 (SnRK2D), SnRK2.3 (SnRK2I) and OST1 (SnRK2.6/SnRK2E) are specifically activated by ABA (Boudsocq *et al*., 2004). The SnRK2.2 and SnRK2.3 kinases share high sequence similarity with OST1. While the single *snrk2.2* and *snrk2.3* mutants do not present altered ABA response compared to the wild type, the *snrk2.2snrk2.3* double mutant shows ABA insensitivity in germination, dormancy and seedling growth assays (Fujii *et al*., 2007). *OST1* is highly expressed in guard cells and *ost1* has a clear wilty phenotype under low humidity conditions; however expression data show that this gene is also expressed in other tissues (Mustilli *et al*., 2002). *SnRK2.2* and *SnRK2.3* present an overlapping expression pattern (Fujii *et al*., 2007). Interestingly, the triple mutant *snrk2.2snrk2.3snrk2.6* showed a dramatic ABA-insensitive phenotype, which was stronger that than the single or the double mutants. The triple mutant was affected in all plant responses regulated by ABA, such as stomatal closure, germination, ABA regulation of gene expression, shoot and root growth and water loss (Fujii and Zhu, 2009; Fujita *et al*., 2009). Water loss was little affected in the *snrk2.2snrk2.3* double mutant compared to the wild type and ABA-sensitivity of *ost1* in seed germination and seedling growth assays was similar to the wild type. The additive phenotype of the triple mutant shows that these three kinases are involved in all plant responses to ABA. Moreover, the phenotype of *ost1* and *snrk2.2snrk2.3* shows that each one has a dominant role in some of these responses. ABA-dependent phosphorylation mediated by SnRK2.2, SnRK2.3, and OST1 is essential for ABA signalling. *snrk2.2snrk2.3snrk2.6* plants were impaired in growth and reproduction. This phenotype contributes to confirm the suggested role of ABA in plant growth and reproduction (Barrero *et al*., 2005; Cheng *et al*., 2002). Whereas the SnRK2s were differentially activated by NaCl, Mannitol and ABA, all of them, except SnRK2.9, were activated by osmotic stress (Boudsocq *et al*., 2004). Thus, the generation of a decuple mutant comprising the ten SnRK2s (*snrk2.1/2/3/4/5/6/7/8/9/10*) has proved the essential role of these proteins in this process (Fujii *et al*., 2011). This mutant is very hypersensitive to osmotic stress showing reduced fresh weight, root growth, less ABA accumulation, reduced expression of genes activated under these conditions and less proline accumulation than the wild type. Interestingly, the IP3 levels (whose accumulation is induced by osmotic stress but not by ABA (Takahashi *et al*., 2001)) in the decuple mutant were not increased under osmotic stress reflecting that these kinases also participate in this response independently of ABA. The analysis of a septuple mutant affected in the SnRK2s that are not strongly activated by ABA (*snrk2.1/4/5/7/8/9/10*) suggests a singular role of these proteins in ABA-dependent proline accumulation. Whereas the decuple mutant and *snrk2.2snrk2.3snrk2.6* mutant accumulated less proline than the wild type, the septuple mutant showed an increase in the amount of this compound,

suggesting a negative role in ABA dependent proline accumulation. Further experiments are required to elucidate the specific role of these proteins in the pathways that regulate the osmotic stress response.

Several targets of the SnRK2 kinases have been isolated. The regulation of stomatal aperture by OST1 is due partially to the regulation of two ions channels, SLAC1 and KAT1. OST1-mediated phosphorylation activates the slow anion channel SLAC1, whereas it inactivates the inward-rectifying potassium channel KAT1, promoting loss of turgor and stomatal closure (Geiger *et al*., 2009; Lee *et al*., 2009; Sato *et al*., 2009). ROS production is involved in ABA-dependent stomatal regulation (Schroeder *et al*., 2001) and Sirichandra *et al*. (2009) have described that OST1 interacts and phosphorylates the plasma membrane NADPH oxidase AtrbohF. Finally, the isolation of transcription factors (TFs) activated by phosphorylation by the SnRK2s has allowed to describe the cascade of events that result in the modification of gene expression after ABA perception (Choi *et al*., 2000; Uno *et al*., 2000). The ABRE-responsive elements binding (AREB) or ABRE-binding factors (ABFs) belong to the group-A subfamily basic-domain leucine zipper (bZIP) TFs, which comprises nine homologues in Arabidopsis. These proteins are able to activate the expression of gene promoters containing ABA-responsive DNA elements (ABRE; PyACGTGG/TC) such as LEA genes, PP2Cs, MYB transcription factor genes, etc (Yoshida *et al*., 2010). Several works have revealed that the ABA-dependent phosphorylation of some AREB/ABFs by the SnRK2s regulates its activation (Fujii *et al*., 2007; Furihata *et al*., 2006; Kobayashi *et al*., 2005). In vegetative tissues, *ABF2/ AREB1*, *ABF3* and *ABF4/ AREB2* are induced by dehydration, high salinity, or ABA treatment and its expression patterns are highly overlapping (Fujita *et al*.,2005; Kang *et al*., 2002). The triple mutant *abf2abf3abf4* shows clear insensitivity to ABA inhibition of root grow, reduced drought tolerance and decreased expression of ABA-mediated responsive genes, suggesting a pivotal role of these proteins in the ABRE-dependent gene expression under conditions of water stress during the vegetative stage (Yoshida *et al*., 2010). In addition, the expression level of *ABI5*, another AREB/ABFs of the group-A subfamily, is severely reduced in the *snrk2.2snrk2.3snrk2.6* triple mutant at different stages suggesting that these kinases also regulate ABI5 (Nakashima *et al*., 2009). *ABI5* expression is the most abundant in dry seeds, decreases during postgermination development and is induced by drought during vegetative development. Finally, the isolation of these transcription factors has allowed reproducing ABA signaling cascade from the perception until the modulation of gene expression (Fujii *et al*., 2009). In protoplast transactivation assays or alternatively in *in vitro* assays, in the presence of ABA, the PYR/PYL/RCAR receptors where able to inactivate the PP2Cs and prevent the PP2C-mediated dephosphorylation of the SnRK2s. As a consequence the activation of a reporter gene under the control of the promoter of an ABA-responsive gene (RD29B) or alternatively, the phosphorylation of ABF2

was significantly recovered compared with the same experiments in the absence of the receptor. Moreover, these results are in agreements with the constitutive activation of the SnRK2.2, SnRK2.3 and SnRK2.6 kinases in the *abi1-2hab1-1pp2ca* triple mutant and with previous results obtained with the *pyr1pyl1pyl2pyl4* quadruple mutant (Park *et al*., 2009). Although the ABA signaling pathway has been considered complex and regulated by multiple factors this work demonstrates the existence of core elements able to complete the ABA regulation of gene expression.

SnRK2 kinases present the catalytic domain typical of eukaryotic Ser-Thr kinases at the N-terminal part of the protein and a regulatory domain at the C-terminal (Figure 1.5). The regulatory domain can be divided in two subdomains; the DI or SnRK-box and the DII or ABA-box. The SnRK-box is conserved in all the SnRK2s proteins, is necessary for the kinase activity and is responsible of its activation in response to osmotic stress independently of ABA. The ABA-box is responsible of the activation of the kinase in response to ABA but does not affect kinase activity (Ng *et al*., 2011; Yunta *et al*., 2011). This domain participates in the interaction with the PP2Cs and classifies the family on two subgroups according to the ability to be activated by this hormone. The mechanism by which these kinases are activated has been subject of controversy. Recombinant SnRK2.2, SnRK2.3 and SnRK2.6 are able to be activated by autophosphorylation of S175 (OST1) *in vitro* in *cis* and in *trans* and it has been suggested that these kinases are activated by default unless a PP2C blocks its activity (Fujii *et al*., 2009; Ng *et al*., 2011). However OST1 autophosphorylation is 5-10 fold greater than in the case of SnRK2.2 and SnRK2.3 suggesting that upstream kinases could also phosphorylate more efficiently the SnRK2s and activate them. Unfortunately no kinase has yet been isolated as an upstream kinase of the SnRK2s. Recently structural data have revealed the structure of these proteins and have described the molecular interactions that trigger their inactivation by the PP2Cs (Figure 1.6).

The catalytic domain is a canonical kinase fold with the N- and C-lobe linked by a catalytic cleft that contains the ATP and substrate binding sites. The junction between the two lobes is flexible and allows the transition between the active (closed) and the inactive (open) conformation (Soon *et al*., 2012). SnRK2 inhibition is caused by both biochemical and physical inactivation of the phosphorylation activity. Kinase activity is reduced by the dephosphorylation of the S175 in the activation loop (Yoshida *et al*., 2006a) and, at the same time, the W385 of the phosphatase is positioned in the catalytic cleft of the kinase physically blocking the access of the substrate to the active site. The surface of interaction of the PP2Cs with the SnRK2s overlaps with the one established with the PYR/PYL/RCAR receptors and reveals a similar system of recognition that prevents these two families of proteins from interacting with the PP2Cs simultaneously. Despite the importance of the

ABA-box in the regulation of the SnRK2s through the ABA pathway, all the structural data published to date lack this domain.

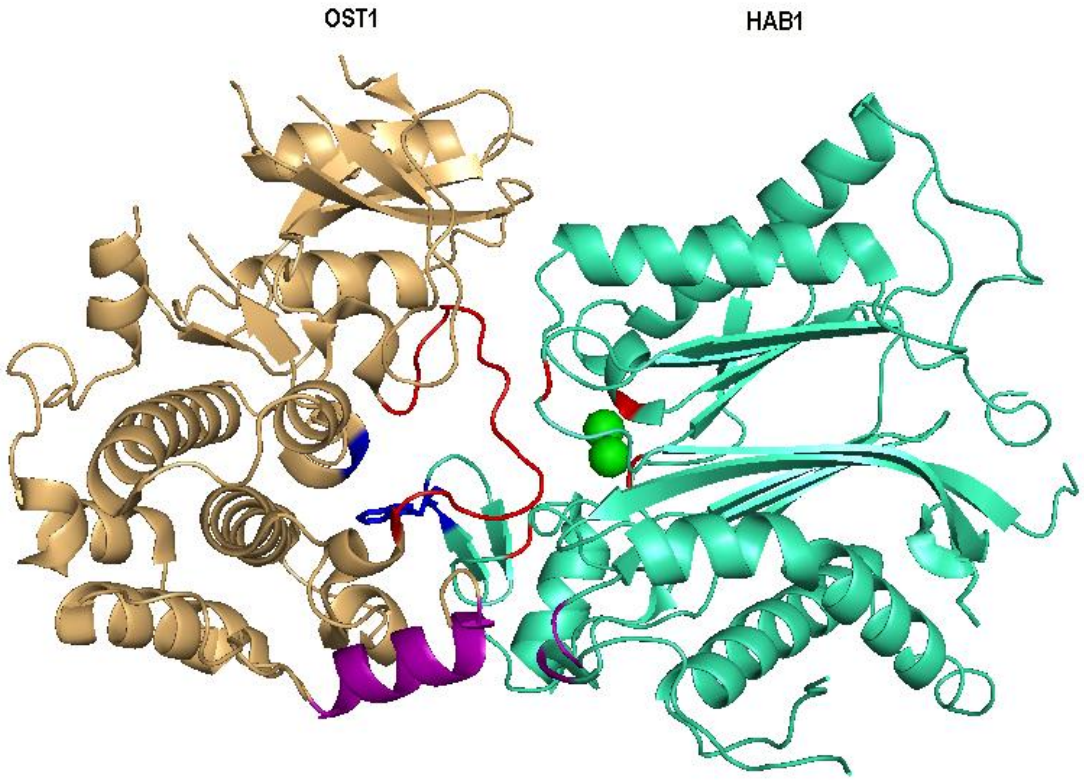


Figure 1.6. Three regions of interaction between the PP2Cs and the catalytic domain of the SnRK2s can be observed. In red, the catalytic cleft of the PP2Cs establishes contact with the activation loop of the kinase and desphosphorilates the S175. In blue, the second region of interaction comprises the Trp-385 located at the flap subdomain of the phosphatase and the catalytic cleft of the kinase. As a consequence the catalytic activity of the kinase is blocked by the incapability of the substrate to reach the kinase active site. In purple, the third region of interaction includes the αG in the kinase and the region adjacent to the Trp-385 in the PP2C.

The ABA-box is located at the C-terminus of the kinase (331-362 in OST1) and is necessary for the ABA-dependent activation of the SnRK2s. Deletion of this domain blocks the inactivation of the kinases by ABA but doesn't blocks its activation by osmotic stress or its catalytic activity. BiFC and Y2H assays using deletion forms of OST1 mapped the region of the interaction with ABI1 in this domain confirming that there is another interaction point between these two proteins apart from the contacts established in the kinase domain of the SnRK2s (Vlad *et al*., 2009; Yoshida *et al*., 2006a).

Moreover, the deletion of this domain abolishes the ABA-dependent activation of these kinases in *in gel* kinase assays (Yoshida *et al*., 2006a). Based on these results, some groups have speculated that this domain could serve to maintain in close proximity SnRK2s and PP2Cs in the presence of ABA, so that after a decrease of the ABA concentration phosphatases could inactivate the kinases very rapidly. According to this hypothesis, it could be possible to isolate *in vivo* complexes formed by ABA-PYL/PYL/RCARs-PP2Cs-SnRK2s. However, to date, no quaternary complex has been isolated. Using YFP-ABI1, Yoshida *et al*. (2006a) performed affinity column-based protein complex purifications from Arabidopsis plants to isolate ABI1-interacting proteins. Between the proteins isolated were found several PYR/PYL/RCAR receptors and SnRK2s. The authors also performed co-inmunoprecipitation experiments and found that YFP-PYR1 co-inmunoprecipitaded with HA-SnRK2.3 in an ABA-independent manner. Since the direct interaction between SnRK2s and PYR/PYL/RCAR has not been described, this result could be explained by the involvement of PP2Cs in this interaction. However, in the presence of ABA, the observed interaction is not consistent with the molecular mechanisms described so far, where the interaction of SnRK2s with PP2Cs is blocked by the receptors in a competitive way. In this direction, Antoni *et al*., (2013) have isolated PP2Cs but none of the ABA-activated SnRK2s in TAP experiments (Tandem Affinity Purification) using PYL8 as a bait. It is possible that the binding to the PP2C only through the ABA-box is not sufficient to recover the kinase in *in vitro* assays. Thus, further approaches would be required to demonstrate if the ABA-box could participate as a domain in charge of keeping in close proximity kinases and phosphatases, or alternatively is in charge of stabilizing the binding of these two families of proteins only in the absence of ABA.

1.3 MECHANISM OF ACTION OF THE ABSCISIC ACID AGONIST PYRABACTIN

The discovery of the PYR/PYL/RCAR proteins by Park *et al*. (2009) was possible through the use of a new agonist of abscisic acid, pyrabactin. This compound was isolated from a chemical library by its capacity to inhibit germination in wild type plants and subsequently, *pyr1-1* was isolated as mutant insensitive to the effect of pyrabactin. Afterwards, biochemical and structural data revealed that the activity of pyrabactin was related to its capacity to promote PP2C inhibition by the PYR/PYL/RCAR proteins. Single loss of function mutants impaired in PYR/PYL/RCAR receptors do not show a clear ABA-insensitive phenotype in germination assays because of functional redundancy.

Pyrabactin, acting as an ABA agonist only for a subset of receptors, was a critical tool to bypass genetic redundancy and isolate PYR1 (Figure 1.7, C). A mutant impaired in PYR1 shows a pyrabactin-resistant phenotype because PYR1 mediates most of the pyrabactin-induced germination inhibition. However, this mutant does not show an ABA-insensitive phenotype because other receptors, such as PYL4, can perceive ABA and inhibit germination.

While for PYR1, PYL1, PYL5, PYL6, PYL9-12 and to lesser extent for PYL8, pyrabactin acts as an ABA agonist, for other receptors pyrabactin is not an agonist (Yuan *et al*., 2010). The study of the structures of PYR1 and PYL1 in complex with ABA or pyrabactin have confirmed that pyrabactin activity is due to its capacity to enter into receptor cavity and trigger a closed conformation that allows the interaction with the PP2Cs (Hao *et al*., 2010; Melcher *et al*., 2010; Peterson *et al*., 2010). Pyrabactin doesn't shares chemical similarity with ABA but in the PYR/PYL/RCAR hydrophobic cavity adopts a U-shaped conformation that directs the different groups of the molecule in a similar way as ABA does (Figure 1.7, A and B).

The two polar modules of pyrabactin (the pyridil nitrogen and the sulfonamide group) are oriented to the bottom of the receptor pocket in a similar manner to the carboxylate and the hydroxyl polar groups of ABA. Similarly to ABA, the two hydrophobic modules of pyrabactin are located close to the gating loops of the receptor. The ciclohexene group in ABA is located in close proximity to the gate loop of the receptor and stabilizes the closed conformation trough hydrophobic and polar interactions. The bromonaphthalene ring in pyrabacting is orientated in the same way indicating that this group performs the same function. Finally the methyl group of ABA and the pyridine ring in pyrabactin are also positioned in similar orientation and are coordinated by hydrophobic residues in the receptor (Hao *et al*., 2010; Peterson *et al*., 2010).

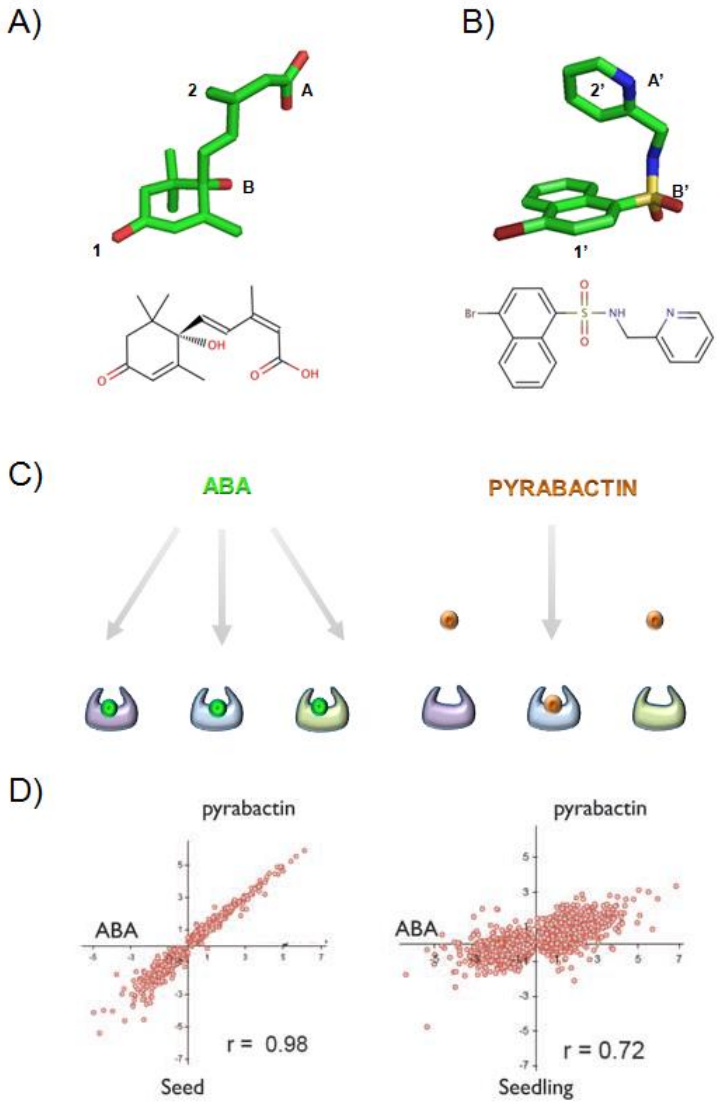


Figure 1.7. Pyrabactin acts as a seed selective agonist of ABA. Chemical structure of ABA (A) and pyrabactin (B). Although these two molecules share no apparent chemical or structural similarity their polar and hydrophobic groups adopt a similar location in the cavity of PYL1 or PYR1 producing a conformational change in the receptor that makes it capable of interacting with the PP2Cs. Polar regions are indicated by letters and hydrophobic regions by numbers. Pyrabactin regions performing the equivalent function in ABA are indicated with apostrophes. C) Schematic representation of pyrabactin mode of action. Pyrabactin acts as an ABA agonist only for a subset of PYR/PYL/RCAR receptors. As a consequence the *pyr1* mutant does not present an ABA-insensitive phenotype but is pyracatin-insensitive in the pyrabactin-mediated inhibition of germination. D) Microarray experiments on pyrabactin and ABA treated seeds and seedlings. In seeds, both compounds induce highly correlated transcriptional responses. Conversely, in seedlings ABA and pyrabactin responses show poorer correlation and few ABA-responsive genes significantly respond to pyrabactin (From Park *et al*., 2009).

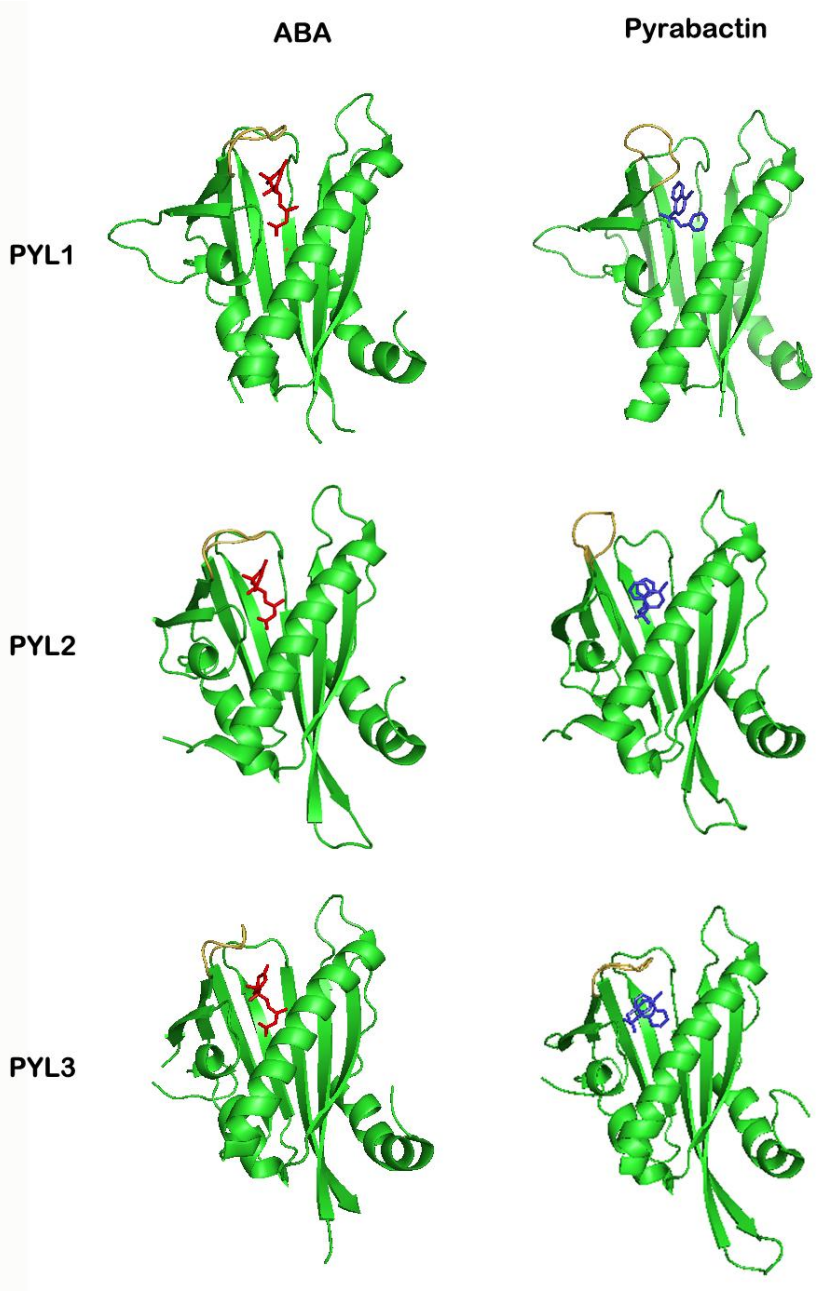


Figure 1.8. Different pyrabactin selectivity by PYR/PYL/RCAR proteins. Pyrabactin (in blue) functional groups share similar spatial geometry to ABA (in red) inside the PYL1 cavity. In the PYL2 cavity pyrabactin is rotated 90º compared to PYL1. As a consequence the receptor remains in its open conformation blocking the inhibition of the PP2Cs. In PYL3 pyrabactin is also differently rotated and adopts a more compacted conformation. As a result the gate loop (in yellow) enters deeply into the cavity resulting in an inadequate surface for the interaction with the PP2Cs.

Although the PYR/PYL/RCAR proteins share a high degree of sequence similarity, pyrabactin doesn't acts as an ABA agonist for some of them. Structural data have revealed the features of the interactions with PYL2 and PYL3, where a non-productive binding results in the incapacity of these receptors to inhibit the PP2Cs (Figure 1.8). In the case of PYL2 pyrabactin is rotated 90º compared to its location in the PYL1 cavity. This is translated in a different location of the bromonaphthalene ring that prevents its interaction with the gate loop and thus the existence of the receptor in its closed conformation (Peterson *et al*., 2010). In the case of PYL3 the gate loop adopts a closed conformation but remains deeper in the cavity respect to other PYR/PYL/RCARs like PYL1 or PYR1. As consequence the surface of interaction with the PP2C is disrupted even though the receptor presents a closed conformation (Zhang *et al*., 2012). Comparison of the sequences in different receptors has helped to isolate those residues implicated in the selectivity for pyrabactin of some receptors. Surprisingly in the case of PYL2, the replacement of a single residue by the corresponding residue in PYL1 (V114I) makes the protein able to inhibit ABI1 and shows that the specific characteristics of each receptor are due to subtle differences in the primary sequence (Yuan *et al*., 2010).

2. OBJECTIVES

2. OBJECTIVES

1. Physiological and biochemical characterization of the HAI1, PP2CA and AHG1, clade A PP2Cs. Role in ABA signaling and regulation by PYR/PYLs ABA receptors.

2. *In vitro* and *in vivo* analyses of key residues for the interaction between PYR1 and HAB1. Study of the biological relevance of HAB1 Trp-385 residue in transgenic plants.

3. Role of PYR/PYLs and PP2Cs in the root hydrotropic response.

4. Characterization of the expression pattern of PYR/PYL/RCARs in root.

5. Screening of ABA agonists through a biochemical genetic approach.

3. Results: Chapter 1

Modulation of abscisic acid signaling *in vivo* by an engineered receptor-insensitive protein phosphatase type 2C Allele

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3.1 Abstract

The plant hormone abscisic acid (ABA) plays a crucial role in the control of the stress response and the regulation of plant growth and development. ABA binding to PYR/PYL/RCAR intracellular receptors leads to inhibition of key negative regulators of ABA signaling, i.e. clade A protein phosphatases type 2C (PP2Cs) such as ABI1 and HAB1, causing the activation of the ABA signaling pathway. In order to gain further understanding on the mechanism of hormone perception, PP2C inhibition and its implications for ABA signaling, we have performed a structural and functional analysis of the PYR1-ABA-HAB1 complex. Based on structural data, we generated a gain-of-function mutation in a critical residue of the phosphatase, hab1^{W385A}, which abolished ABA-dependent receptor-mediated PP2C inhibition without impairing basal PP2C activity. As a result, hab1^{W385A} caused constitutive inactivation of the protein kinase OST1 even in the presence of ABA and PYR/PYL proteins, in contrast to the receptor-sensitive HAB1, and therefore hab1^{W385A} qualifies as a hypermorphic mutation. Expression of *hab1*^{W385A} in *Arabidopsis thaliana* plants leads to a strong, dominant ABA-insensitivity, which demonstrates that this conserved Trp residue can be targeted for the generation of dominant clade A PP2C alleles. Moreover, our data highlight the critical role of molecular interactions mediated by Trp385 equivalent residues for clade A PP2C function *in vivo* and the mechanism of ABA perception and signaling.

3.2 Introduction

Abscisic acid (ABA) is required for biotic and abiotic stress responses as well as the control of plant growth and development. Plant growth can be severely impaired by adverse environmental conditions like drought, salinity, cold or high temperature, which can reduce average productivity of crops by 50% to 80% (Bray *et al.,* 2000). ABA plays a key role in orchestrating the adaptive response of the plant to cope with these forms of abiotic stress (Cutler *et al.,* 2010; Verslues *et al.,* 2006). Under drought stress, cleavage of ABA from ABA conjugates stored in the vacuole or apoplastic space (Lee *et al.,* 2006) as well as *de novo* ABA biosynthesis (Nambara and Marion-Poll, 2005) are stimulated, leading to a sharp increase in the cellular concentration of the hormone. This elicits a plant response that limits water loss and, under prolonged stress, the hormone response adapts plant metabolism to the low water potential of the cellular environment.

A large number of cellular components have been implicated in the ABA signaling pathway (Hirayama and Shinozaki, 2007). However, recently it has become clear that just three types of proteins constitute the so-called "core ABA pathway" (Cutler *et al.,* 2010). These include the family of PYR/PYL/RCAR ABA receptors, the clade A of protein phosphatases type 2C (PP2Cs) and three ABA-activated protein kinases from the sucrose non-fermenting1-related subfamily 2 (SnRK2) (Cutler *et al.,* 2010). Under non-stress conditions clade A PP2Cs can interact with and dephosphorylate three SnRK2s, i.e. 2.2, 2.3 and 2.6/OST1, reducing their catalytic activity (Umezawa *et al.,* 2009; Vlad *et al.,* 2009). The increase of ABA levels in the plant cell leads to the PYR/PYL/RCAR receptor-mediated inhibition of the PP2C activity which results in the activation of the three SnRK2s and ultimately of the ABA signaling pathway (Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.,* 2009; Vlad *et al.,* 2009). Upon activation, the SnRK2s directly phosphorylate transcription factors that bind to ABA-responsive promoter elements (ABREs), named ABFs/AREBs for ABRE-binding factors, and components of the machinery regulating stomatal aperture like the anion channel SLAC1 (Fujii *et al.,* 2009; Fujita *et al.,* 2009; Geiger *et al.*, 2009; Lee *et al.,* 2009).

To date, three receptors, i.e. PYR1, PYL1 and PYL2, and two receptor-ABA-phosphatase complexes, i.e. PYL1-ABI1 and PYL2-HAB1, have been studied at a structural level, which has contributed to the understanding of the molecular interactions between receptor, hormone and phosphatase (Melcher *et al.,* 2009; Miyazono *et al.,* 2009; Nishimura *et al.,* 2009; Santiago *et al.*, 2009a; Yin *et al.,* 2009). The PYR/PYL/RCAR proteins belong to the super-family of START/Bet v proteins, whose members are widespread in eukaryotes and are characterized by the presence of a cavity able to accommodate hydrophobic ligands (Iyer *et al.,* 2001; Radauer *et al.,* 2008). This cavity represents the hormone-binding pocket and is flanked by two flexible loops (b3-b4 and b5-

b6), the so-called gating loops, which close over the hormone once inside the binding pocket. In the two structures available from ternary complexes, the ABA-bound receptor contacts the PP2C through the gating loops that cover the ABA-binding pocket. Thus, the side-chains of Ser112 of PYL1 and the Ser89 of PYL2, located in the b3-b4 loop, insert into the PP2C active site and presumably occlude the access of the substrates (Melcher *et al.,* 2009; Miyazono *et al.,* 2009; Yin *et al.,* 2009). These conserved Ser residues establish contacts with Gly180 of ABI1 or Gly246 of HAB1, next to the PP2C active site, and the metal-coordinating residue Glu142 of ABI1 or Glu203 of HAB1, respectively. Another important feature of the ternary complex, involves a key water-mediated interaction between the ABA´s ketone group and the Trp300 or Trp385 residue of ABI1 or HAB1, respectively. Indeed, this is the only residue of the PP2C approaching the ABA molecule and accordingly, this interaction has been postulated to play a key role in the stabilization of the whole ternary complex, contributing to the higher ABA affinity measured for PYR/PYL/RCAR receptors in the presence of the PP2Cs (Ma *et al.*, 2009; Santiago *et al.*, 2009b). However, beyond the structural data, no *in planta* evidence has been provided for its direct role in ABA signaling. Moreover, the ternary complexes analyzed at a structural level have not included PYR1, which plays a predominant role in germination (Park *et al.,* 2009).

Plants harbouring *abi1*^{G180D}, *abi2*^{G180D} and *hab1*^{G246D} dominant mutations have represented valuable tools to dissect ABA signaling (Leung *et al.,* 1994; Leung *et al.,* 1997; Meyer *et al.,* 1994; Robert *et al.,* 2006; Rodriguez *et al.,* 1998). Their ABA-insensitive phenotypes are in agreement with a reduced capacity of the mutant PP2Cs to interact with PYR/PYL/RCAR receptors (Park *et al.,* 2009; Santiago *et al.,* 2009b; Umezawa *et al.,* 2009). In spite of their utility, these alleles bear mutations close to the phosphatase catalytic site and have reduced basal PP2C activity (Bertauche *et al.,* 1996; Leube *et al.,* 1998; Leung *et al.,* 1997; Robert et al., 2006) Rodriguez *et al.,* 1998), which has complicated the interpretation of their *in vivo* phenotypes. Mutations in the conserved Trp residue described above have not been isolated by forward genetic screens, or engineered in Arabidopsis plants, and the functional relevance of this residue has been documented uniquely on *in vitro* studies for the case of ABI1 (Miyazono *et al.,* 2009). Since mutations in the Trp residue are expected to affect the stability of the ternary complex without compromising the phosphatase catalytic activity, they represent an ideal tool for studying *in planta* the effect of de-coupling the receptor and phosphatase interaction.

Here we present a combined structural and functional analysis of the ternary complex formed by PYR1-ABA-HAB1. We analyzed the effect of PYR1-HAB1 mutations on OST1 kinase activity *in vitro,* since this SnRK2 is a key target of HAB1 (Vlad *et al.,* 2009). We also performed *in planta* analysis of a *hab1*^{W385A} mutation that de-couples receptor and phosphatase interaction

without impairing PP2C activity. These transgenic plants show an acute ABA-insensitivity demonstrating the importance of ABA-mediated PYR/PYL/RCAR-PP2C contacts for receptor function *in vivo*, and enabling a new method for probing PP2C function with dominant receptor-insensitive mutations.

3.3 Results

Architecture of the PYR1-ABA-ΔNHAB1 ternary complex

The PYR1 receptor and the catalytic domain of the HAB1 phosphatase (residues 179-511, ΔNHAB1) were separately overexpresed in *E. coli*, purified and mixed in equimolar amounts in the presence of 1 mM (+)-ABA. The resulting complex was assayed for crystallization at the high throughput crystallization facility of the EMBL Grenoble Outstation (https://embl.fr/htxlab) (Dimasi *et al.,* 2007). X-ray diffraction data was collected from orthorhombic crystals at the ID14-4 beam line of the ESRF to 1.8 Å resolution. Initial phases were obtained by the molecular replacement method using the two central β-sheets of the catalytic domain of the human PP2Cα protein (1A6Q) (Das *et al.,* 1996) as a search model. The initial phases provided an easily interpretable electron density map extending outside the search model region. Successive rounds of automatic refinement and manual building resulted in a refined model with a Rwork and Rfree of 17.4% and 21.8 % respectively. In the refined model, the crystallographic asymmetric unit contains one molecule of PYR1 one molecule of ΔNHAB1, one molecule of ABA and three manganese ions (Fig. 3.1 and n3.I).

The structure of PYR1 in the complex is very similar to that of the ABA-bound subunit in dimeric PYR1 (Nishimura *et al.,* 2009; Santiago *et al.,* 2009a). The ABA molecule is located in the receptor cavity stabilized by both polar and hydrophobic interactions and the gating loops are in the closed conformation, as described previously (Nishimura *et al.,* 2009; Santiago *et al.,* 2009a) (Fig. 3.1). Subtle differences between the two PYR1 structures likely induced by interaction with HAB1 are found around Ser85 in one of the gating loops, and the loop β7/α5, adjacent to the gating loops (Fig. 3.1, B and C). The structure of the HAB1 catalytic domain is similar to those of Arabidopsis ABI1 (Melcher *et al.,* 2009; Miyazono *et al.,* 2009; Yin *et al.,* 2009) and the human PP2Cα protein phosphatase (Das *et al.,* 1996). It is formed by a central 10-strand antiparallel β-sandwich flanked by two long α-helices at each side. A 55 amino acid α/β domain, which has been named the flap sub-domain in some bacterial PP2Cs (Schlicker *et al.,* 2008) is inserted between strands β8 and β12 of HAB1. This sub-domain contains the HAB1 Trp385 (Fig. 3.1A), which is highly conserved in

plant clade A PP2Cs. Small conformational differences between the three phosphatases are found at the β2-β3 and α1-α2 loop regions of HAB1. In addition to this, HAB1 displays a 16 amino acid insertion at the α3/β4 loop not found in ABI1 and the human PP2Cα (Supplemental Fig. S1).

The catalytic site of HAB1 is located inside a deep channel formed at the top of the β-sandwich and flanked by the flap sub-domain (Fig. 3.2; Supplemental Fig. S2). In our structure, the catalytic site of HAB1 contains three metal ions designated here as M1, M2 and M3 according to Alzari and co-workers (Wehenkel *et al.*, 2007) (Fig. 3.2). While some protein phosphatases contain two metal ions at the catalytic site, a few bacterial phosphatases have been shown to display a third conserved metal ion site, M3 (Pullen *et al.,* 2004; Schlicker *et al.,* 2008; Wehenkel *et al.,* 2007). The M3 site, is located at the exit of the catalytic channel and is typically coordinated by one conserved aspartic residue also involved in coordination of the metal at M1 (Asp432 for HAB1), and one residue from the flap domain. In some bacterial PP2Cs coordination of the third metal ion at M3 has been correlated with a change in position of the flap sub-domain (Wehenkel *et al.*, 2007), however, this site displays low metal binding affinity and has been shown to be dispensable for catalysis (Wehenkel *et al.*, 2007). To our knowledge, HAB1 is the first eukaryotic PP2C with three metal sites.

Molecular interactions stabilizing the PYR1-ABA-HAB1 complex

The PYR1-HAB1 interface comprises a total protein buried surface area of 1691 $Å^2$. As in the case of the PYL2-HAB1 and PYL1-ABI1 structures (Melcher *et al.,* 2009; Miyazono *et al.,* 2009; Yin *et al.,* 2009), HAB1 docks into the ABA-bound receptor establishing interactions with the gating loops (loops β3/β4 and β5/β6), the N-terminal part of the α5 helix and the α4/β2 loop of PYR1 (Fig. 3.1, A-C). The HAB1 residues involved in those interactions are located both in the flap sub-domain including Trp385 and the phosphatase active site including the β1/β2 , β3/α1 and α2/β4 loops (Fig. 3.1, A-C; Fig. 3.2; Supplemental Fig. S3). The HAB1 Trp385 residue is inserted between the PYR1 gating loops with the nitrogen in the indole group establishing a hydrogen bond with the water located at the channel between the gating loops (Fig. 3.1B). This water molecule represents a critical point in the ternary complex, establishing hydrogen bonds not only with HAB1 Trp385 but also with the receptor gating loops (with the backbone carbonyl and amine groups of Pro88 and Arg116 respectively) and with the hormone itself, through its ketone group. Comparison of the present structure with the previously reported structures of isolated PYR1 reveals a conformational rearrangement in the β7/α5 loop of PYR1 upon binding to HAB1. This loop moves forward towards the flap domain of HAB1 (Fig. 3.1B), establishing new interactions that stabilize both the closed conformation of the gating loops and the receptor-phosphatase complex. These

interactions involve Asn151 of PYR1, which is hydrogen bonded to both the carbonyl group of HAB1 Gln384 in the flap domain and PYR1 Arg116, located in one of the gating loops. At the same time, in the present structure the side chain of PYR1 Ser152 is involved in a helix capping interaction (Presta and Rose 1988) that stabilizes the forward movement of the β7/α5 loop.

Another important interaction region involves the PYR1 β3/β4 loop containing Ser85 and the catalytic site of the phosphatase (Fig. 3.1C). PYR1 Ser85 takes part in a hydrogen bond network with the backbone amine of Gly246 and the carboxylic group of Glu203 at the catalytic site of HAB1. This interaction is likely to be responsible for the inhibition of the phosphatase activity, as the β3/β4 loop containing Ser85 seems to block access to the phosphatase catalytic site (Fig. 3.2). The structure of the human PP2Cα contains a phosphate ion at the catalytic site, which is likely mimicking the position of the phosphorylated amino acid substrate (Das *et al.*, 1996). Interestingly, when PP2Cα and HAB1 catalytic cores are superimposed the phosphate ion of human PP2Cα is 2.9 Å away from the Cβ carbon of Ser 85 of PYR1 (Fig. 3.2; Supplemental Fig. S4), which suggests that a phosphoserine substrate might enter the catalytic site in a similar manner.

Mutational analysis of the PYR1-HAB1 interaction and effect on the HAB1-dependent inhibition of OST1 activity

To test the biological relevance of the interactions observed in the PYR1-HAB1 complex, we analyzed the effect of a number of single point mutations on both proteins. In the case of PYR1, we mutated key amino acid residues involved in either direct ABA-binding (Glu94Lys, Glu141Lys and Tyr120Ala) or both ABA-binding and PP2C interaction, particularly residues located in the gating loops (Ser85Ala, Leu87Ala, Pro88Ser, Arg116Ala) and the loop β7-α5 (Ser152Leu). For HAB1 we chose the Gly246Asp mutation, equivalent to *abi1-1D* and *abi2-1D* mutations, since expression of *hab1*^{G246D} *in planta* leads to a dominant ABA-insensitive phenotype (Robert *et al.*, 2006) and Trp385Ala, due to its critical interactions with the PYR1 gating loops and ABA. For each PYR1 mutant we first tested both its capacity to interact with HAB1 and inhibit its activity through yeast two hybrid (Y2H) interaction and *in vitro* phosphatase activity assays, respectively (Fig. 3.3, A and B; Supplemental Fig. S5). In general, the PYR1 mutations abolished or severely reduced the ABA-mediated interaction and the inhibition of HAB1 phosphatase activity as compared to the wt. An exception is the PYR1^{R157H} variant. Although this mutation confers resistance to pyrabactin, a seed ABA-agonist (Park *et al.*, 2009), it shows very limited effect in both the Y2H and phosphatase activity assays.

In vitro reconstitution of an ABA signaling cascade can be achieved by combining PYR1, PP2C, SnRK2.6/OST1 and ABF2 in a test tube (Fujii *et al.,* 2009). In this system, OST1 activity is measured as auto-phosphorylation as well as trans-phosphorylation of its natural substrate ABF2. We used this assay to determine how the different mutations affect the control of the OST1 activity. Figure 3.3 shows that HAB1 dephosphorylates OST1 and inhibits its kinase activity (lanes 1 and 2, Fig. 3.3, C and D). However, if ABA and PYR1 are added, HAB1 is inactivated, and consequently a significant recovery of OST1 activity is observed (lane 5, Fig. 3.3, C and D). All the PYR1 mutants assayed, except R157H, showed a strongly decreased capacity to antagonize the HAB1-mediated dephosphorylation of OST1 and were unable to promote ABA-dependent recovery of the OST1 protein kinase activity.

Both HAB1 Trp385Ala and Gly246Asp mutations abolished the ABA-dependent interaction between HAB1 and PYR1, as revealed by the Y2H and *in vitro* phosphatase activity assays (Fig. 3.4, A and B). In agreement with these results and in contrast to wild type HAB1, both mutant PP2Cs were able to dephosphorylate OST1 in the presence of ABA and PYR1 (Fig. 3.4C). Thus, both mutant PP2Cs were refractory to inhibition by PYR1 under these experimental conditions. This result indicates that both hab1^{W385A} and hab1^{G246D} qualify as hypermorphic mutants compared to wild type HAB1 in the presence of ABA and PYR1 (Wilkie, 1994). However, the basal dephosphorylation of OST1 by hab1^{G246D} was less-effective than wild type in the absence of ABA and PYR1(Vlad, *et al.,* 2009; this work), which can be explained because this mutation is located close to the PP2C active site. Indeed, using p-nitrophenol as substrate, hab1^{G246D} showed 4 times lower specific activity as compared to wt HAB1 (4.86 ± 0.43 and 18.76 ± 2.13 nmoles Pi/min · mg, respectively). Instead, the activity of hab1^{W385A} was similar to wild type both in the pNPP (20.52 ± 2.53 nmoles Pi/min · mg) and the OST1 dephosphorylation assays (Fig. 3.4C).

In summary, the mutational analysis of both PYR1 and HAB1 confirms that the interactions revealed by the structural analysis of the ternary complex are crucial for the inhibition of HAB1 activity. Additionally, these results illustrate that certain mutations in the PP2C lead to escape of the inhibitory ABA-mediated PYR/PYL mechanism. The results obtained for hab1^{G246D} provide additional support to the model proposed by Merlot and co-workers (Vlad *et al.,* 2009) to explain the negative regulation of OST1 activity by HAB1 and the strong ABA-insensitive phenotype of *35S:hab1^{G246D}* plants (Robert *et al.,* 2006), assuming that a general escape from PYR/PYL receptors occurs in these plants. Indeed, we have demonstrated *in vitro* that hab1^{G246D} phosphatase, as well as hab1^{W385A}, are refractory to inhibition by different PYR/PYL proteins (Fig. 3.4D).

Expression of *hab1*^{W385A} in Arabidopsis plants leads to reduced ABA sensitivity

To test the biological relevance of the PYR1-ABA-HAB1 interaction mediated by the residue Trp385 of HAB1, we generated *35S:hab1*^{W385A} transgenic lines and examined their ABA response compared to *35S:HAB1* plants (Fig. 3.5). For this analysis, we selected three *35S:hab1*^{W385A} transgenic lines that showed expression levels of the recombinant protein similar to those of the previously described *35S:HAB1* plants (Saez *et al.*, 2004), as determined by immunoblot analysis against the HA-epitope added to each protein (Fig. 3.5C). Germination and early seedling establishment of *35S:HAB1* and *35S:hab1*^{W385A} seeds were less sensitive to ABA-mediated inhibition than wild type seeds (Fig. 3.5, A and B). Moreover, *35S:hab1*^{W385A} seeds were able to germinate and establish seedlings at 10 μM ABA, which is an inhibitory concentration for establishment of *35S:HAB1* seeds (Fig. 3.5, A and B).

Stomatal closing is a key ABA-controlled process that preserves water under drought conditions. We mimicked drought by exposing plants to the drying atmosphere of a flow laminar hood and under these conditions we measured water-loss in two-week old seedlings (Fig. 3.5, D and E). Both *35S:HAB1* and *35S:hab1*^{W385A} plants showed a higher transpiration rate than wild type, and water-loss in plants over-expressing the mutated phosphatase was higher than in the wild type PP2C. The increased insensitivity to ABA of the *35S:hab1*^{W385A} plants as compared to *35S:HAB1*, is consistent with the inability of the PYRL/PYL/RCAR receptors to inhibit *in vitro* the activity of hab1^{W385A} (Fig. 3.4D). Finally, the expression of ABA-inducible genes was severely reduced in *35S:hab1*^{W385A} plants as compared to the wild type (Fig. 3.5F). The accumulation of these transcripts was also impaired in *35S:HAB1* plants; in some cases, *RAB18*, *RD29B*, the effect was similar to *35S:hab1*^{W385A} plants, however, ABA induction of other transcripts, *KIN1*, *RD29A*, *P5CS* and *RD22*, was less affected (Fig. 3.5F).

3.4 Discussion

The structure of the PYR1-ABA-HAB1 complex presented here and those of the ternary complexes studied previously (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Yin *et al.*, 2009) contribute to explain how ABA binding induces the interaction between receptor and phosphatase and its inhibitory nature on phosphatase activity. Interestingly, these complexes show a 1:1 receptor:phosphatase stoichiometry. Since it has been shown that PYR1 forms a dimer *in vivo* (Nishimura *et al.*, 2009), evidence that is not yet available for PYL1 and PYL2, our data confirm that PYR1 dimer dissociation is required for the formation of the ternary complex, as Yan and co-

workers have suggested (Yin *et al.,* 2009). However, a detailed understanding of the dimer dissociation process is not available yet.

Once the hormone enters the receptor cavity, the cyclic moiety of the ABA molecule establishes interactions with the receptor gating loops, which favours their closed conformation. This closed conformation offers an optimal surface for the docking of the phosphatase, which contributes in turn to the stability of the ternary complex by locking the gating loops in their closed conformation and trapping the hormone inside the binding cavity. For instance, PYL9 and PYL5 bind to ABA with a Kd of 0.70 μM and 1.1 μM, respectively, whereas inclusion of ABI2 and HAB1 in the binding assay leads to a Kd of 64 nM and 38 nM, respectively (Ma *et al.,* 2009; Santiago *et al.,* 2009b). The HAB1 Trp385 residue plays a major role in this stabilization process by inserting between the gating loops, and additionally via an indirect contact with the ABA´s ketone group through a hydrogen bond network mediated by a critical water molecule. This water molecule establishes hydrogen bonds not only with HAB1 Trp385 and the hormone, but also with key residues (Pro88 and Arg116) of the receptor gating loops. This complex network of interactions provides a mechanism through which the phosphatase is able to monitor hormone occupancy of the ABA binding cavity, and therefore ensuring that the conserved Trp residue will only contribute to the stabilization of the receptor-phosphatase complex if the hormone is present. The *in vitro* data presented here for hab1^{W385A} and by Miyazono *et al.,* (2009) for abi1^{W300A} support this conclusion. Moreover, our results show that this hormone sensing mechanism is critical for ABA response *in planta*. Thus, expression of *hab1*^{W385A} in Arabidopsis plants leads to a strong ABA-insensitive phenotype, which can´t be explained solely by enhanced PP2C gene dosage, since *35S:HAB1* plants, although less sensitive to ABA than wt, show milder phenotypes. The reduced sensitivity to ABA-mediated inhibition of seed germination and seedling establishment enhanced water-loss and reduced expression of ABA-responsive genes in *35S:hab1*^{W385A} plants support the relevance of this locking interaction, postulated by structural studies. Additionally, these plants represent a valuable tool to dissect the ABA pathway by using dominant receptor-insensitive PP2C mutants that do not compromise the intrinsic phosphatase activity. Taking into account the large number of screenings performed to identify ABA-insensitive plants, the failure to isolate mutants harbouring missense mutations in this Trp residue is somehow surprising. However, since EMS mutagenesis usually leads to G →A transitions, such mutation in the Trp codon (UGG) would lead to stop codons and presumably loss-of-function alleles. The locking mechanism provided by the Trp residue appears to be a particular evolution of the plant clade A PP2Cs, since with the exception of AHG1, they are the unique plant PP2Cs that present this residue in the appropriated position of the flap PP2C sub-

domain. Interestingly, AHG1 was less-sensitive to ABA-dependent PYL8-mediated inhibition than other clade A PP2Cs, such as PP2CA and At5g59220 (Supplemental Fig. S6).

This work and previous structural analyses indicate that the insertion of the PYR1 Ser85-containing β3-β4 loop (Ser112 of PYL1 and Ser89 of PYL2) into the phosphatase catalytic site could account for the inhibition of PP2C catalytic activity by blocking access of potential substrates to the phosphatase catalytic site in a competitive manner. However, although this mechanism looks plausible, the phosphatase catalytic channel remains open in its lower part in the ternary complexes formed by both HAB1 and ABI1 (Supplemental Fig. S2). This lower part of the phosphatase catalytic groove might represent an alternative entry site for substrates and indeed initial studies based on biochemical assays with a non-peptidic substrate, suggested that inhibition of the PP2C activity by PYR/PYL/RCAR proteins occurs by a non-competitive, rather than competitive mechanism (Ma *et al.*, 2009). In contrast, in other studies the inhibition of HAB1 by ABA-bound PYL2 was overcome by increasing concentrations of an OST1 phosphopeptide containing residues of the kinase activation loop (Melcher *et al.*, 2009). Unfortunately the structure of a PP2C in complex with a natural peptide substrate is lacking, which could contribute to resolve this issue. However, one striking observation arising from the present structural analysis is the proximity of Ser85 in the gating loop of the PYR1 receptor to the position expected to be occupied by the phosphoryl group of the substrate of the phosphatase reaction. Superposition of the present structure and the catalytic domain of human PP2Cα shows that the β-carbon of PYR1 Ser85 is next to the phosphate ion oxygen atom that Barford and co-workers have proposed as the seryl/threonyl oxygen in their analysis of the PP2Cα catalytic site (Das *et al.*, 1996). This would suggest that the PYR1 Ser85, and its equivalent in other PYR/PYL proteins, might act as a product mimic and occupy a similar position as the phosphorylated serine residues in SnRK2s and other PP2C targets. In our view, this important observation lends weight to the interpretation that the formation of the receptor-phosphatase complex prevents access of natural PP2C substrates to the catalytic site, supporting the competitive nature of the inhibition mechanisms. At the same time it would support the catalytic mechanism proposed by Barford (Das *et al.*, 1996), where the water molecule linked to the metal at the M2 site and Glu37 of human PP2Cα (Glu203 in HAB1) would contribute to catalysis by facilitating the protonation of the oxygen atom in the P-O scissile bond.

Since Ser85 of PYR1, Ser112 of PYL1 and Ser89 of PYL2 insert into the PP2C active site and establish contacts with Gly180 of ABI1 or Gly246 of HAB1, the structural data provide a framework to explain the effect of *abi1*^{G180D} and *hab1*^{G246D} mutations. However, no direct biochemical evidence had been previously provided in the case of *hab1*^{G246D}. The present analysis shows that hab1^{G246D} is insensitive to inhibition by various PYR/PYL proteins, which leads to the

escape from the ABA-dependent PYR/PYL inhibitory mechanism and the subsequent constitutive inhibition of OST1 activity. Therefore, these data are in agreement with the notion that hab1^{G246D} behaves as a hypermorphic mutation in the presence of ABA, as noted by Schroeder and co-workers (Robert *et al.,* 2006). Paradoxically, in the absence of ABA, hab1^{G246D} shows lower intrinsic phosphatase activity than wild type HAB1, probably because this mutation perturbs the PP2C active site to some extent.

Even though other ABA receptors have been identified (Pandey *et al.,* 2009, Shang *et al.,* 2010) and therefore other input sources exist for ABA signaling, the phenotypes of both *35S:hab1^{G246D}* and *35S:hab1^{W385A}* plants indicate that constitutive activation of the PP2Cs (and the consequent inactivation of the SnRK2s) leads to a severe blockade of ABA signaling. Therefore, the action of the SnRK2s is likely localized downstream of the other putative inputs and could represent a core ABA signaling component shared by all ABA receptors. This would be in agreement with the extreme ABA insensitivity of triple *snrk2.2/2.3/2.6* mutant plants (Fujii and Zhu 2009).

3.5 Material and methods

Construction of plasmids

Plasmids pETM11 or pET28a were used to generate N-terminal His_6-tagged recombinant proteins. The cloning of *6xHis-ΔNHAB1* (lacking residues 1-178), *PYR1*, *PYL4*, *PYL5* and *PYL8* constructs was previously described (Santiago *et al.,* 2009b). Using a similar approach, *PYL1* and *PYL6* were cloned in pETM11, whereas *PYL9* was cloned in pET28a. *HAB1(W385A)*, *HAB1(G246D)*, *PYR1(S85A)*, *PYR1(R116A)*, *PYR1(L87A)* and *PYR1(Y120A)* mutants were produced using the overlap extension procedure (Ho *et al.,* 1989) and cloned into pETM11. *PYR1(S152L)*, *PYR1(P88S)*, *PYR1(R157H)*, *PYR1(E141K)* and *PYR1(E94K)* mutants were obtained from the *pyr1-2*, *pyr1-3*, *pyr1-4*, *pyr1-5* and *pyr1-6* alleles, respectively (Park *et al.,* 2009) and cloned into pET28a. The coding sequence of *OST1* and a C-terminal deletion of *ABF2* (*ΔCABF2*, amino acids 1-173) were cloned into pET28a.

Protein expression and purification

BL21(DE3) cells transformed with the corresponding constructs in pETM11 or pET28a vectors were grown in LB medium to an OD_{600} of 0.6-0.8. At this point 1 mM IPTG was added and the cells were harvested after overnight incubation at 20ºC. Proteins used for crystallization were purified as described (Santiago *et al.,* 2009a). For small scale protein preparations, the following protocol was used. Pellets were resuspended in lysis buffer (50mM Tris pH 7.5, 250mM KCl, 10%

Glycerol, 1 mM β-mercaptoethanol) and lysed by sonication with a Branson Sonifier 250. The clear lysate obained after centrifugation was purified by Ni-affinity. A washing step was performed using 50mM Tris, 250 mM KCl, 20% Glycerol, 30 mM imidazole and 1mM β-mercaptoethanol washing buffer, and finally the protein was eluted using 50mM Tris, 250 mM KCl, 20% Glycerol, 250mM imidazole and 1mM β-mercaptoethanol elution buffer.

Crystallization and structure solution

The PYR1-ABA-HAB1 ternary complex was prepared by mixing PYR1, ΔNHAB1 and 1mM ABA to a final concentration of 3 mg/ml, 5 mg/ml and 1 mM respectively in 20mM Tris pH7.5, 150mM NaCl, 1mM $MnCl_2$, 1mM βmercaptoethanol. Crystallization conditions for the complex were identified at the High Throughput crystallization Laboratory of EMBL Grenoble Outstation (https://htxlab.embl.fr) as described in (Marquez *et al.,* 2007). The crystals used for data collection were obtained by vapour diffusion method in 0.25M NaCl, 19% Peg 3350 at 20^{o}C. X-ray diffraction data was collected at the ID14-4 beam line of the ESRF to 1.8 Å resolution. Initial phases were obtained by the molecular replacement method using the two central β-sheets of the catalytic domain of the human PP2Cα protein (1A6Q) (Das *et al.,* 1996) as a search model and the program Phaser (McCoy *et al.,* 2007). Successive rounds of automatic refinement and manual building were carried out with RefMac5 (Murshudov *et al.,* 1997) and Coot (Emsley and Cowtan 2004). Atomic coordinates from the final model have been deposited in the Protein Data Bank under accession code 3QN1.

PP2C and OST1 *in vitro* activity assays

Phosphatase activity was measured using either the Ser/Thr Phosphatase assay system (Promega) using the RRA(phosphoT)VA peptide as substrate or pNPP (p-nitrophenyl phosphate). In the first case assays were performed in a 100 µl reaction volume containing 25 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 25 µM peptide substrate and the PP2C. When indicated, PYR-PYL recombinant proteins and ABA were included in the PP2C activity assay. After incubation for 60 min at 30ºC, the reaction was stopped by addition of 30 µl molybdate dye (Baykov *et al.,* 1988) and the absorbance was read at 630 nm with a 96-well plate reader. For the pNPP phosphatase activity assays a 100 µl solution containing 25 mM Tris-HCl pH 7.5, 2 mM $MnCl_2$ and 5mM pNPP substrate and the indicated amount of the PP2Cs was used. Measurements were taken with a ViktorX5 reader at 405nm every 60 seconds over 30 minutes.

Phosphorylation assays were done basically as described previously (Belin *et al.,* 2006; Vlad *et al.*, 2009). Assays to test recovery of OST1 activity were done by previous incubation for 10 min of the protein phosphatase HAB1 together with the PYR1 wt or PYR1 mutant proteins in the presence of the indicated concentration of (+)-ABA. Next, the reaction mixture was incubated for 50 min at room temperature in 30 µl of kinase buffer: 20 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, and 3.5 µCi of γ-^{32}ATP (3000 Ci/mmol). The reaction was stopped by adding Laemmli buffer. When indicated, ΔCABF2 recombinant protein (100 ng) was added as substrate of OST1. After the reaction proteins were separated by SDS-PAGE using an 8% acrylamide gel and transferred to an Immobilon-P membrane (Millipore). Radioactivity was detected using a Phosphorimage system (FLA5100, Fujifilm). After scanning, the same membrane was used for Ponceau staining. The data presented are averages of at least three independent experiments.

Yeast two-hybrid assays

Protocols were similar to those described previously (Saez *et al.*, 2006).

Generation of *35S:hab1*^{W385A} transgenic lines

The mutated *hab1*^{W385A} was cloned into pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the gateway compatible ALLIGATOR2 vector (Bensmihen *et al.,* 2004). This construct drives expression of *hab1*^{W385A} under control of the 35S CaMV promoter and introduces a triple HA epitope at the N-terminus of the protein. Selection of transgenic lines is based on the visualization of GFP in seeds, whose expression is driven by the specific seed promoter At2S3. The *ALLIGATOR2-35S:3HA-hab1*^{W385A} construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere *et al.,* 1985) by electroporation and used to transform Columbia wild type plants by the floral dip method. T1 transgenic seeds were selected based on GFP visualization and sowed in soil to obtain the T2 generation. Homozygous T3 progeny was used for further studies and hab1^{W385A} protein level was verified by immunoblot analysis using anti-HA-peroxidase (Roche). The generation of *35S:HAB1-dHA* lines was described previously (Saez *et al.,* 2004).

Seed germination and seedling establishment assays

After surface sterilization of the seeds, stratification was conducted in the dark at 4ºC for 3 d. Next, approximately 200 seeds per experiment were sowed on solid medium composed of Murashige and Skoog basal salts, 1% sucrose and supplemented with different ABA concentrations. To score seed germination, radical emergence was analysed at 72 h after sowing. Seedling establishment was

scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves at 7 d.

Water loss assays

2-3 weeks-old seedlings growing in MS plates were used. Three seedlings per genotype with similar growth were submitted to the drying atmosphere of a flow laminar hood. Kinetic analysis of water-loss was performed and represented as the percentage of initial fresh weight loss at each scored time point. Data are averages ± SE from two independent experiments.

RNA analyses

ABA treatment, RNA extraction and RT-quantitative PCR amplifications were performed as previously described (Saez *et al.*, 2004).

Data Collection and Refinement

Data collection		**Refinement**	
Space group	$P2_12_12_1$	Resolution range (A)	28.24–1.8
Unit cell a, b, c,	45.849, 65.857, 170.867	No. reflections	340.949
α, β, γ	90, 90, 90	No. unique reflections	47.524
Resolution	30.0–1.80	Rwork (%)	17.386
Highest resolution shell	(1.9–1.8)	Rfree (%)	21.760
Rsym	6.2% (19.6%)	No. atoms	4,17
Completeness	97% (91%)	Protein	3,72
I/σ (I)	22.6 (5.2)	Ligand	21
		Solvent	475
		R.m.s. deviations	
		Bond length	0.02
		Angles	1.655

Table 3.1. Crystallographic data collection and refinement statistics

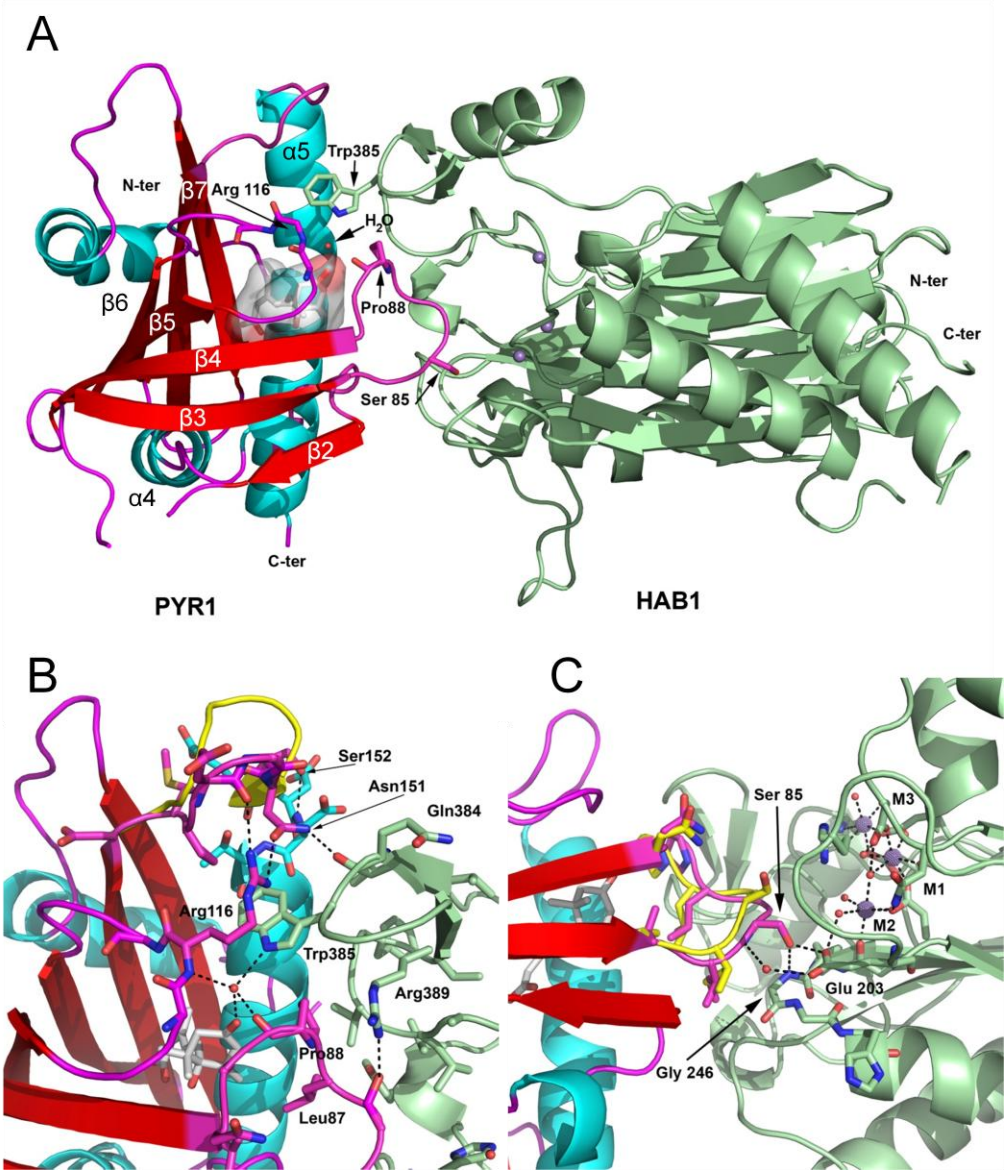

Figure 3.1. Structure of the PYR1-ABA-HAB1 complex. A, The PYR1 receptor is shown with strands in red, loops in magenta and helices in cyan. The HAB1 catalytic domain is shown in green. The (+)-ABA molecule is shown as stick model with semi-transparent surface. The three metal ions at the phosphatase catalytic site are depicted (blue spheres). The gating loops containing Pro88, Ser85 and Arg116 are indicated. The flap sub-domain containing Trp385 can be easily appreciated. The water molecule (red sphere) at the narrow channel between the gating loops is hydrogen bonded to the ketone group of the hormone, the backbone atoms of PYR1 Pro88 and Arg116 and the side chain of HAB1 Trp385. B, Detail of the interaction between HAB1 Trp385 region and the PYR1 gating loops. C, Detail of the interaction between the β3-β4 loop containing Pro88 and Ser85 and the phosphatase catalytic site. Relevant amino acids are shown as sticks, hydrogen bonds are indicated by dotted lines. The conformation rearrangements in the β7/β5 and β3/β4 loops of PYR1 upon binding to the phosphatase (magenta) as compared to the ABA-bound subunit of the PYR1 dimer (yellow) can be appreciated.

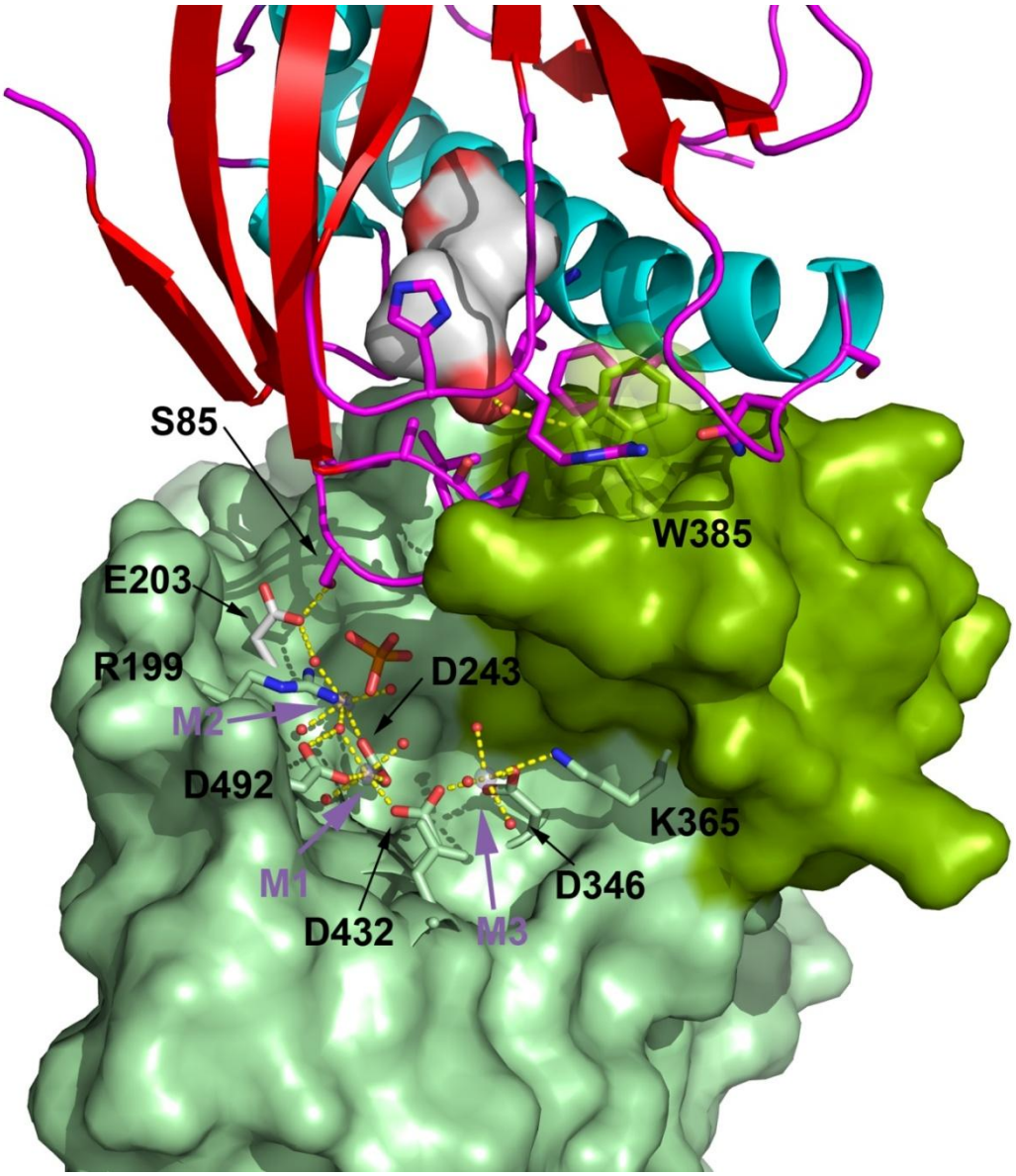

Figure 3.2. The PYR1 β3/β4 loop docks at the catalytic site of HAB1. The ABA-bound PYR1 receptor is shown as in Fig. 3.1. The accessible surface of the HAB1 phosphatase is depicted in light green with the flap sub-domain containing Trp385 in dark green. Residues coordinating the three metal ions at the catalytic site were excluded in the calculation of the molecular surface and are depicted as stick models. The water molecules involved in metal coordination are depicted as red spheres. The human PP2Cα structure (not shown), which contains a phosphate ion (shown as stick model) in the active site, was superposed on HAB1 to transfer the position of the phosphate ion into the catalytic site of HAB1.

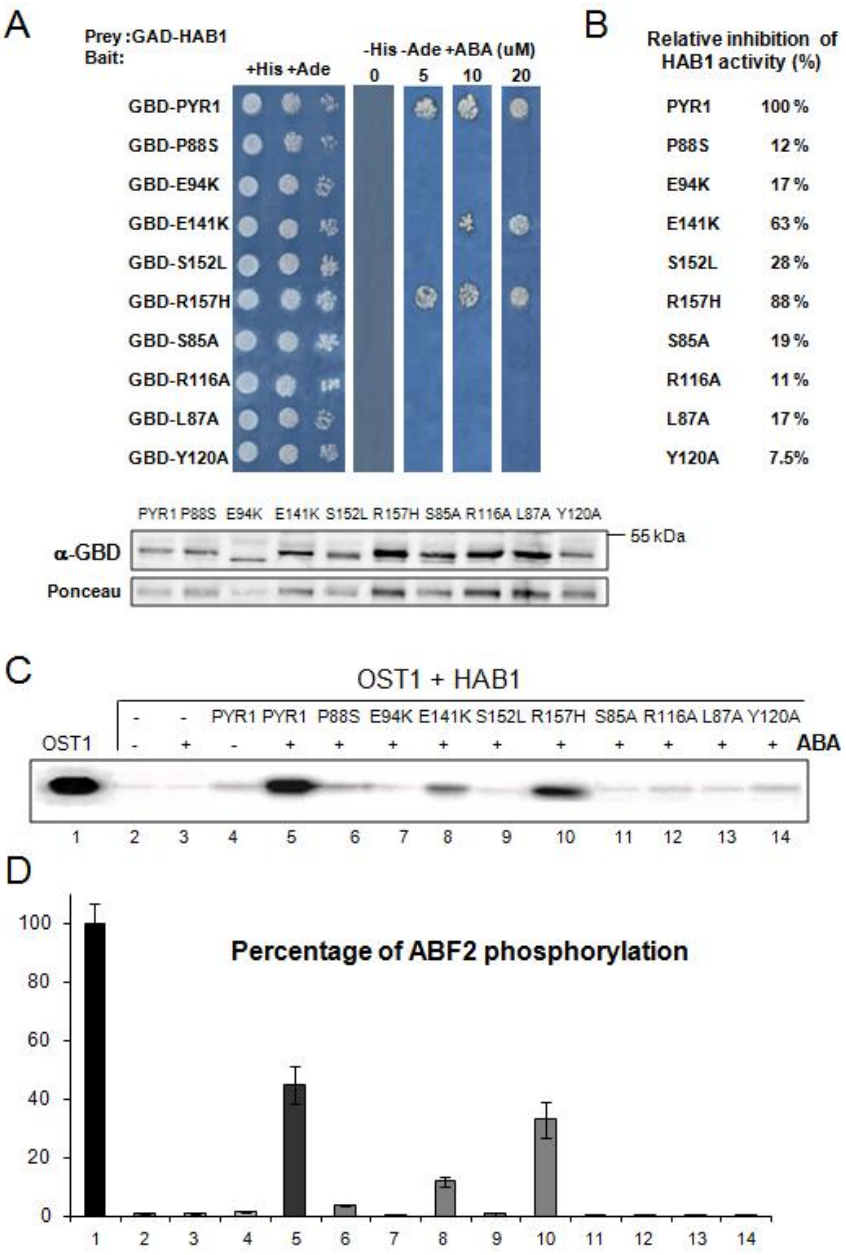

Figure 3.3. Analysis of the PYR1 mutations and their effect on the HAB1-dependent inhibition of OST1 activity. A, Interaction between HAB1 and PYR1 variants was analysed by the yeast two-hybrid (Y2H) growth assay on medium lacking His and Ade in the presence of 5, 10 or 20 μM (+)-ABA. Immunoblot analysis using antibody against the Gal4 binding domain (GBD) verifies the expression of the different fusion proteins in the Y2H assay. Ponceau staining from a representative yeast protein is shown as loading control. B, Relative inhibition of HAB1 activity by the different PYR1 variants in the presence of 8 μM ABA with respect to wt PYR1 (100%; SD was below 7%,). C, OST1 *in vitro* kinase activity assay in the presence of HAB1, PYR1 wt and mutated versions, ΔCABF2 and 10 μM ABA, when indicated. The autoradiography shows the levels of auto-phosphorylation of OST1. D, Quantification of ΔCABF2 phosphorylation levels in the previous assay using the phosphoimager Image Gauge V.4.0. Standard error measurements are shown (*n=3*).

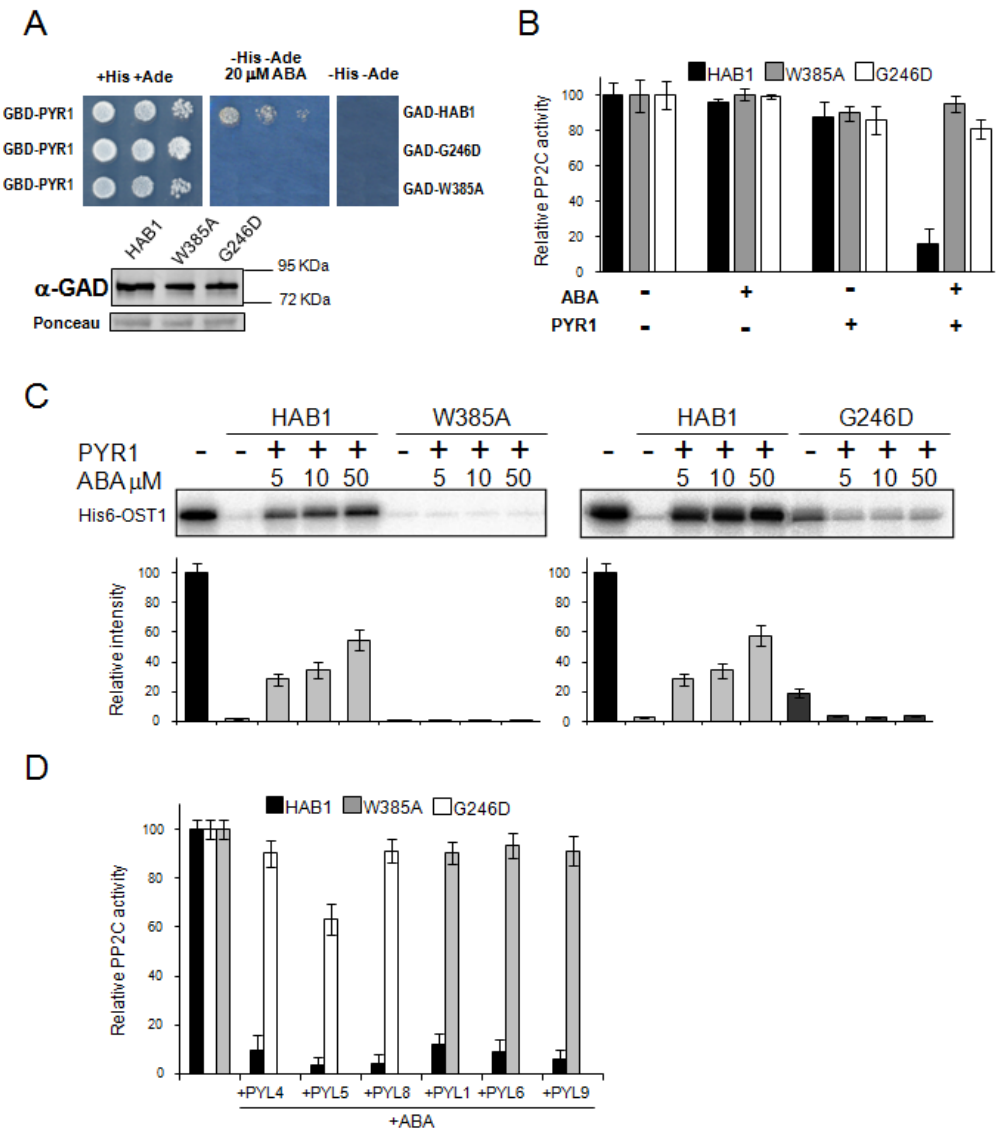

Figure 3.4. The hab1^{W385A} and hab1^{G246D} PP2Cs are refractory to inhibition by PYR1 and dephosphorylate OST1 in the presence of ABA and PYR1. A, The HAB1 mutations Trp385Ala and Gly246Asp abolish the interaction of the PP2C and PYR1 in a Y2H assay. Immunoblot analysis using antibody against the Gal4 activation domain (GAD) is shown to verify the expression of the different fusion proteins. Ponceau staining from a representative yeast protein is shown as loading control. B, Phosphatase activity of HAB1, hab1^{W385A} and hab1^{G246D} proteins was measured *in vitro* using p-nitrophenyl phosphate as substrate in the absence or presence of PYR1 and ABA, as indicated. Assays were performed in a 100 µl reaction volume containing 2 µM phosphatase and, when indicated, 4 µM HIS_6-PYR1 and 1 µM (+)-ABA. Data are averages ± SD from three independent experiments. C, *In vitro* OST1 kinase activity in the presence of wt and mutated versions of HAB1, PYR1 and ABA, as indicated. The autoradiography shows the level of autophosphorylation of OST1 in each reaction. The graphs show the quantitative analysis of the autoradiogram. D, hab1^{W385A} and hab1^{G246D} proteins are resistant to ABA-mediated inhibition by different PYR/PYLs. The assay was performed as described in B.

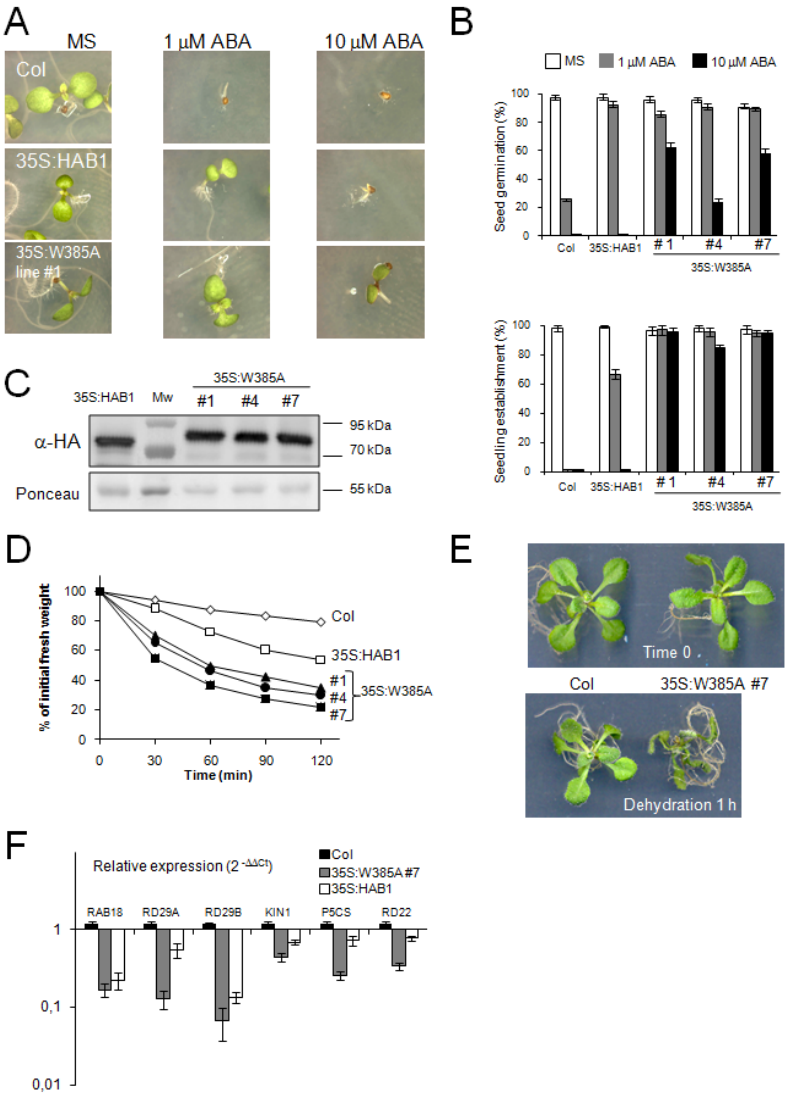

Figure 3.5. Constitutive expression of *hab1*^{W385A} leads to reduced ABA sensitivity. A, Seed germination and seedling establishment of representative Columbia wt, *35S:HAB1* and *35S:hab1*^{W385A} plants in medium lacking or supplemented with ABA. Photographs were taken 7 d after sowing. B, Inhibition of seed germination and seedling establishment by ABA in Columbia wt, *35S:HAB1* and *35S:hab1*^{W385A} plants. C, Immunoblot analysis using antibody against HA tag to quantify phosphatase expression in transgenic lines. Ponceau staining from the large subunit of RuBisCO is shown as loading control. D, Enhanced water loss measured in detached leaves of *35S:HAB1* and *35S:hab1*^{W385A} plants as compared to Columbia wt. Values are averages from two independent experiments (n=10), and SD (not shown) was below 7%. E, The photograph illustrates the severe phenotype observed in *35S:hab1*^{W385A} plants after 60 minutes of water loss. F, Reduced expression of ABA-inducible genes in *35S:hab1*^{W385A} (line #4) and *35S:HAB1* plants compared with Columbia wt. Values are expression levels reached in the transgenic lines with respect to wt (value 1) as determined by RT-qPCR analysis. Expression of gene markers was analyzed in 10-days-old seedlings treated with 10 µM ABA for 3h. Values are averages ± SD for two independent experiments (n=30 to 40 seedlings per experiment).

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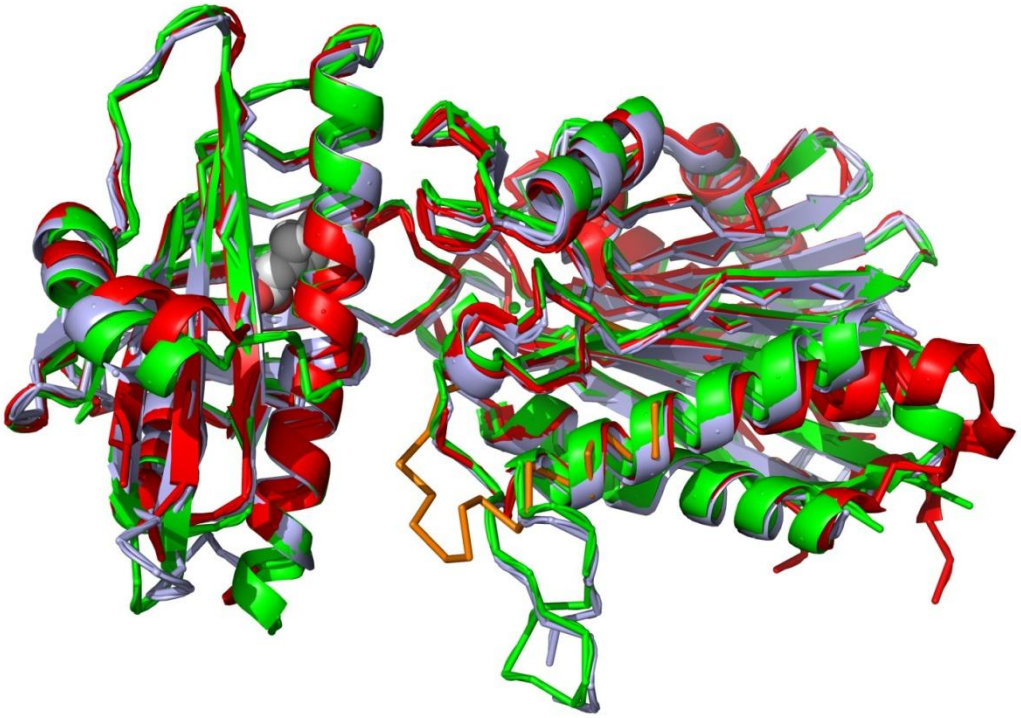
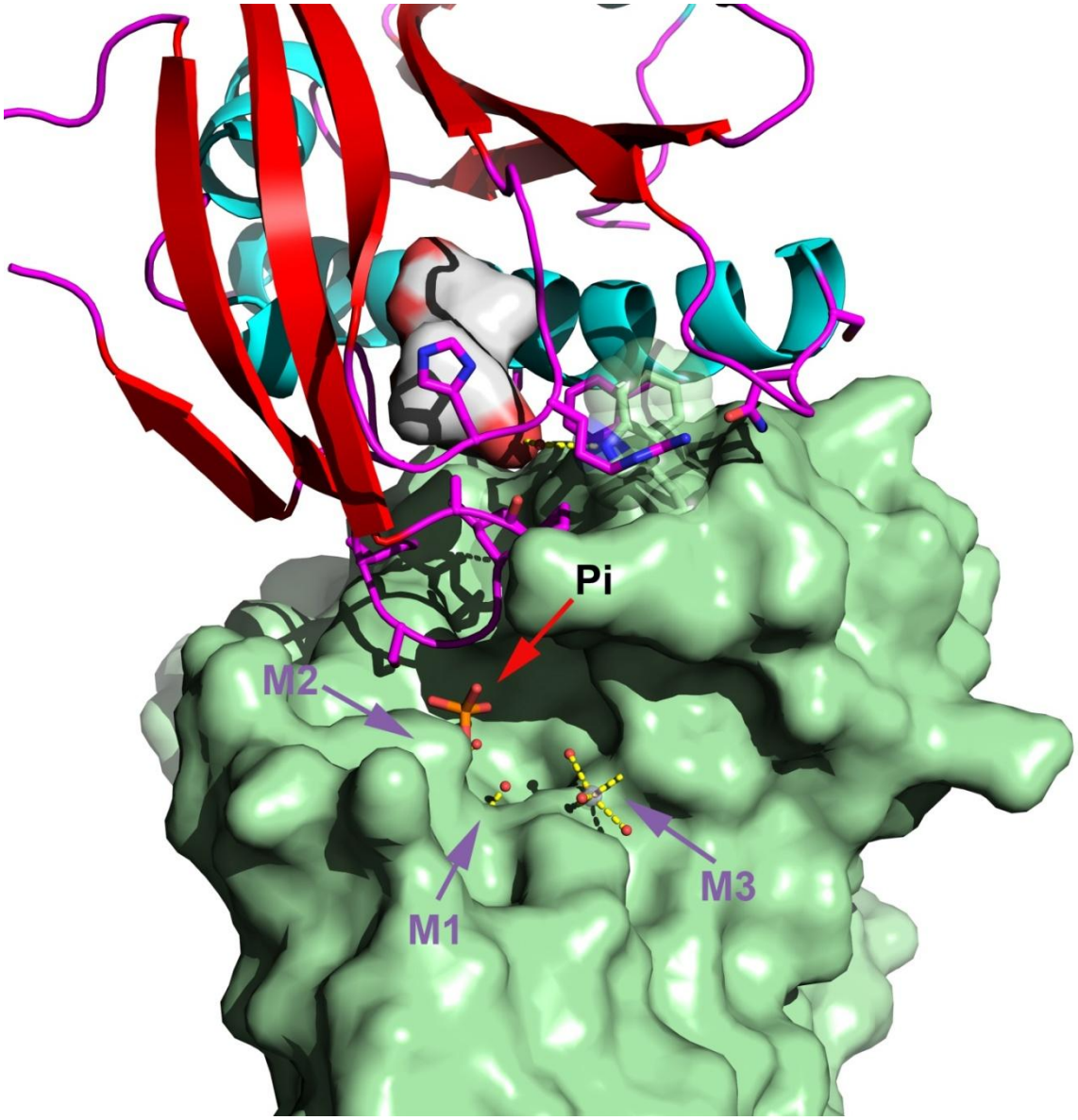
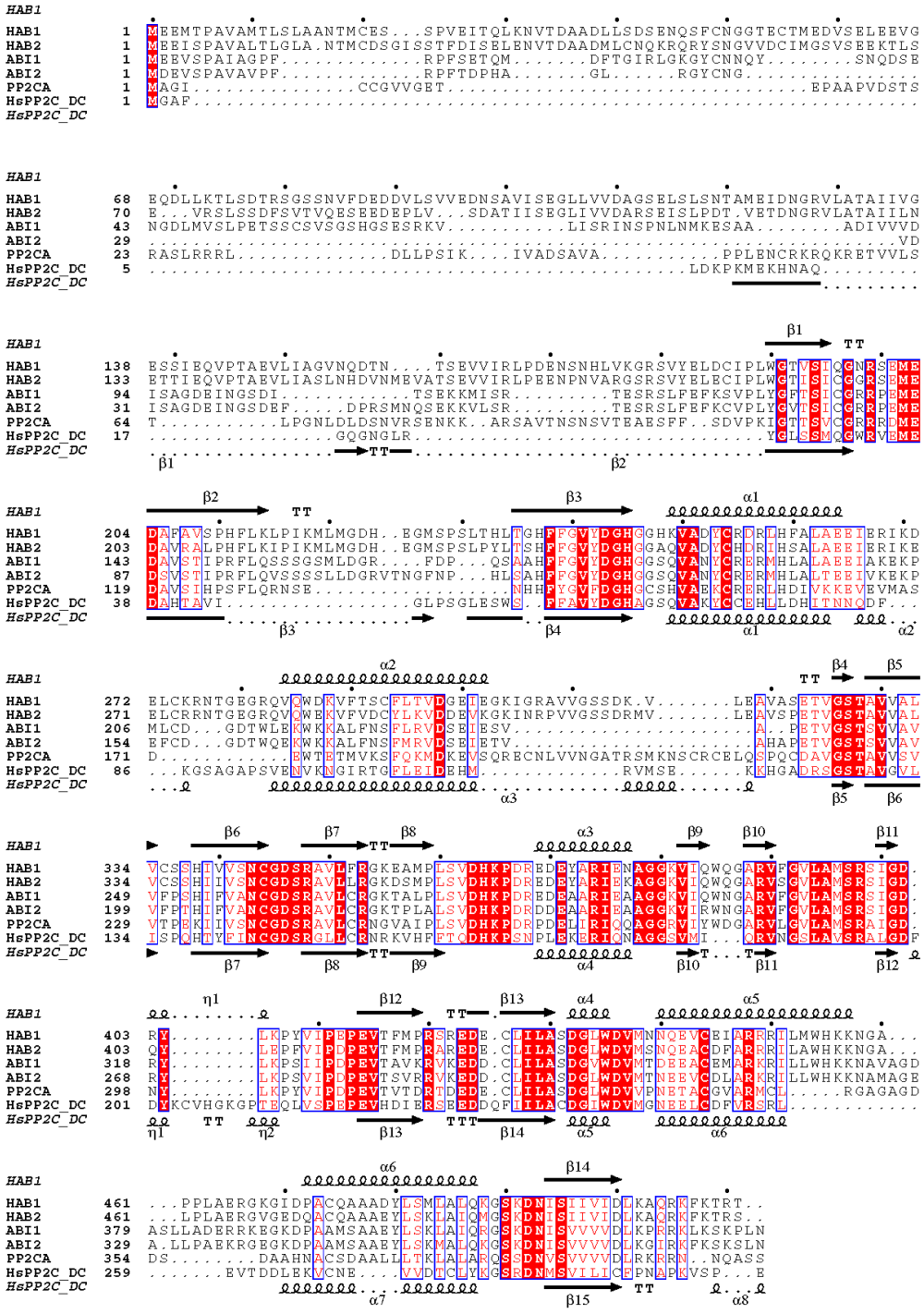
3.7 Supplemental material



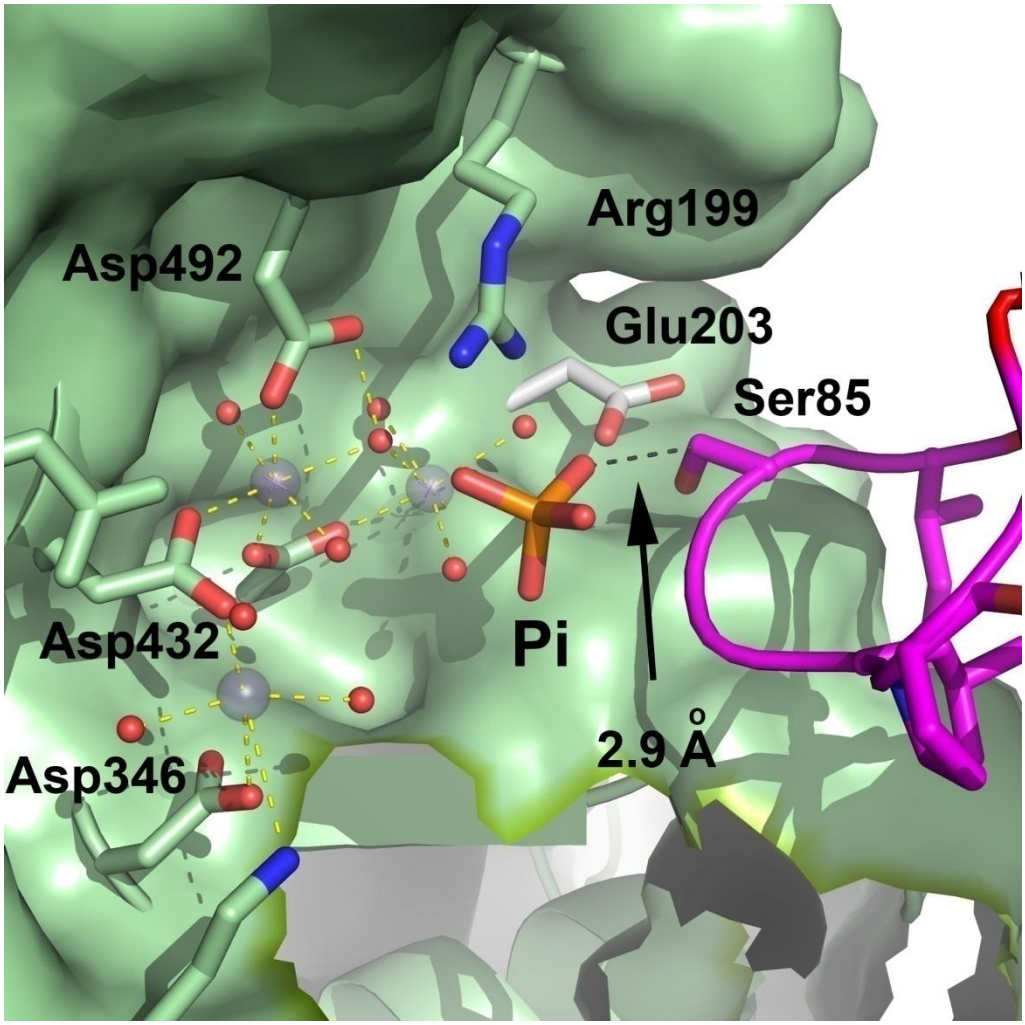
Supplemental Figure S1. Structural superposition of ternary receptor complexes. Superposition of the PYR1-HAB1 (green, this work), PYL1-ABI1 (red, 3JRQ), PYL2-HAB1 (blue, 3KB3) and human PP2Ca ((orange, 1A6Q) structures. For human PP2Ca only the residues 105 to 129 are shown as backbone trace for clarity. The PYR1 ABA molecule is shown as spheres. The overall similarity in the four structures can be appreciated. An insertion at the a3/b4 loop of HAB1 (green and blue, bottom side) relative to ABI1 and human PP2Ca can be appreciated. The orientation of this view is similar to that shown in Figure 3.1.



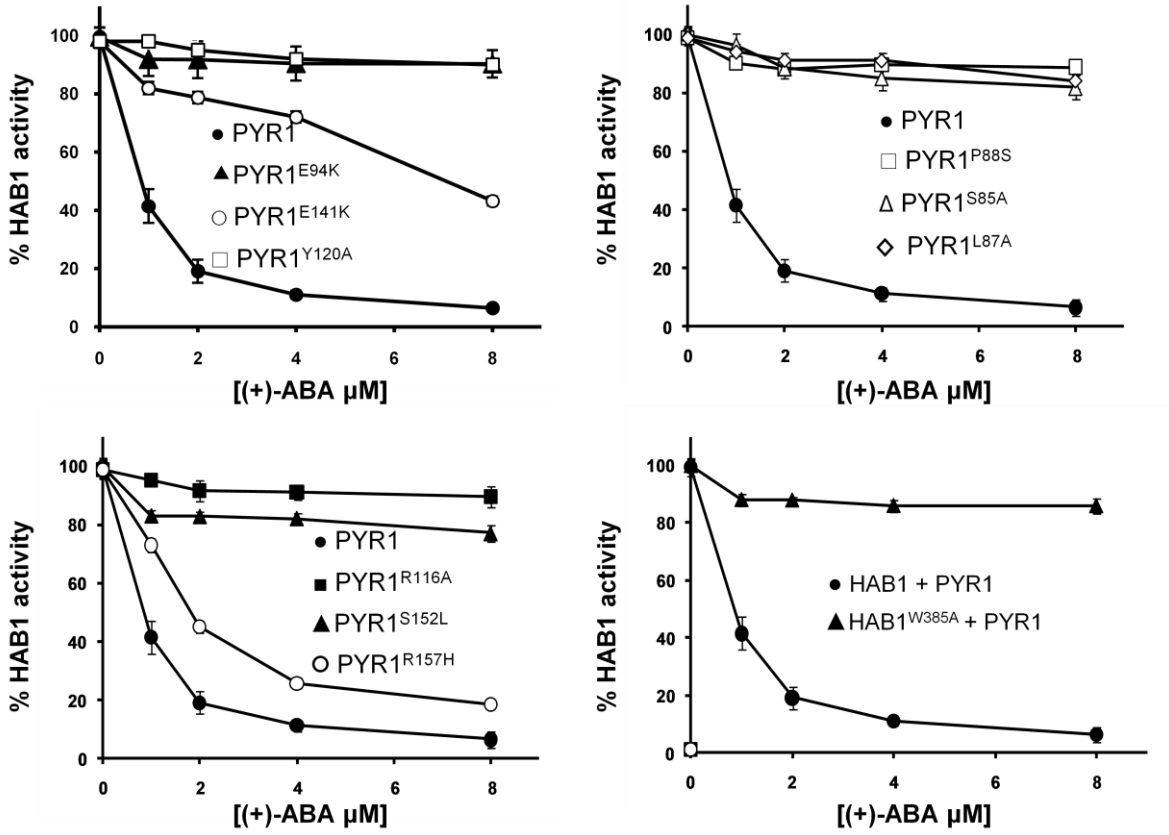
Supplemental Figure S2. Detail of the catalytic groove of HAB1. The ABA-bound PYR1 receptor is shown as in Figure 3.2. The accessible surface of the HAB1 phosphatase is depicted in light green. In this case no amino acids have been excluded for the calculation of the PP2C accessible surface and the long active site channel, with its two openings (one occupied by PYR1) can be more easily appreciated. The approximate positions of the three metal ions (of which only M3 is directly visible, grey sphere) are indicated by pink arrows and water molecules coordinated to the three metal ions are depicted as red spheres. The structures of human PP2Ca (not shown) and HAB1 have been superimposed using the Cα backbones and the phosphate ion found at the PP2Ca catalytic site is shown as stick model.



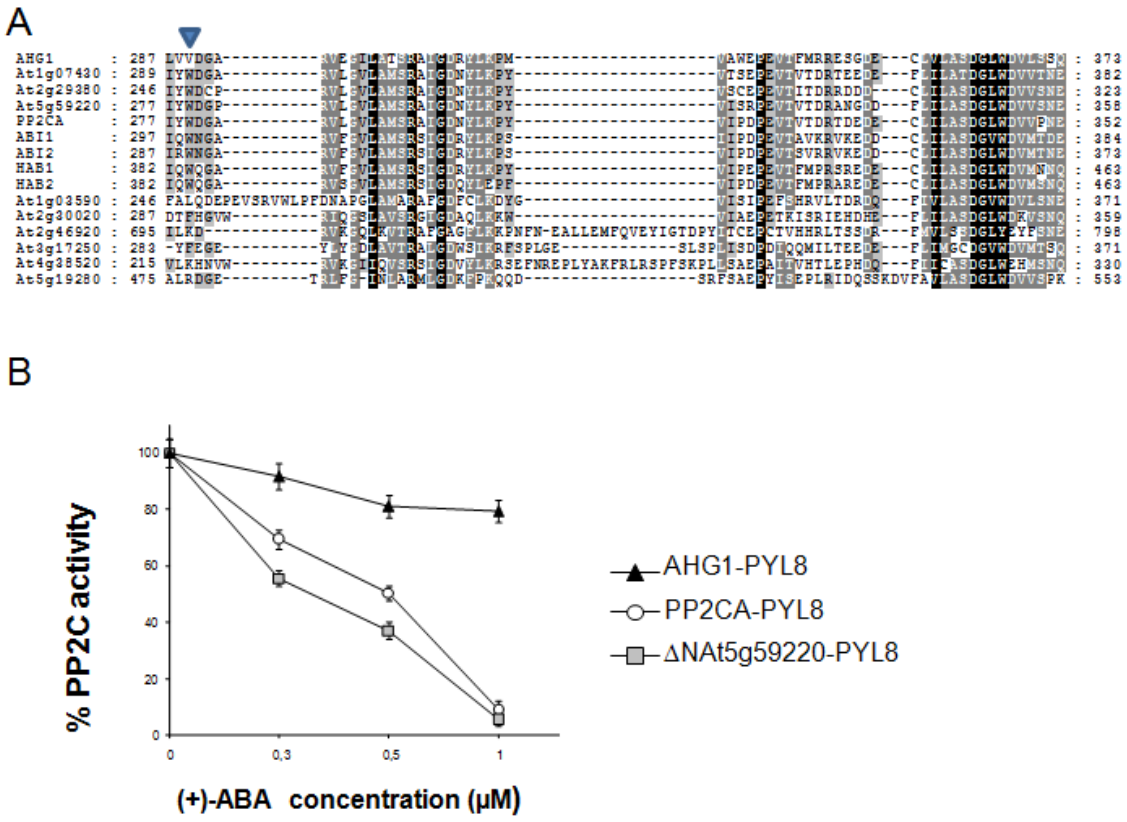
Supplemental Figure S3. Multiple amino acid sequence and secondary structure alignment of plant PP2Cs with the catalytic core of human PP2Cα (residues 1-300). Colour codes indicate the amino acid residues involved in the interaction with ABA receptors and contact points with phosphate, metal, ABA and hypermorphic mutations. This figure was generated with ESPript (Gouet *et al*. 1999. *Bioinformatics*. **15** 305-8)



Supplemental Figure S4. Detail of the HAB1 catalytic site around the PYR1 Ser85. The PYR1 β3/β4 loop containing Ser85 is shown in magenta. The accessible surface of the HAB1 phosphatase is depicted in light green. Residues coordinating the three metal ions at the catalytic site were excluded in the calculation of the molecular surface and are depicted as stick models. The water molecules involved in metal coordination are depicted as red spheres. The structures of human PP2Ca (not shown) and HAB1 have been superimposed using the Cα backbones and the phosphate ion found at the PP2Ca catalytic site is shown as stick model (Pi).



Supplemental Figure S5. Comparison of the ABA-dependent inhibitory effect of PYR1 and pyr1 mutant proteins on HAB1 activity. HAB1^{W385A} is refractory to inhibition by PYR1. Phosphatase activity was measured using the Ser/Thr Phosphatase assay system (Promega) and the RRA(phosphoT)VA peptide as substrate. Data are averages ± SE from three independent experiments. The HIS6-HAB1 and HIS6-PYR1 proteins were obtained as described in methods. Phosphatase assays were performed in a 100 µl reaction volume containing 1 µM phosphatase and 4 µM HIS6-PYR1 proteins, respectively. The indicated (+)-ABA concentration was included in the PP2C activity assay. The activity of HIS6-HAB1 in the absence of ABA (100 % activity) was 4.6±0.3 nmoles Pi/min · mg. The activity of HIS6-HAB1 and HIS6-HAB1^{W385A} in the absence of ABA (100 % activity) was 4.6±0.3 and 4.5±0.35 nmoles Pi/min · mg, respectively.



Supplemental Figure S6. A, Amino acid sequence alignment of clade A PP2Cs and representative PP2Cs from other groups. The Trp residue involved in the interaction with ABA (blue triangle) is only present in clade A PP2Cs, with the exception of AHG1. B, AHG1 is less-sensitive to ABA-dependent PYL8-mediated inhibition than other clade A relatives, such as PP2CA and At5g59220. Phosphatase activity was assayed as described in Suppl. Figure S5. The activity of HIS6-AHG1, His6-PP2CA and HIS6-DNAt5g59220 in the absence of ABA (100 % activity) was 4.8±0.3, 4.7±0.3 and 5.9±0.4 nmoles Pi/min · mg, respectively.

4. Results: Chapter 2

Selective inhibition of Clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors

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4.1 Abstract

Clade A protein phosphatases type 2C (PP2Cs) are negative regulators of abscisic acid (ABA) signalling that are inhibited in an ABA-dependent manner by PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/ REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) intracellular receptors. We provide genetic evidence that a previously uncharacterized member of this PP2C family, At5g59220, is a negative regulator of osmotic stress and ABA signalling and this function was only apparent when double loss-of-function mutants with *pp2ca-1/ahg3* were generated. *At5g59220-GFP* and its close relative *PP2CA-GFP* showed a predominant nuclear localization, however, hemagglutinin (HA)-tagged versions were also localized to cytosol and microsomal pellets. At5g59220 was selectively inhibited by some PYR/PYL ABA receptors, and close relatives of this PP2C, such as PP2CA/ABA-HYPERSENSITIVE GERMINATION3 (AHG3) and AHG1, showed a contrasting sensitivity to PYR/PYL inhibition. Interestingly, AHG1 was resistant to inhibition by the PYR/PYL receptors tested, which suggests that this seed-specific phosphatase is still able to regulate ABA signalling in the presence of ABA and PYR/PYL receptors and therefore to control the highly active ABA signalling pathway that operates during seed development. Moreover, the differential sensitivity of the phosphatases At5g59220 and PP2CA to inhibition by ABA receptors reveals a functional specialization of PYR/PYL ABA receptors to preferentially inhibit certain PP2Cs.

4.2 Introduction

ABA is a key phytohormone that regulates plant response to abiotic and biotic stress as well as plant development and growth. In seeds, ABA regulates several processes essential for seed viability and germination, including the accumulation of protein and lipid reserves, the induction of dormancy and the acquisition of tolerance to desiccation (Cutler *et al*., 2010). Recently, a core signalling pathway has been established that connects ABA perception, inactivation of PP2Cs and activation of three SUCROSE NON-FERMENTING 1-RELATED SUBFAMILY 2 (SnRK2s) protein kinases, i.e. SnRK2.2/D, 2.3/I and 2.6/OST1/E (Cutler *et al*., 2010). Under basal ABA levels, at least 6 clade A PP2Cs (Schweighofer *et al*., 2004), ABA-INSENSITIVE1 (ABI1), ABI2, ABA-HYPERSENSITIVE1 (HAB1), HAB2, AHG1 and PP2CA/AHG3, act as negative regulators of ABA signalling, either through dephosphorylation of SnRK2s or interaction with other targets (Sheen, 1998; Gosti *et al*., 1999; Merlot *et al*., 2001; Tahtiharju and Palva, 2001; Himmelbach *et al*., 2002; Saez *et al*., 2004; Leonhardt *et al*., 2004; Yoshida *et al*., 2006; Kuhn, *et al*., 2006; Saez *et al*., 2006; Miao *et al*., 2006; Nishimura *et al*., 2007; Saez *et al*., 2008; Umezawa *et al*., 2009; Vlad *et al*., 2009). When ABA levels rise, the PYR/PYL ABA receptors inactivate PP2Cs in an ABA-dependent manner, which leads to activation of SnRK2.2, 2.3 and 2.6/OST1, and subsequent phosphorylation of downstream targets, e.g. members of the ABF/AREB transcription factors that recognize ABRE promoter sequences or regulatory components of the stomatal aperture, such as the anion channel SLAC1 (Fujii *et al*, 2009; Fujita *et al*, 2009; Geiger *et al*, 2009; Lee *et al*, 2009). In *Xenopus laevis* oocytes and *in vitro* studies, it has been also shown that ABI1 inhibits the calcium-dependent kinases CPK21 and CPK23, which have the anion channels SLAC1 and SLAH3 as substrates, and RCAR1/PYL9 restores the SLAC1 and SLAH3 phosphorylation through ABA-dependent inhibition of ABI1 (Geiger *et al*, 2010 and 2011). Finally, genetic evidence has largely supported the negative role of PP2Cs in ABA signalling, and indeed, certain triple loss-of-function pp2c mutants display partial constitutive response to ABA (Rubio *et al*., 2009).

According to sequence alignment of the catalytic phosphatase core, the clade A of PP2Cs is arranged in two subgroups, one including ABI1, ABI2, HAB1 and HAB2, and a second one formed by PP2CA/AHG3, AHG1, At5g59220, At1g07430 and At2g29380 (Schweighofer *et al*., 2004; Supplemental Figure S1). These three latter PP2Cs are also known as HAI1, HAI2 and HAI3, respectively, for HIGHLY ABA-INDUCED PP2C genes and interaction of HAI1 with SnRK2.2 has been reported (Fujita *et al*., 2009). However, At1g07430 had been previously named AIP1 (Lee *et al*., 2007), for AKT1-INTERACTING PP2C, and later on At2g29380 and At5g59220 were named AIPH1 and AIPH2, for AIP1 HOMOLOGUES, respectively (Lee *et al*., 2009). Therefore,

current nomenclature on At5g59220 reflects connection with either ABA signalling or regulation of K^+ transport. Intriguingly, it has been reported that At5g59220, named now as PP2CA2, plays a positive role in ABA signalling because the corresponding T-DNA loss-of-function mutant shows an ABA hyposensitive phenotype in ABA-mediated inhibition of germination and post-germinative growth (Guo *et al*., 2010). In that case, At5g59220 would represent a singular member of clade A PP2Cs, showing an opposite function to other members of the group.

According to sequence alignment, *At5g59220* is closely related to *PP2CA/AHG3* (Supplemental Figure S1), which has been implicated as a key negative regulator of ABA signalling since *pp2ca* mutant alleles show ABA-hypersensitive phenotypes in germination, growth and stomatal assays. In addition to dephosphorylation of ABA-activated SnRK2s (Umezawa *et al*., 2009; Lee *et al*., 2009), PP2CA has been reported to interact with two ion transporters localized to plasma membrane, i.e. the K^+ channel AKT2 and the anion channel SLAC1 (Cherel *et al*., 2002; Lee *et al*., 2009). Finally, both PP2CA/AHG3 and AHG1 appear to play an essential role for ABA signaling during seed development and germination (Kuhn, *et al*., 2006; Yoshida *et al*., 2006; Nishimura *et al*., 2007), but in contrast to *pp2ca-1*, the *ahg1-1* mutant has no ABA-related phenotype in adult plants and expression of *AHG1* is restricted to seed (Nishimura *et al*., 2007). In this work, we have analysed loss-of-function mutants of *At5g59220*, either single or double mutants with *pp2ca-1*, and we found evidence At5g59220 is also a negative regulator of osmotic stress and ABA signalling. Additionally, analysis of the biochemical regulation of At5g59220, PP2CA/AHG3 and AHG1 reveals a differential sensitivity to inhibition by ABA and PYR/PYL receptors. Since AHG1 appears to be immune to PYR/PYL-mediated inhibition, this PP2C might control ABA signalling during seed development even in the presence of ABA and PYR/PYL receptors.

4.3 Results

An *At5g59220* loss-of-function mutant reinforces the ABA-hypersensitive phenotype of the *pp2ca-1* mutant

At5g59220 is not expressed in seeds (Nakabayashi *et al*., 2005; Yoshida *et al*., 2006; Nishimura *et al*., 2007) but it is expressed in seedlings or different tissues of adult plants according to public microarray data (Winter *et al*., 2007; Supplemental Figure S2). Basal transcript levels of *At5g59220* are lower than those reported for other clade A PP2Cs; however, its expression is highly induced by ABA or osmotic stress (Fujita *et al*., 2009; Yoshida *et al*., 2010; Supplemental Figure S2), and this induction was dramatically impaired in the *areb1areb2abf3* triple mutant (Yoshida *et al*., 2010). In order to investigate the relative contribution of At5g59220 to ABA signalling, we

analysed ABA-response of the At5g59220 loss-of-function mutant SALK_142672, which we have named *hai1-1* (Figure 4.1A). ABA-mediated inhibition of seedling establishment was similar in single *hai1-1* mutant compared to wild type, and a double *pp2ca-1 hai1-1* mutant did not show enhanced response to ABA compared to *pp2ca-1*, even at low ABA concentrations (Figure 4.1B, Supplemental Figure S3). However, a double *pp2ca-1 hai1-1* mutant was more sensitive to glucose or mannitol-mediated inhibition of seedling establishment than single parental mutants (Figure 4.1B, Supplemental Figure S3).

We also generated Arabidopsis transgenic lines expressing HA-tagged versions of the PP2Cs driven by the 35S promoter. With respect to PP2CA, we confirmed previous results obtained by Kuhn *et al*., (2006), which have shown that over-expression of *PP2CA* leads to an ABA-insensitive phenotype (Figure 4.1C). Likewise, *35S:At5g59220* lines showed diminished sensitivity to ABA mediated inhibition of seedling establishment and root growth, enhanced water-loss and diminished expression of ABA-inducible genes compared to wt (Figure 4.1C; Supplemental Figure S4). Conversely, a characteristic feature of *pp2ca-1 hai1-1* double mutant was an enhanced sensitivity to ABA-mediated inhibition of growth compared to single mutants, which suggests that At5g59220 attenuates ABA signalling in vegetative tissue (Figure 4.2A and 4.2B). Therefore, the very moderate ABA-hypersensitivity of *pp2ca-1* in root assays described by Kuhn *et al*., (2006) is likely explained by partial redundancy with *At5g59220* or other PP2Cs (Rubio *et al*., 2009).

We also analysed transcriptional regulation of ABA responsive genes in the double *pp2ca-1 hai1-1* mutant compared to wt and single parental mutants. ABA-mediated induction of the genes *KIN1*, *RAB18* and *RD29B* was >2-fold higher in the double mutant compared to the other genetic backgrounds (Figure 4.2C). Expression of these genes in the single parental mutants showed less than 2-fold difference with respect to wt. Finally, by measuring loss of fresh weight of detached leaves, we could observe a reduced water loss of the double *pp2ca-1 hai1-1* mutant compared to wt and single parental mutants (Figure 4.2D)

Subcellular localization of PP2CA and At5g59220

While the catalytic core of *At5g59220* is closely related to *PP2CA*, the N-terminal sequence shows a clear divergence (Supplemental Figure S1). Several clusters rich in arginine residues are present at the N-terminal sequence of *At5g59220*. Different programs for prediction of subcellular localization reveal the presence of nuclear targeting signals in this region, indeed two nuclear localization patterns are present, both the pattern of 4 basic residues (type SV40 T antigen) and the bipartite nuclear localization signal (Supplemental Figure S1). Instead, PP2CA only displays the pattern of 4 basic residues, which is localized at the C-terminus of the protein (Supplemental Figure

S1). In experiments where GFP fusion proteins were transiently expressed in tobacco epidermal cells, both PP2CA and At5g59220 appeared to be predominantly localized to the nucleus, although some cytosolic expression was also observed (Figure 4.3A). Deletion of the N-terminal region of At5g59220 (construct expressing residues 98-413) led to a subcellular localization of the catalytic phosphatase core similar to GFP, whereas fusion of the residues 1-97 of At5g59220 to GFP rendered a nuclear GFP protein (Figure 4.3A).

Proper elucidation of the subcellular localization of clade A PP2Cs is an important goal to better understand their role in plant physiology, however, biochemical fractionation studies have been only reported for HAB1 (Saez *et al*., 2008). Since interaction of PP2CA with the plasma membrane transporters AKT2 and SLAC1 has been reported (Cherel *et al*., 2002; Lee *et al*., 2009) and interaction of PP2CA and At5g59220 with SnRK2s was localized to both nucleus and cytosol (Fujita *et al*., 2009), we further investigated the subcellular localization of both PP2Cs by fractionation studies. To this end, we used the Arabidopsis transgenic lines that express HA-tagged versions of the PP2Cs (see above Figure 4.1C). Both HA-PP2CA and HA-At5g59220 proteins appeared to be functional with respect to ABA signalling since their constitutive expression led to reduced sensitivity to ABA (Figure 4.1C). Both proteins showed cytosolic and nuclear localization, and, interestingly, part of the protein pool was localized to either the microsomal or nuclear insoluble (chromatin associated) fractions (Figure 4.3B). A relative quantification of the subcellular distribution of HA-PP2CA and HA-At5g59220 indicated that most of the protein was localized at the cytosol, although these data also confirmed that a significant fraction of the protein, 13% and 28% for HA-PP2CA and HA-At5g59220, respectively, was localized in the nucleus. The apparently predominant nuclear localization of transiently expressed GFP-tagged PP2Cs might be explained because the lower volume of the nucleus, compared to the cytosol, leads to a higher concentration of GFP fusion proteins, enhancing the GFP fluorescent signal (Figure 4.3A).

Selective inhibition of At5g59220, PP2CA and AHG1 by PYR/PYL ABA receptors

Since both At5g59220 and PP2CA regulate different aspects of ABA signalling, we analysed its possible regulation by PYR/PYL ABA-receptors. Co-expression of these PP2Cs and PYR/PYLs in seedlings, root or guard cells could be documented in public microarray databases (Figure S2). Thus, we analysed phosphatase activity of PP2CA and At5g59220 in the presence of seven PYR/PYL receptors, which represent the dimeric class, i.e. PYR1, PYL1 and PYL2, and the monomeric class, i.e. PYL4, PYL5, PYL6, PYL8 (Hao *et al*., 2011; Dupeux *et al*., 2011). Using a 100:1 ratio of receptor:PP2CA, between 40-80% ABA-independent inhibition of PP2CA by some monomeric receptors was recently reported (Hao *et al*., 2011). However, in our hands using either a

4:1 ratio in phosphopeptide-based activity assays (Figure 4.4A) or 10:1 ratio in OST1 dephosphorylation assays (Figure 4.4B), we did not detect such ABA-independent inhibition of PP2CA by the PYR/PYL receptors tested. In the best case, only a 10-15% ABA-independent inhibition of PP2CA by PYL8 was found (Figure 4.4A and 4.4B). In the presence of ABA, PP2CA was inhibited by all the receptors, although important differences could be observed depending on the receptor considered. For instance, PP2CA inhibition by PYL8 was much more effective than by PYR1 (inhibitory concentration to obtain 50% inhibition, IC_{50}=0.5 and 25 μM, respectively), whereas IC50 values for the other receptors ranged between 4-10 μM ABA. Such differences were not noted previously, but it is likely that they were masked by the high concentration of receptor used with respect to the PP2C by Hao *et al*., (2011). At5g59220 was relatively resistant to inhibition by PYL4 and PYL6 (IC_{50}>50 μM), and IC_{50} for dimeric receptors, such as PYR1, PYL1, PYL2, was approximately 30 μM ABA, whereas PYL5 and PYL8 where the most effective inhibitors (IC_{50}=8 and 0.8 μM, respectively). Indeed, both PYL5 and PYL8 were the most effective inhibitors of PP2CA as well (IC_{50}=3.7 and 0.5 μM, respectively).

Structural and genetic studies have showed the importance for the locking mechanism of the ternary receptor:ABA:PP2C complex of a conserved Trp residue of clade A PP2Cs that establishes a water-mediated hydrogen bond with the ketone group of ABA in ternary complexes (Melcher *et al*., 2009; Miyazono *et al*., 2009; Dupeux *et al*., 2011; Supplemental Figure S1). Interestingly, AHG1 is the only clade A PP2C that lacks this conserved Trp (Dupeux *et al*., 2011). Therefore, we wondered whether this seed-specific PP2C would be subjected to PYR/PYL regulation. As can be observed in Figure 4.4A, AHG1 phosphatase activity was not significantly affected by PYR/PYL receptors even at 50 μM ABA. This result indicates that AHG1 could negatively regulate ABA signalling even in the presence of high levels of ABA and PYR/PYL receptors.

To gain additional evidence on the biochemical regulation of the above described PP2Cs, we also performed *in vitro* reconstitution of the ABA signalling cascade and tested protection of OST1 activity by PYL4, PYL5, PYL6 and PYL8 in the presence of the different PP2Cs and ABA (Figure 4.4B). Both PP2CA and At5g59220 efficiently dephosphorylated OST1, whereas AHG1 was less effective (Figure 4.4). Co-incubation of PP2CA in the presence of ABA either with PYL4, PYL5, PYL6 or PYL8 or At5g59220 with PYL5 or PYL8 notably protected OST1 activity. PYL4 and PYL6 only modestly recovered OST1 activity when co-incubated with At5g59220 in the presence of ABA. In agreement with the phosphatase assays described in Figure 4.4A, co-incubation of AHG1 with PYR/PYL receptors did not prevent OST1 dephosphorylation. Finally, we used ABF2 as a substrate of OST1 and after generation of phosphorylated ABF2, we incubated it

with PP2CA and At5g59220 (Figure 4.4C). Both PP2Cs efficiently dephosphorylated ABF2, whereas co-incubation with PYL8 in the presence of ABA abolished their activity against the transcription factor. Taking into account that a significant portion of PP2CA and At5g59220 is localized at the nucleus, these results suggest ABFs might also be substrates of these PP2Cs.

4.4 Discussion

In this work we provide novel insights on the role of clade A PP2Cs in ABA signalling and their regulation by PYR/PYL receptors. Genetic analysis of *hai1-1* indicates that At5g59220 functions as a negative regulator of ABA signalling, although this role has been likely masked by functional redundancy with other PP2Cs. Thus, compared to wt and single parental mutants, the *pp2ca-1 hai1-1* double mutant showed enhanced ABA-mediated inhibition of growth, induction of ABA-responsive genes and diminished water loss. Taking into account that both *PP2CA* and *At5g59220* transcripts are themselves strongly induced by ABA or osmotic stress (Fujida *et al*., 2009), their up-regulation under these conditions likely exerts a negative feedback on ABA and osmotic stress signalling. Glucose-mediated inhibition of seedling establishment was also notably enhanced in the *pp2ca-1 hai1-1* double mutant compared to single mutants and wt. Although part of this effect could be attributed to enhanced osmotic stress sensitivity of the double mutant, early seedling growth in medium supplemented with glucose was more severely inhibited than by an isosmotic concentration of mannitol. Indeed, 0.2 M glucose (3.6%) was relatively well tolerated by parental single mutants or wild type seedlings, whereas a strong inhibition of early seedling growth was found in the double mutant.

Subcellular localization studies of these PP2Cs indicated that they are present both at the nucleus and cytosol, which is in agreement with their reported interaction with SnRK2s (Fujita *et al*., 2009). In addition to dephosphorylation of SnRK2s (Umezawa *et al*., 2009; this work), these PP2Cs might efficiently dephosphorylate ABFs (Figure 4.4C), although further studies are required to firmly establish this point. Interestingly, a minor portion of these PP2Cs co-localized with the nuclear insoluble fraction (chromatin associated) and interaction of PP2CA with SWI3B, a putative component of chromatin-remodelling complexes, was previously reported (Saez *et al*., 2008). Although a major portion of PP2CA was localized in the cytosol, the presence of PP2CA was also detected in microsomal membranes, where two PP2CA-interacting proteins are localized, i.e. AKT2 and SLAC1.

High ABA levels and presumably active ABA signalling are temporarily correlated with the onset of maturation and prevention of precocious germination during mid-embryo development

(Kanno *et al*., 2010). Thus, ABA levels reach a maximum in the middle of seed development (around 9-10 days after flowering, DAF), and a second peak of ABA accumulation takes place late in development (15-16 DAF) (Kanno *et al*., 2010). This latter peak appears to be required to regulate the synthesis of proteins involved in desiccation tolerance and the development of seed dormancy. The ABA-induced LEA (late embryogenesis-abundant) proteins have been proposed to play a key role in protecting proteins and membranes from the severe water loss that occurs during seed desiccation. Different PP2Cs are expressed during seed development to regulate ABA signalling, however both AHG3/PP2CA and AHG1 are supposed to play a major role according to the seed phenotype of *ahg1* and *pp2ca* mutants and expression levels in seed (Nishimura *et al*., 2007). Even though AHG1 shares many features with AHG3/PP2CA, detailed characterization of *ahg3-1*/*pp2ca* and *ahg1-1* mutants has revealed important differences, particularly enhanced ABA-hypersensitivity of *ahg1* in radical emergence and deeper seed dormancy compared to *ahg3* (Nishimura *et al*., 2007). Figure 4.4 shows an additional key difference since PP2CA is regulated by PYR/PYL receptors whereas AHG1 seems to be immune to such regulation. Interestingly, whereas expression of *PP2CA* remained steady during seed development, the expression of *AHG1* was detected at 8 DAF and increased until 16 DAF (Nishimura *et al*., 2007). This expression pattern is similar to that of *ABI5*, which plays a key role for ABA signalling during seed development, and genetic analysis indicated that AHG1 functions upstream of ABI5 and ABI3 in the ABA pathway (Nishimura *et al*., 2007). The biochemical assays performed here for AHG1 indicate that this PP2C could partially dephosphorylate a SnRK2 even in the presence of high levels of ABA and PYR/PYL receptors. The presence of a PP2C resistant to inhibition might represent an adaptive response to partially control the highly active ABA signalling pathway that operates during mid and late seed development. Otherwise, since the rest of clade A PP2Cs are inhibited by PYR/PYL receptors, seed ABA signalling would operate in the absence of the negative control imposed by PP2Cs, which might impair the interplay with other hormonal pathways that also operate during seed development, such as cytokinins, auxins and gibberellins (Kanno *et al*., 2010). Indeed, inactivation of AHG1 leads to extreme hypersensitivity to ABA-mediated inhibition of germination and *ahg1* shows a delayed germination in the absence of exogenous ABA (Nishimura *et al*., 2007).

The analysis of the interaction between PP2Cs and PYR/PYLs has shown that receptor complexes differ in their sensitivity to ABA-mediated inhibition (Santiago *et al*., 2009; Szostkiewicz *et al*., 2010). However, PP2Cs such as ABI1, ABI2 and HAB1, all appear to be inhibited at least more than 50% by the different PYR/PYLs tested. This situation also applies to PP2CA but not to At5g59220 (Figure 4.4A), which reveals that receptors can discriminate among closely related PP2Cs and preferentially inhibit some of them. Finally, AHG1 represents an

exception to the general mechanism of clade A PP2C inhibition based on ABA and PYR/PYL receptors.

ABA-independent inhibition of some PP2Cs has been recently reported for some monomeric receptors using *in vitro* phosphatase assays (Hao *et al*., 2011). However, in order to achieve high inhibition of the PP2Cs, a 100:1 ratio of receptor to PP2C was used in these assays, and only PYL10, which is not expressed in the Arabidopsis transcriptome database **(**http://signal.salk.edu/cgi-bin/atta?GENE=At4g27920), was effective at 1:1 ratios (Hao *et al*., 2011). At the ratios used in this work, we did not find evidence for a meaningful inhibition of PP2CA or At5g59220 in the absence of ABA by PYR/PYL receptors either using phosphopeptide-based or OST1 dephosphorylation assays. These latter assays are particularly valuable in this context, because monomeric PYLs compete with SnRK2s to interact with PP2Cs (Melcher *et al*., 2009; Soon *et al*., 2011). Indeed, the recent elucidation of a SnRK2-PP2C complex reveals a striking similarity in PP2C recognition by SnRK2 and ABA-bound receptors (Soon *et al*., 2011). Therefore, it was important to test whether the ABA-independent interaction of monomeric PYLs with PP2Cs was strong enough as to efficiently block PP2C-mediated dephosphorylation of SnRK2s (Figure 4.4B). In our hands, major restoration of OST1 activity by PYL-mediated inhibition of PP2Cs was dependent on ABA, which is in agreement with *in vivo* results obtained through protoplast transfection assays (Fujii *et al*., 2009). In the absence of ABA, dimerization in receptors like PYR1, PYL1 and PYL2 prevents basal interactions with the PP2Cs, while monomeric receptors are able to form low-affinity complexes with PP2Cs, but these complexes lack the network of interactions that occur in the ternary complex with ABA (Dupeux *et al*., 2011). For instance, they lack the hydrogen bonds established among the conserved Trp residue of clade A PP2Cs, key residues of the receptor gating loops and the key water molecule that contacts the ketone group of ABA (Melcher *et al*., 2009; Miyazono *et al*., 2009; Dupeux *et al*., 2011). Finally, biochemical analysis of a natural PP2C version lacking the conserved Trp residue, namely AHG1, or the mutants abi1^{W300A} and hab1^{W385A} further support the structural mechanism of ABA signalling, which indicates that ternary receptor:ABA:phosphatase complexes are required to fully inhibit PP2C activity (Melcher *et al*., 2009; Miyazono *et al*., 2009; Dupeux *et al*., 2011).

4.5 Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For plants grown under growth chamber conditions, seeds

were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4ºC for 3 days. Then, seeds were sowed on Murashige-Skoog (1962) (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 μE m^{-2} sec^{-1}.

Subcellular localization studies

Constructs to investigate the subcellular localization of PP2CA (*At3g11410*) and At5g59220 were generated in Gateway-compatible vectors. To this end, the coding sequences of *PP2CA, At5g59220*, the N-terminal extension (residues 1-97) and the catalytic core (residues 98-413) of *At5g59220* were PCR-amplified using the following primer pairs, respectively: FPP2CANcoI, RPP2CAnostopSalI; F5g59220 and Rnostop5g59; F5g59220 and RNterm2CB; FMSTV2CB and Rnostop5g59. The sequences of all primers used in this work are provided as Supplemental Table S1. The PCR products were cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pMDC83 destination vector (Saez *et al*., 2008). The different binary vectors where introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) by electroporation (Deblaere *et al*., 1985). Transformed cells were grown in liquid LB medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM morpholinoethanesulphonic (MES) acid-KOH pH 5.6 containing 10 mM MgCl2 and 150 mM acetosyringone to an OD600 nm of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1 (pCH32 35S:p19) expressing the silencing suppressor p19 of tomato bushy stunt virus (Voinnet *et al*., 2003) so that the final density of Agrobacterium solution was about 1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old *N. benthamiana* plants. Leaves were examined after 3-4 days under a Leica TCS-SL confocal microscope and laser scanning confocal imaging system.

Generation of overexpressing lines for *PP2CA* and *At5g59220*

The coding sequence of *PP2CA* was amplified by PCR using the primers FPP2CANcoI and RPP2CASalI. The coding sequence of *At5g59220* was amplified by PCR using the primers F5g59220 and Rnosto5g59. Next, both were cloned into pCR8/GW/TOPO and recombined by LR reaction into the ALLIGATOR2 destination vector (Bensmihen *et al.,* 2004). The *ALLIGATOR2-35S:3HA-PP2CA* or *35S:3HA-At5g59220* constructs were transferred to *Agrobacterium tumefaciens*

C58C1 (pGV2260) (Deblaere *et al.*, 1985) by electroporation and used to transform Col wt plants by the floral dip method. T1 transgenic seeds were selected based on GFP visualization and T3 progenies homozygous for the selection marker were used for further studies.

Seedling establishment and root growth assays

To determine sensitivity to inhibition of seedling establishment either by ABA, glucose or mannitol, the MS medium was supplemented with the indicated concentration of these compounds. The percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. Approximately 200 seeds of each genotype were sowed in each medium and scored for germination and early growth at 3, 5, 7 and 10 days later. For root growth assays, seedlings were grown on vertically oriented MS medium plates for 4 to 5 days. Afterwards, 20 plants were transferred to new plates containing MS medium lacking or supplemented with the indicated concentrations of ABA. After the indicated period of time, the plates were scanned on a flatbed scanner to produce image files suitable for quantitative analysis using the NIH Image software ImageJ v1.37.

Biochemical fractionation

A nuclear fractionation was performed according to techniques described by Bowler *et al*., (2004) and Cho *et al*., (2006). Arabidopsis leaves of epitope HA-tagged PP2CA or At5g59220 transgenic lines were ground in lysis buffer, 20 mM Tris-HCl pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl2, 250 mM sucrose, containing protease inhibitor cocktail (Roche) and 1 mM PMSF. The lysate was filtered through four layers of miracloth (Calbiochem) and centrifuged at 1000 g for 10 min to pellet the nuclei. The cytosolic fraction was removed and the pellet was washed in nuclei resuspension buffer, 20 mM Tris-HCl, 25% glycerol, 2.5 mM MgCl2 and 0.5% Triton X-100, to solubilize most proteins from the organelles. The nuclear pellet was resuspended in five volumes of medium salt buffer (Bowler *et al*., 2004), 20 mM Tris-HCl, 0.4 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.5 mM PMSF and protease inhibitor cocktail (Roche), and then frozen and thawed. After incubation with gently mix for 15 min at 4ºC, the nuclear insoluble fraction containing the major protein histones was precipitated by centrifugation at 10000 g for 10 min, whereas the supernatant contained the nuclear soluble fraction. Detection of PP2CA or At5g59220 was performed using anti-HA-peroxidase conjugate (Roche). The purity of the different fractions was demonstrated using antibodies against histone H3 (Abcam, UK), plasma membrane H^+-ATPase (Dr. Ramón Serrano, Universidad Politecnica de Valencia) and Ponceau staining of the ribulose 1,6-bisphosphate carboxylase (RBC).

A second fractionation procedure was used to analyze the presence of PP2CA and At5g59220 in cytosol or microsomal pellets (Hua *et al*., 2010). *Arabidopsis* leaves of epitope HA-tagged PP2CA or At5g59220 transgenic lines were ground in lysis buffer, 50mM Tris pH8, 2mM EDTA, 20% Glycerol, 5mM $MgCl_2$, 1mM DTT, 25Mm $CaCl_2$, containing protease inhibitor cocktail (Roche) and 1 mM PMSF. The lysate was filtered through miracloth and centrifuged at 5000g for 5 min to remove organelles and debris. Supernatants were centrifuged at 100000g for 45min to pellet microsomal membranes and to obtain the cytosolic soluble fraction. The resulting microsomal pellet was solubilized in resuspension buffer (25mM Tris pH7.2, 10% sucrose, 2mM EDTA, 5mM $MgCl_2$, 1mM DTT, protease inhibitor cocktail, 0.1 mM PMSF and 25mM $CaCl_2$) using a 2 ml glass homogenizer.

RNA analysis

After mock- or ABA-treatment, plant material was collected and immediately frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit and 1 μg of the RNA obtained was reverse transcribed using 0.1 μg oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40 μl cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems) and they were monitored using the Eva-Green™ fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. Gene induction ratios were calculated as the expression ratio between ABA treated plantlets vs mock treated plantlets. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent experiments. The sequences of the primers used for RT-qPCR amplifications have been previously described (Rubio *et al*., 2009)

Purification of recombinant proteins

The coding sequence of *PP2CA* was amplified by PCR using the primers F2CA*Sal*I*Nde*I and R2CA*Sma*I*Sal*I. The coding sequence of $At5g59220_{98\text{-}413}$ was amplified by PCR using the primers FMSTV2CB and RHAstopP2B. Both were cloned into pCR8/GW/TOPO, next the coding sequence of *PP2CA* was excised from this plasmid using *Nde*I *Sal*I double digestion and subcloned into pET28a, whereas the coding sequence of $At5g59220_{98\text{-}413}$ was excised using *Eco*RI digestion and subcloned into pET28a. The coding sequence of *AHG1* was excised from a pACT2 construct (kindly provided by Dr JF Quintero, CSIC, Sevilla) using *Nco*I *Bam*HI double digestion and

subcloned into pETM11. BL21 (DE3) cells transformed with the corresponding pET28a/pETM11 construct were grown in 50 ml of LB medium supplemented with 50 μg/ml kanamycin to an OD at 600 nm of 0.6-0.8. Then, 1 mM IPTG was added and the cells were harvested 3 h after induction and stored at -80ºC before purification. The pellet was resuspended in 2 ml of buffer HIS (50 mM Tris-HCl pH 7.6, 250 mM KCl, 10 % glycerol, 0.1 % Tween-20, 10 mM mercaptoethanol) and the cells were sonicated in a Branson Sonifier. A cleared lysate was obtained after centrifugation at 14000 g for 15 min, and it was diluted with two volumes of buffer HIS. The protein extract was applied to 0.5 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose column and the column was washed with 10 ml of buffer HIS supplemented with 20 % glycerol and 30 mM imidazol. Bound protein was eluted with buffer HIS supplemented with 20 % glycerol and 250 mM imidazol.

PP2C and OST1 *in vitro* activity assays

Phosphatase activity was measured using the RRA(phosphoT)VA peptide as substrate, which has a Km of 0.5-1 μM for eukaryotic PP2Cs (Donella Deana *et al*., 1990). Assays were performed in a 100 μl reaction volume containing 25 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 25 μM peptide substrate and 0.5 μM PP2C. When indicated, PYR-PYL recombinant proteins and ABA were included in the PP2C activity assay. ABA concentrations were 0.5, 1, 5, 10, 20, 40 and 50 μM. After incubation for 60 min at 30ºC, the reaction was stopped by addition of 30 μl molybdate dye (Baykov *et al.*, 1988) and the absorbance was read at 630 nm with a 96-well plate reader.

Phosphatase activity was also measured using phosphorylated OST1 and ΔCABF2 (amino acids 1-173, containing the C1, C2 and C3 protein kinase targets) as substrates (Vlad *et al.*, 2009; Dupeux *et al*., 2011). Auto-phosphorylated OST1 or trans-phosphorylated ΔCABF2 were prepared in a 60 min reaction. Dephosphorylation of OST1 or ΔCABF2 was achieved by incubation with the different PP2Cs. Assays to test recovery of OST1 activity were done by previous incubation of the PP2C for 10 min in the absence or the presence of 30 μM ABA and the indicated PYR/PYL. Next, the reaction mixture was incubated for 50 min at room temperature in 30 μl of kinase buffer: 20 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, and 3.5 μCi of γ-^{32}ATP (3000 Ci/mmol). The reaction was stopped by adding Laemmli buffer. After the reaction proteins were separated by SDS-PAGE using an 8% acrylamide gel and transferred to an Immobilon-P membrane (Millipore). Radioactivity was detected using a Phosphorimage system (FLA5100, Fujifilm). After scanning, the same membrane was used for Ponceau staining. The data presented are averages of at least three independent experiments.

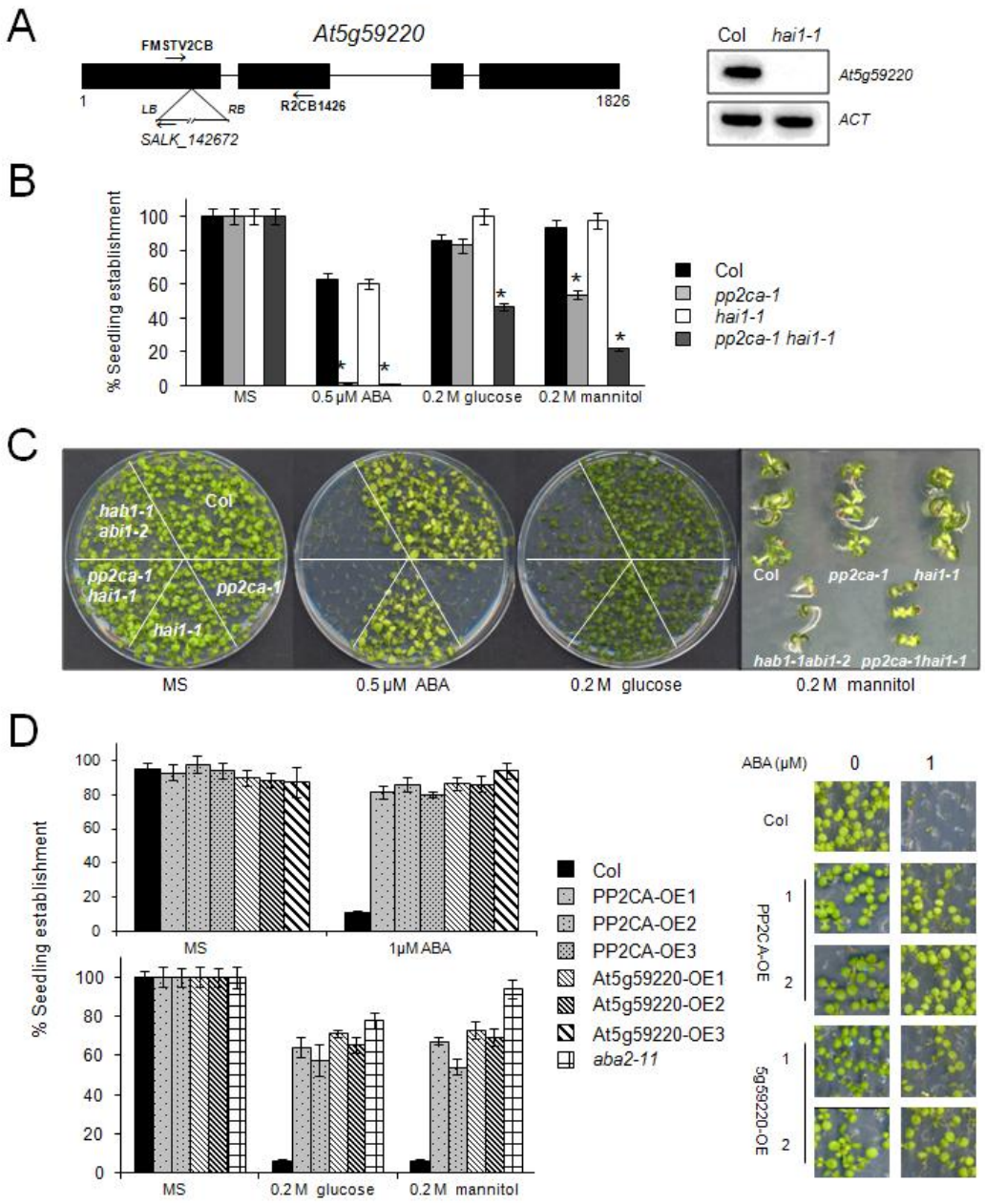

Figure 4.1. A, Schematic diagram of the *At5g59220* gene showing the position of the T-DNA insertion in *hai1-1* mutant. RT-PCR analysis of mRNAs from wt and *hai1-1* mutant seedlings. Primers FMSTV2CB and R2CB1426 were used to partially amplify the *At5g59220* cDNA. B, Seedling establishment of Col wt, *hai1-1*, *pp2ca-1* and double mutant in medium supplemented with ABA, mannitol or glucose. Percentage of seeds that germinated and developed green cotyledons in the different media at 5 days. Values are averages ±SE for three independent experiments (200 seeds each). The asterisk indicates P<0.01 (Student´s t test) with respect to wt. C, Photograph of a representative experiment taken 10 days after sowing. Magnification of representative seedlings grown on MS plates supplemented with 0.2 M mannitol. D, Seedling establishment of wt, *35S:HA-PP2CA* and *35S:HA-At5g59220* lines in medium supplemented with either 1 mM ABA (top panel), 0.2 M glucose or 0.2 M mannitol (bottom panel). Approximately 200 seeds of each genotype were sowed on each plate and scored 4 d later. Photographs were taken after 8 d.

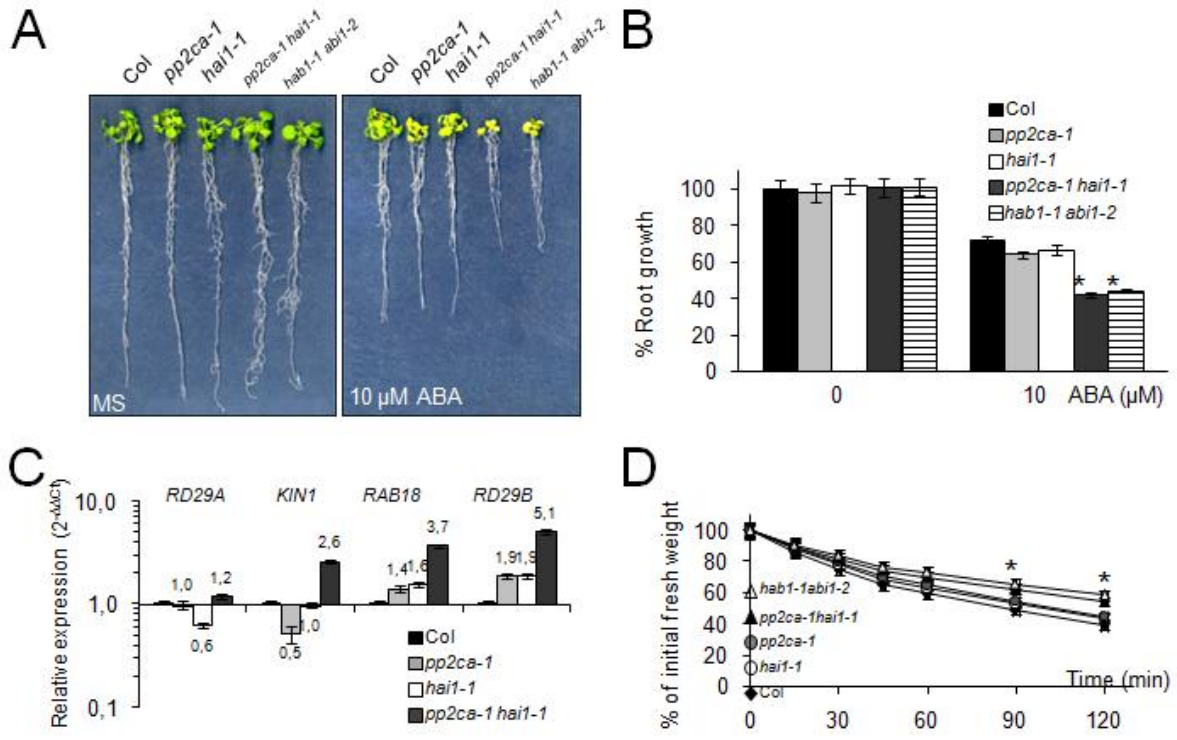

Figure 4.2. A, ABA-hypersensitive growth inhibition of *pp2ca-1 hai1-1* and *hab1-1 abi1-2* double mutants compared to wt and single parental mutants. Photograph of representative seedlings 10 days after the transfer of 4-day-old seedlings from MS medium to plates lacking or supplemented with 10 µM ABA. B, Quantification of ABA-mediated root growth inhibition of *pp2ca-1 hai1-1* and *hab1-1abi1-2* double mutants compared to wt and single parental mutants. Data are averages ±SE from three independent experiments (n =15 each). The asterisk indicates P<0.01 (Student´s t test) with respect to wt. C, Relative expression of ABA-responsive genes in *pp2ca-1 hai1-1* double mutant compared to wt and single parental mutants. RT-qPCR analyses were made in triplicate on RNA samples of 2-week-old seedlings that were either mock or 10 µM ABA-treated for 3 h. Numbers indicate the induction level of the genes in each mutant genotype with respect to the wt (value 1).

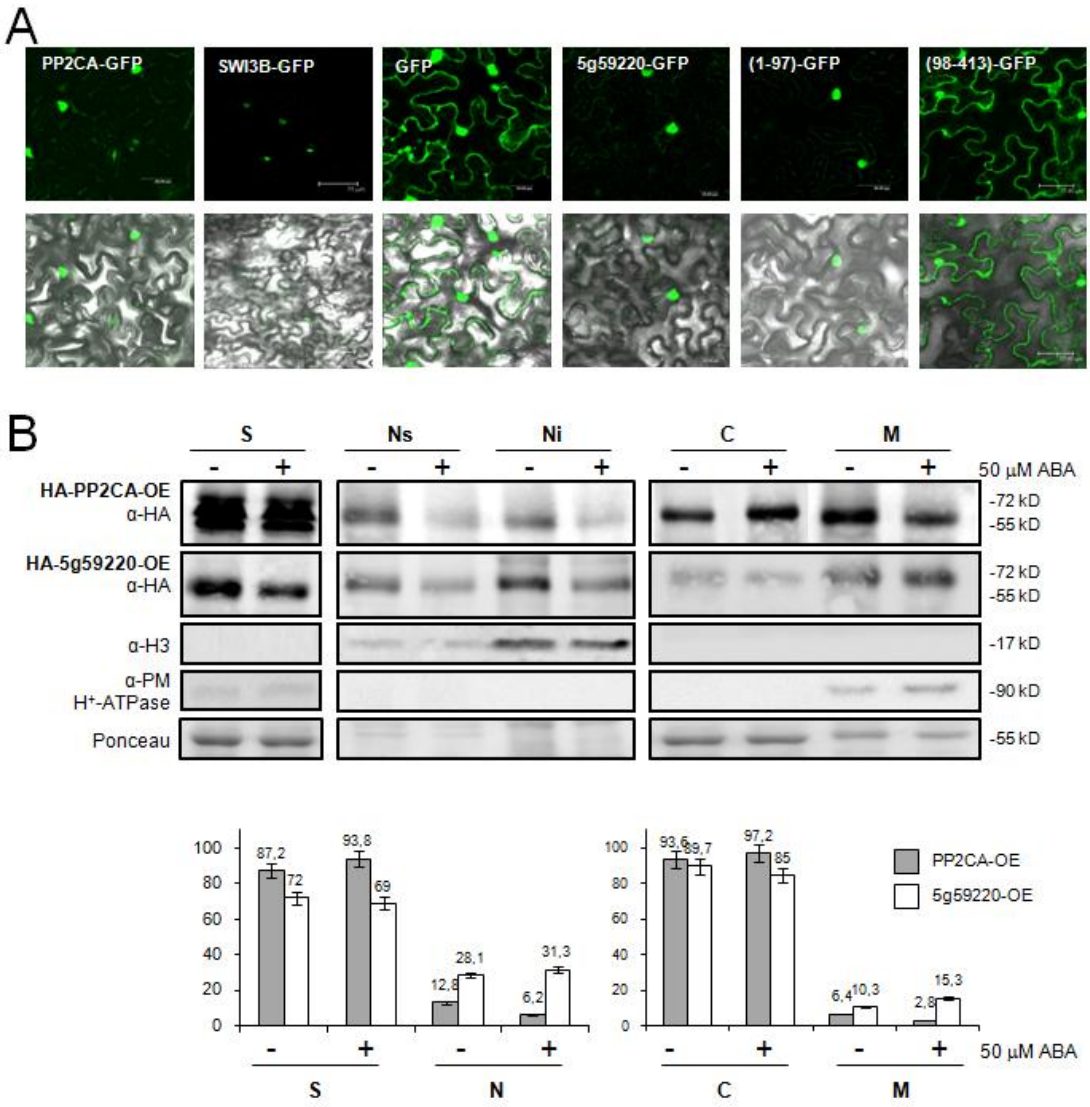

Figure 4.3. Subcellular localization of PP2CA and At5g59220. A, Subcellular localization of PP2CA-GFP and At5g59220-GFP proteins transiently expressed in tobacco cells. Epifluorescence and bright-field images of epidermal leaf cells infiltrated with a mixture of Agrobacterium suspensions harboring the indicated constructs and the silencing suppressor p19. SWI3B is a nuclear protein that forms part of SWI/SNF chromatin-remodeling complexes (Saez *et al*., 2008). The N-terminal extension (residues 1-97) and the catalytic core (residues 98-413) of *At5g59220* were expressed as fusions with GFP. B, Biochemical fractionation of HA-PP2CA and HA-At5g59220 proteins. Plant material was obtained from epitope HA-tagged PP2CA or At5g59220 transgenic lines after mock- or 50 mM ABA treatment for 1 h. Samples were analyzed using anti-HA, anti-histone 3 (H3), anti plasma membrane H^+-ATPase antibodies and Ponceau staining of the ribulose-1,5-bis-phosphate carboxylase/oxygenase. Localization of HA-PP2CA and HA-At5g59220 proteins in soluble (S), total nuclear (N), nuclear soluble (Ns), nuclear insoluble (Ni), cytosolic (C) or microsomal (M) fractions is indicated. Histograms show the relative amount of each protein in the different fractions. OE indicates overexpression lines.

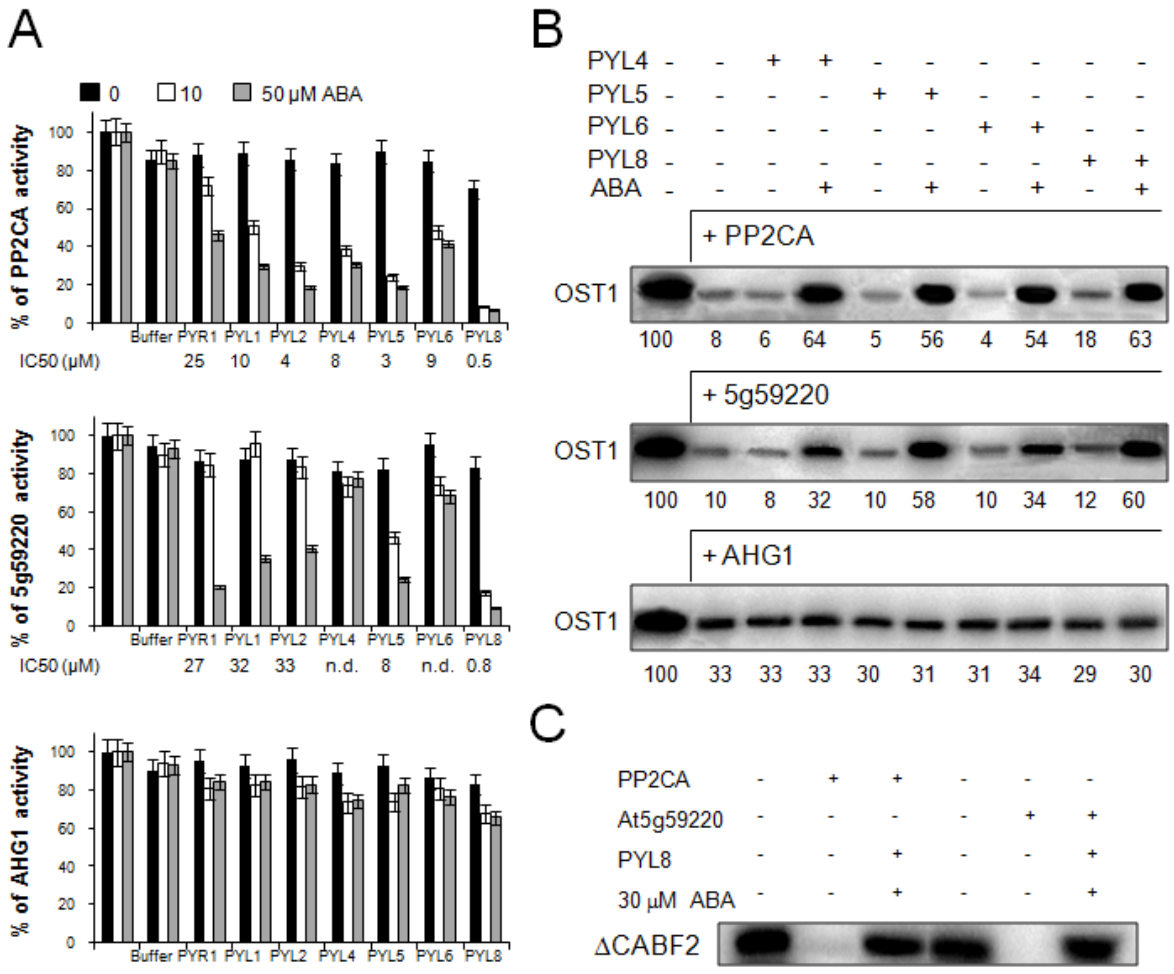

Figure 4.4. Differential sensitivity of PP2CA, At5g59220 and AHG1 to ABA-dependent PYR/PYL-mediated inhibition. A, Phosphatase activity of the different PP2Cs was measured *in vitro* using a phosphopeptide substrate in the absence or the presence of 10 or 50 mM ABA and the indicated receptors. Data are averages ±SD for three independent experiments. Phosphatase assays were performed in a 100 µl reaction volume containing either 2.3 µg His_6-PP2CA, 2.1 µg His_6-ΔNAt5g59220 or 2.5µg of His_6-AHG1 and between 5 to 5.7 µg of the different His_6-PYR/PYL proteins in order to obtain an 1:4 phosphatase:receptor stoichiometry. The activities of the PP2C recombinant proteins in the absence of ABA (100% activity) were 12.2 ±0.3, 13.3 ± 0.2 and 11.0 ± 0.2 nmol $Pi \cdot min \cdot mg^{-1}$, respectively. In order to check the effect of the HIS elution buffer on the PP2C activity we performed an assay lacking PYR/PYL proteins but adding an equivalent volume of HIS elution buffer. B, *In vitro* OST1 kinase activity in the absence or the presence of 30 mM ABA and the indicated receptors. An 1:10 phosphatase:receptor stoichiometry was used in this assay. The quantification of the autoradiography (numbers below) shows the percentage of phosphorylation of OST1 in each reaction relative to the first reaction (100%, phosphorylation of OST1 in the absence of PP2Cs). C, Dephosphorylation of DCABF2 by PP2CA and At5g59220 in the absence or the presence of 30 mM ABA and PYL8.

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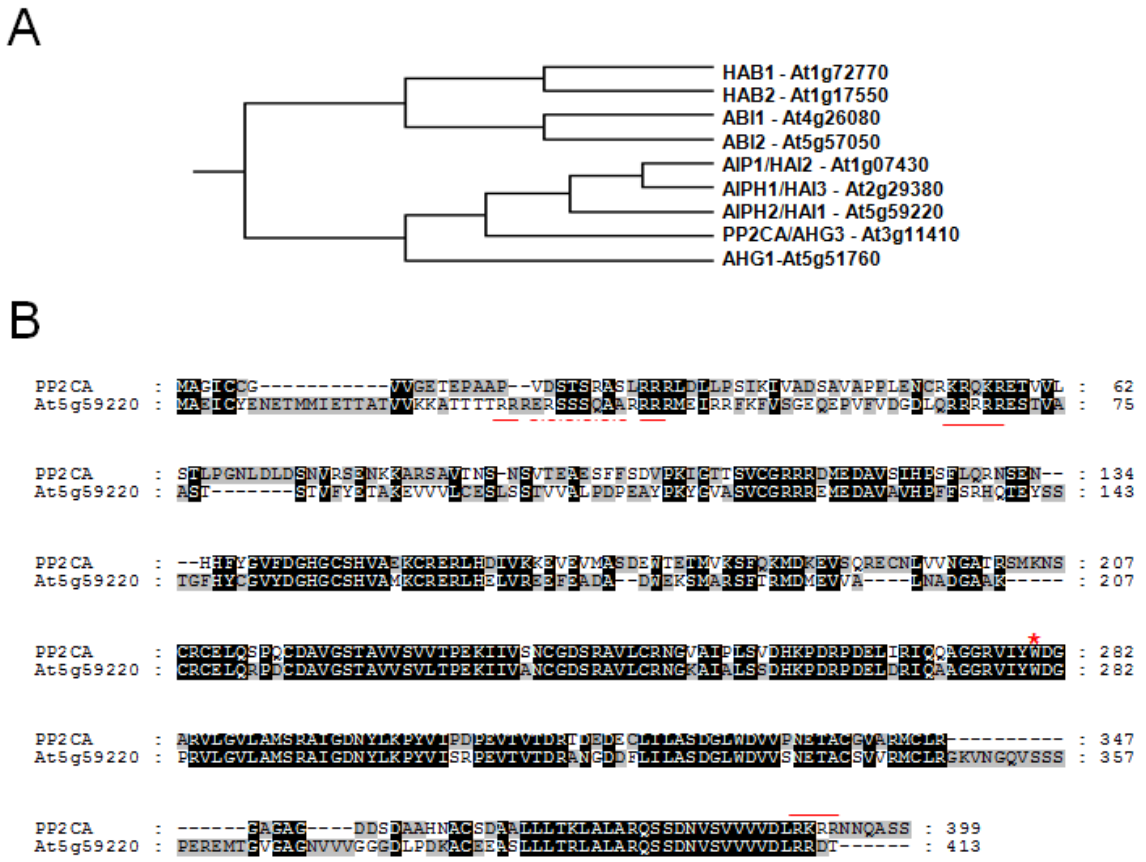
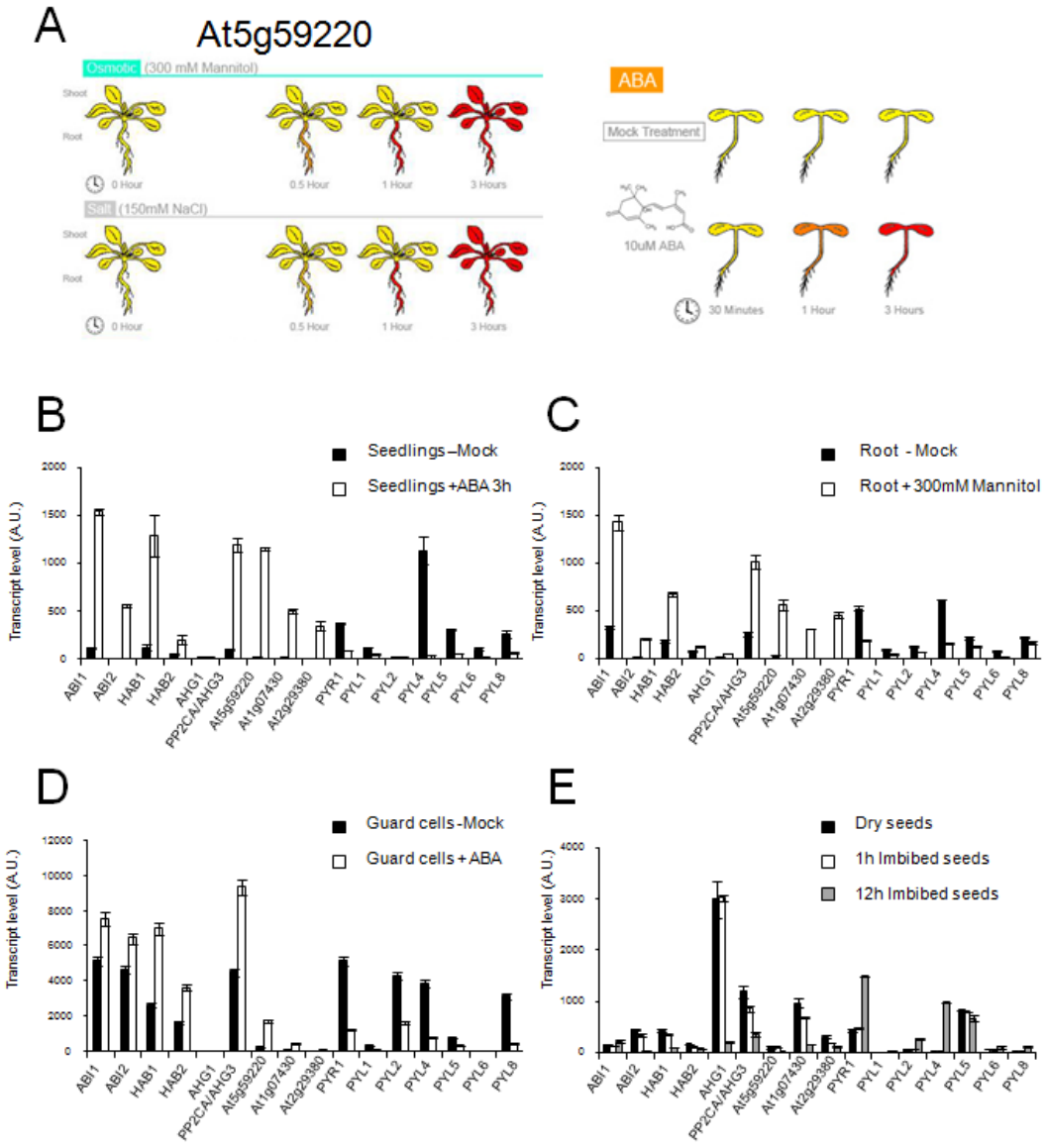
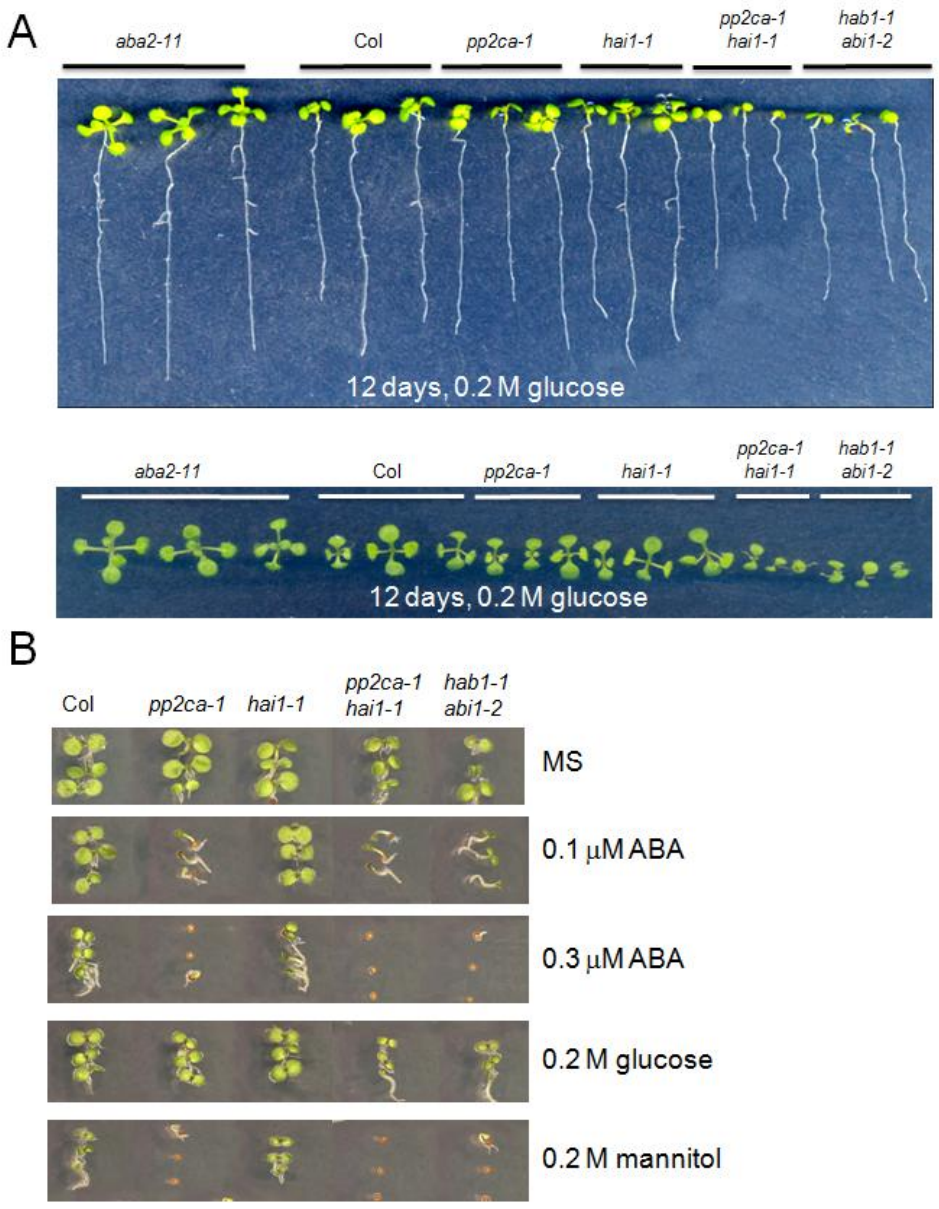
4.7 Supplemental material



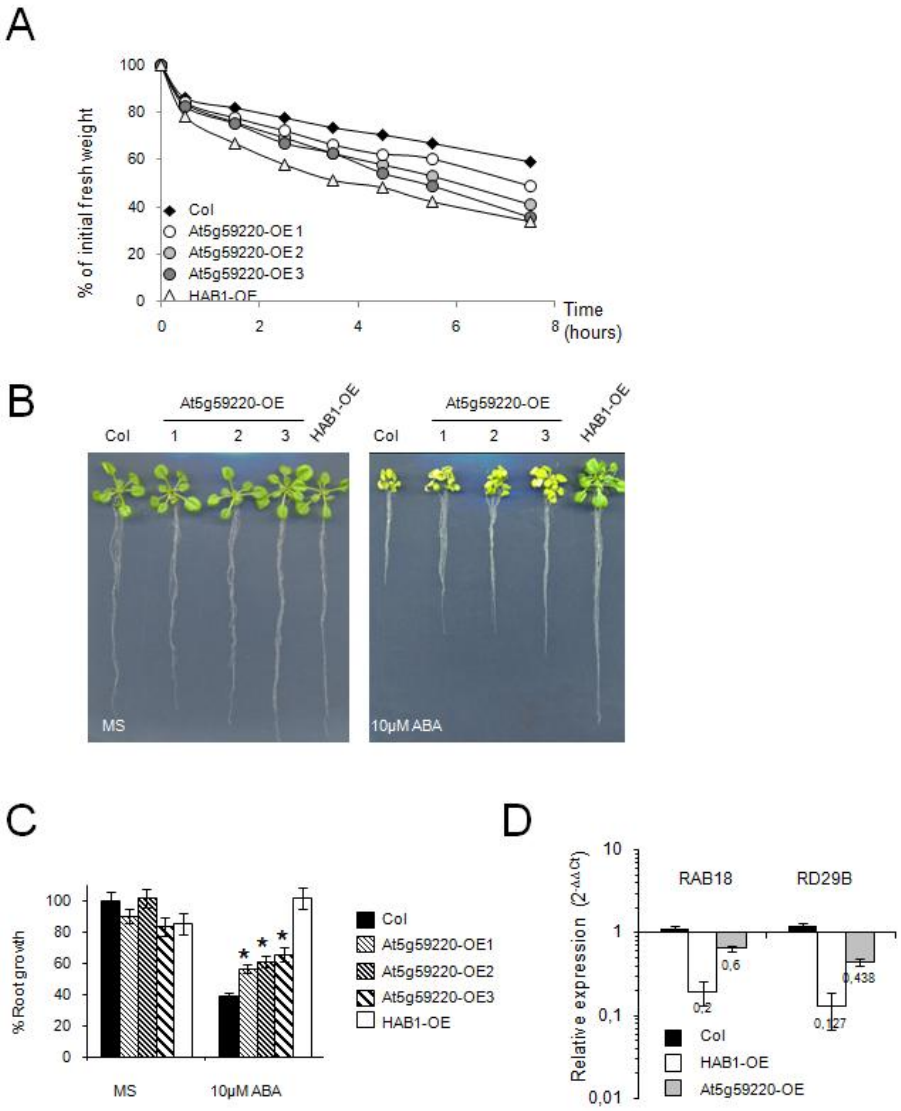
Supplemental Figure S1. Cladogram and nomenclature of Clade A PP2Cs. Position of nuclear localization signals and the conserved Trp residue in PP2CA and At5g59220. A, Cladogram, according to Schweighofer *et al*., (2004). B, Position of nuclear localization signals and the conserved Trp residue in PP2CA and At5g59220. A discontinuous line indicates the bipartite nuclear localization of At5g59220, whereas continuous lines mark the four basic residues of both PP2CA and At5g59220. An asterisk indicates the position of the conserved Trp residue described in the text.



Supplemental Figure S2. Upregulation of *At5g59220* gene expression by osmotic stress (300 mM mannitol or 150 mM NaCl) and ABA. Expression levels of clade A PP2Cs and seven PYR/PYLs in whole 7-day-old seedlings, root, guard cells and seeds. A, Mannitol, NaCl and ABA induce At5g59220 expression. Data were obtained from the Bio-Array Resource for Arabidopsis Functional Genomics (http://bar.utoronto.ca) (Winter *et al*., 2007). B, Expression levels of clade A PP2Cs in whole 7-day-old seedlings that were either mock- or ABA-treated for 3 h (data produced by the AtGen-Express Consortium; http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm). C, Expression levels in roots that were either mock- or 300 mM mannitol treated (Kilian *et al*., 2007). D, Expression levels in guard cells that were either mock- or ABA-treated (Yang *et al*., 2008). E, Expression levels in dry seeds, or 1 and 12h imbibed seeds (Nakabayashi *et al*., 2005).



Supplemental Figure S3. Glucose-hypersensitive growth inhibition of *pp2ca-1 hai1-1* and *hab1-1 abi1-2* double mutants compared to wt and single parental mutants. A, Seedling growth after 12 days of *aba2-11*, Col wt, *pp2ca-1*, *hai1-1*, *pp2ca-1 hai1-1* and *hab1-1 abi1-2* double mutants in medium supplemented with 0.2 M glucose. Approximately 200 seeds of each genotype were sowed on MS plates supplemented with 0.2 M glucose. After 12 days, representative seedlings were removed from the medium, rearranged in a new plate and photographed under a Nikon SMZ800 binocular glass. B, Germination and seedling establishment of Col wt, *pp2ca-1*, *hai1-1*, *pp2ca-1 hai1-1* and *hab1-1 abi1-2* double mutants in medium lacking or supplemented with either ABA, glucose or mannitol. The photograph was taken 5 days after sowing.



Supplemental Figure S4. Analysis of water-loss, ABA-mediated growth inhibition and expression of two ABA-responsive genes in *35S:HAB1* and *35S:At5g59220* lines compared to wt. A, Enhanced water loss of *35S :HAB1* and *35S:At5g59220* lines compared to Col wt. Five leaves at the same developmental stage were detached from 21-d-old plants and fresh weight was determined after submitting them to the drying atmosphere of a flow laminar hood (*n* =4 plants per experiment). B, C ABA-hypersensitive root growth inhibition of *35S :HAB1* and three *35S:At5g59220-OE* lines compared to wild-type. B, Photograph of representative seedlings 10 days after the transfer of 4-day-old seedlings from MS medium to plates lacking or supplemented with 10 mM ABA. Root growth was scored after 10 days. Data are averages ±SE from three independent experiments (n=20 seedlings per experiment). Asterisk indicates $P < 0.01$ (Student's t test) when comparing data for each genotype versus the wild-type under the same assay conditions. D, Relative expression of two ABA-responsive genes in *35S:HAB1* and *35S:At5g59220* plants compared to wt. RT-qPCR analyses were made in triplicate on RNA samples of 2-week-old seedlings that were either mock or 10 mM ABA-treated for 3 h. Numbers indicate the induction level of the genes in each over-expression line with respect to the wt (value 1).

6. Results: Chapter 3

PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in Root

5.1 Introduction

Control of abscisic acid (ABA) signaling by PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) ABA receptors involves direct ABA-dependent inhibition of clade A phosphatases type 2C (PP2Cs), such as ABA-INSENSITIVE1 (ABI1), HYPERSENSITIVE TO ABA1 (HAB1), and PP2CA, which are key negative regulators of the pathway (Saez *et al*., 2004, 2006; Rubio *et al*., 2009). Under resting conditions, clade A PP2Cs can interact with and dephosphorylate SnRK2.2, SnRK2.3, and SnRK2.6/OST1, reducing their catalytic activity (Fujii *et al*., 2009; Umezawa *et al*., 2009; Vlad *et al*., 2009). The increase of ABA levels in the plant cell leads to PYR/PYL receptor-mediated inhibition of PP2C activity, which results in the activation of the three SnRK2s and ultimately of the ABA signaling pathway (Ma *et al*., 2009; Park *et al*., 2009; Umezawa *et al*., 2009; Vlad *et al*., 2009; Gonzalez-Guzman *et al*., 2012). Biochemically, SnRK2s are activated through the phosphorylation of certain Ser residues of their activation loop, including Ser-175, either by autophosphorylation or by yet unidentified upstream activating kinases that are staurosporine resistant (Boudsocq *et al*., 2007; Fujii *et al*., 2009; Umezawa *et al*., 2009; Vlad *et al*., 2009, 2010). Next, the SnRK2s directly phosphorylate transcription factors that bind to ABA-responsive promoter elements and components of the machinery regulating stomatal aperture, like the slow anion channel SLAC1, K^+ inward channel KAT1, or reactive oxygen species biosynthetic enzymes such as the NADPH oxidase AtrbohF (Kobayashi *et al*., 2005; Fujii *et al*., 2009; Geiger *et al*., 2009; Lee *et al*., 2009; Sato *et al*., 2009; Sirichandra *et al*., 2009).

PYR/PYL ABA receptors constitute a 14-member family, and all of them (except PYL13) are able to activate ABA-responsive gene expression using protoplast transfection assays (Fujii *et al*., 2009). However, gene expression patterns obtained from public databases and GUS reporter gene analyses have revealed substantial differences among them (Gonzalez-Guzman *et al*., 2012). Thus, the expression of *PYL3* and *PYL10* to *PYL13* is very low to undetectable in different whole-genome microarrays, whereas the expression of *PYR1* and the rest of *PYL1* to *PYL9* could be detected in both vegetative and reproductive tissues, although at different levels (Gonzalez-Guzman *et al*., 2012). From a biochemical point of view, recent studies reveal at least two families of PYR/PYL receptors characterized by a different oligomeric state, some being dimeric (PYR1, PYL1, and PYL2), whereas others are monomeric (PYL5, PYL6, and PYL8; Dupeux *et al*., 2011). The dimeric receptors show a higher dissociation constant for ABA (greater than 50 μM; lower affinity) than monomeric ones (approximately 1 μM); however, in the presence of the PP2C, both groups of receptors form ternary complexes with high affinity for ABA (dissociation constant of 30–60 nM; Ma *et al*., 2009; Santiago *et al*., 2009), and physiological characterization of some ABA

responses in different multiple pyr/pyl mutants did not reveal a clear difference between dimeric and monomeric receptors (Gonzalez-Guzman *et al*., 2012). Finally, both the biochemical properties of the PYR/PYL receptors and *in silico* modeling of the ABA activation pathway reveal adequate coverage of the full spectrum of physiological ABA concentrations, ranging from basal ABA levels (nanomolar range) to high levels induced by water stress (micromolar range; Priest *et al*., 2006).

Gene expression patterns, biochemical analysis of different PP2C-PYL receptor complexes, and genetic analysis of different *pyr/pyl* mutants suggest that the function of PYR/PYL proteins is not completely redundant (Santiago *et al*., 2009; Szostkiewicz *et al*., 2010; Antoni *et al*., 2012; Gonzalez-Guzman *et al*., 2012). However, some functional redundancy exists, since the generation of a *pyr1pyl1pyl2pyl4* quadruple mutant, *1124*, was required to obtain robust ABA-insensitive phenotypes (Park *et al*., 2009), and a *pyr1pyl1pyl2pyl4pyl5pyl8* sextuple mutant, *112458*, is at least 1 order of magnitude more ABA insensitive than 1124 (Gonzalez-Guzman *et al*., 2012). Recently, analysis of mutants lacking three, four, five, or six PYR/PYLs has revealed quantitative regulation of ABA signaling by this family of receptors (Gonzalez-Guzman *et al*., 2012). Finally, GUS reporter analyses of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5*, and *PYL8* promoters has shown both overlapping and differential expression in different tissues (Gonzalez-Guzman *et al*., 2012). For instance, in 5-d-old seedlings, only *PYR1* and *PYL5* were expressed in the cortex of the upper part of the root, whereas *PYL1*, *PYL4*, and *PYL8* were expressed in the columella cells. On the other side, overlapping expression of *PYR1*, *PYL1*, *PYL2*, *PYL4*, and *PYL8* in root vascular tissue was observed (Gonzalez-Guzman *et al*., 2012).

ABA regulates root growth and root architecture, likely interacting with other hormones in these processes, such as auxins, gibberellins, or brassinosteroids (Deak and Malamy, 2005; Swarup *et al*., 2005; Péret *et al*., 2009; Rodrigues *et al*., 2009; Ubeda-Tomás *et al*., 2009, 2012; Hacham *et al*., 2011). ABA signaling in the root is required for different processes, such as the maintenance of primary root elongation and the repression of lateral root formation when water availability is reduced (Sharp *et al*., 2004; Deak and Malamy, 2005). Recent results in 17 natural accessions of Arabidopsis (*Arabidopsis thaliana*) revealed increased root-versus-shoot biomass partitioning as a crucial plant response to cope with water stress (Des Marais *et al*., 2012). Several mechanisms dependent on ABA signaling have been proposed to maintain root elongation at low water potentials, such as osmotic adjustment in the root tip, restriction of ethylene production, and control of K^+ translocation from root to shoot (Gaymard *et al*., 1998; Sharp *et al*., 2004). Enhanced cell wall loosening is required to maintain root elongation at low water potential, and indeed, ABA induces xyloglucan endotransglycosylase, which is a cell wall-degrading enzyme (Wu *et al*., 1994). Thus, the role of ABA in maintaining root growth under water deficits has been well established

(Sharp *et al*., 2004); however, high concentrations of ABA inhibit root growth. Another important function of ABA is the regulation of the hydrotropic response (i.e. a genuine response of roots to a moisture gradient). Results from Takahashi *et al*. (2002) indicate that ABA constitutes an intrinsic signal in hydrotropism, since both *aba1-1* and *abi2-1* mutants were less sensitive to hydrotropic stimulation, whereas the addition of ABA to *aba1-1* restored its capacity to perceive the moisture gradient. Additionally, the *no hydrotropic response1* mutant of Arabidopsis showed reduced ABA sensitivity in root (Eapen *et al*., 2003), and ABA induces the expression of *MIZ1*, a gene essential for hydrotropism (Kobayashi *et al*., 2007). The regulation of root growth by ABA must be closely connected with hydrotropism, since the hydrotropic response likely involves asymmetric transmission of ABA signaling to the root sides that are in contact with different water potentials.

In this work, root expression analyses served to pinpoint relevant ABA receptors in the root, particularly *PYL8.* Finally, we found that PYR/PYLs and clade A PP2Cs play an important role for the ABA-mediated root hydrotropic response.

5.2 Previous results obtained at our laboratory.

The root ABA sensitivity of different *pyr/pyl* single mutants was analyzed. Probably due to the multigenic nature and partial functional redundancy observed in the PYR/PYL family, *pyl8-1* was the only single mutant that showed reduced inhibition of root growth compared with the wild type even at 20µM ABA, whereas the rest of the single mutants did not show significant differences from the wild type in that response. Moreover, using tandem affinity purification (TAP) technology and mass spectrometry (MS) analyses it was able to identify 5 clade A PP2Cs that interact *in vivo* with PYL8.

A detailed reporter gene analysis of *PYR1, PYL1, PYL2, PYL4, PYL5*, and *PYL8* promoters suggested no completely redundant functions for PYR/PYL genes. Expression of *PYR1, PYL1, PYL2, PYL4*, and *PYL8* was detected in the vascular bundle of the primary root, whereas *PYR1* and *PYL5* were expressed in the cortex of the upper part of the root. Interestingly, *PYL1, PYL4*, and *PYL8* were also expressed in the columella cells. Finally, ABA treatment inhibited or strongly attenuated GUS expression driven by these promoters.

5.3 Results

Reporter gene analysis of *PYR1, PYL1, PYL2, PYL4, PYL5, PYL6, PYL7, PYL8* and *PYL9* promoters in root.

We have completed the GUS reporter analysis of the PYR/PYL family by generating additional GUS reporter lines for *PYL6, PYL7,* and *PYL9* promoters (Fig. 5.1). Root expression of GUS driven by the *PYL6* promoter was almost undetectable, expression driven by the *PYL7* promoter was weak and could only be detected after 6 h of incubation with the GUS substrate, whereas *ProPYL9:GUS* lines showed GUS staining after 3 h (Fig. 5.1). Additionally, we used a modified pseudo-Schiff propidium iodide (PS-PI) staining method to get a detailed GUS staining of the apical root (Fig. 5.1). After 3 h of incubation with the GUS substrate, we could detect GUS staining in stele cells of the *ProPYR1:GUS*, *ProPYL1:GUS, ProPYL2:GUS, ProPYL4:GUS, ProPYL8:GUS,* and *ProPYL9:GUS* lines as well as root epidermis and lateral root cap for *PYL8* (Fig. 5.1, A and D). PS-PI staining combined with confocal laser scanning microscopy produced high-resolution images; however, it eliminated GUS staining of columella cells in *ProPYL1:GUS, ProPYL4:GUS,* and *ProPYL8:GUS* lines, which was detected previously (Gonzalez-Guzman *et al*., 2012; Fig. 5.1, E). In order to get an estimation of GUS expression in the whole root, we performed a quantitative GUS activity assay in extracts of root tissue prepared from 15 day old seedlings by using 4-methylumbelliferyl β-D-glucuronide as a substrate (Fig. 5.1, B). GUS activity was particularly high for *ProPYL8:GUS, ProPYL1:GUS, ProPYR1:GUS, ProPYL9:GUS,* and *ProPYL2:GUS* genes, whereas the expression of *ProPYL4:GUS, ProPYL5:GUS,* and *ProPYL7:GUS* genes was lower and *ProPYL6:GUS* was almost undetectable (Fig. 5.1, B). These results were in good agreement with immunoblot analysis of the corresponding protein extracts using anti-GUS antibody (Fig. 5.1C), and they provide a quantitative estimation on the expression of the different PYR/PYL receptors in root. Finally, ABA treatment inhibited or strongly attenuated GUS expression driven by these promoters (figure 5.2, Antoni *et al.*, 2013).

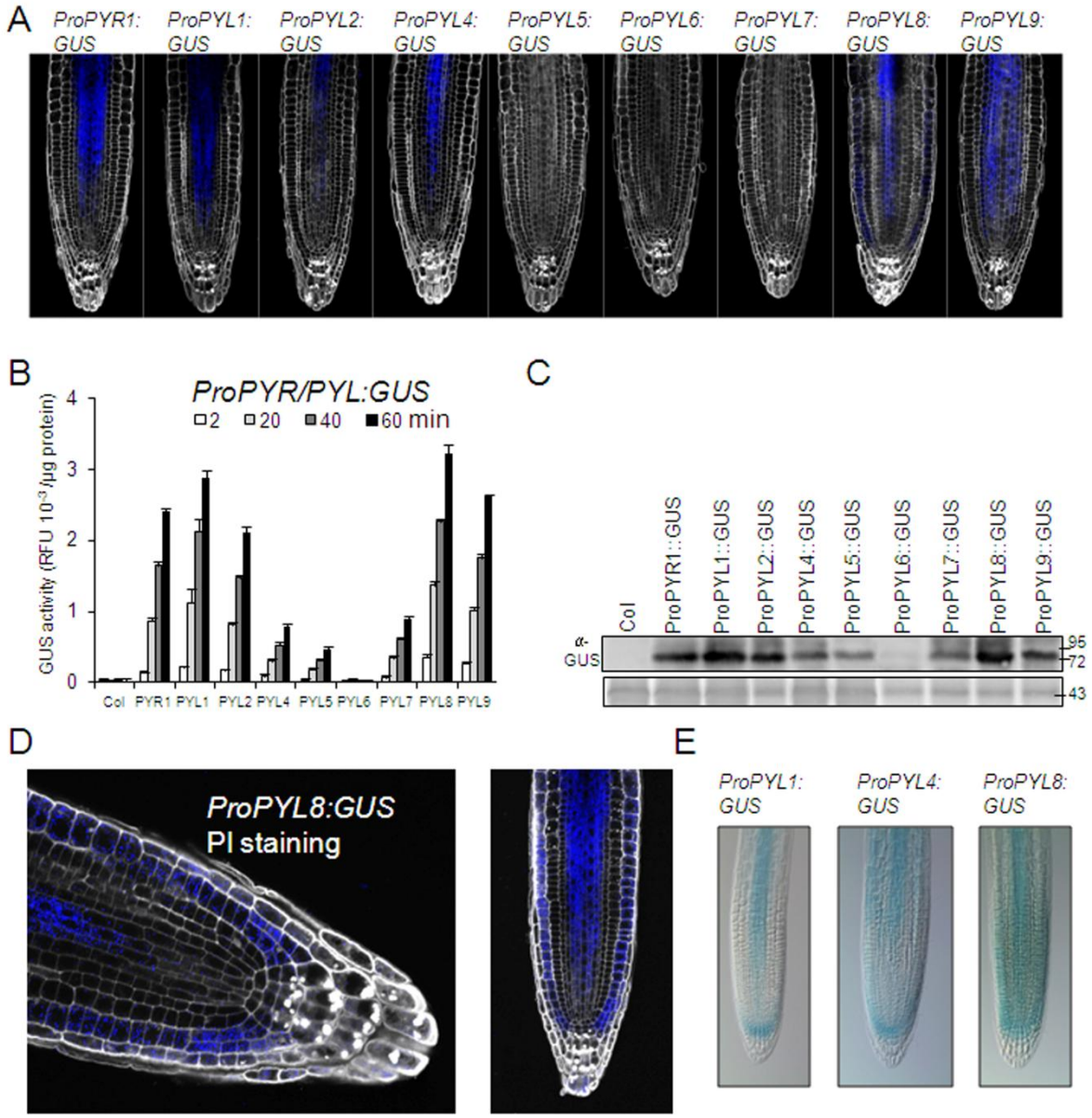


Figure 5.1. GUS expression driven by *ProPYR1:GUS, ProPYL1:GUS, ProPYL2:GUS, ProPYL4:GUS, ProPYL5:GUS, ProPYL6:GUS, ProPYL7:GUS, ProPYL8:GUS*, and *ProPYL9:GUS* genes in the apical root. A, GUS expression visualized using modified PS-PI staining and confocal laser scanning microscopy. B, Quantification of GUS activity in 15-d-old roots using 4-methylumbelliferyl β-D-glucuronide as a substrate. RFU, Relative fluorescence units. C, Immunoblot analysis of protein extracts from 15-d-old roots using anti-GUS antibody. Ponceau staining from a 43-kD protein is shown as a loading control. D, Magnification of the apical root from *ProPYL8:GUS* lines that were stained as described in A. E, GUS expression driven by *ProPYL1:GUS*, *ProPYL4:GUS*, and *ProPYL8:GUS* genes in columella cells. GUS staining was observed in the absence of subsequent PS-PI staining.

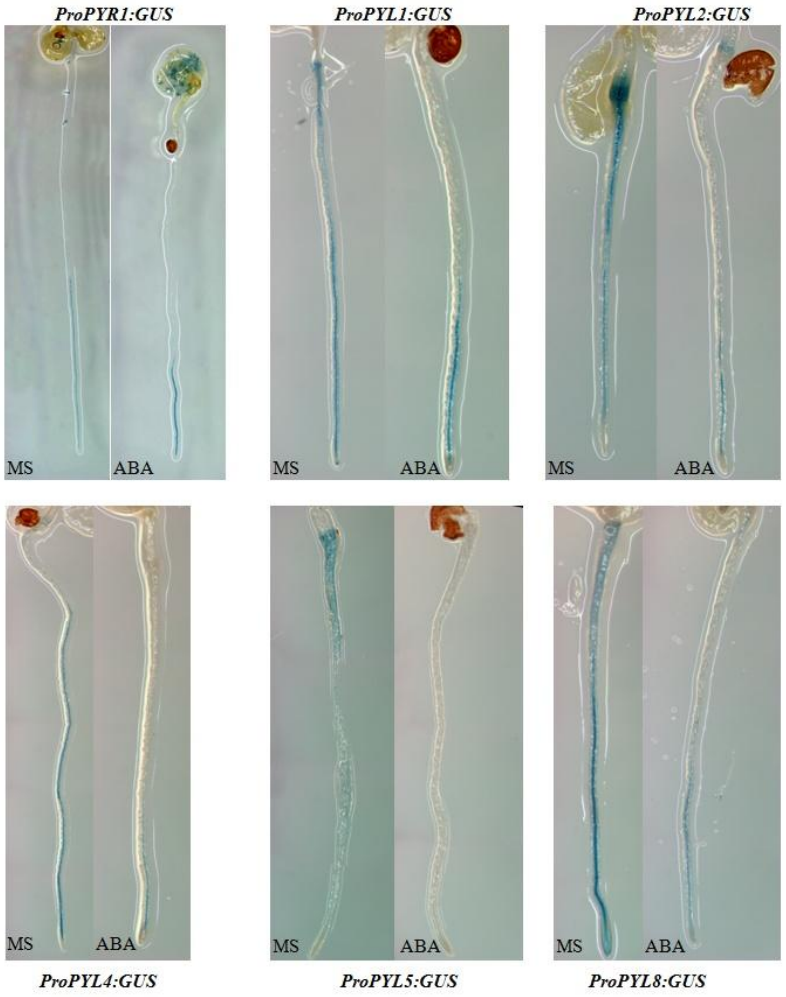


Figure 5.2. ABA treatment inhibits or attenuates GUS expression driven by *ProPYL1, ProPYR1, ProPYL2, ProPYL4, ProPYL5* and *ProPYL8:GUS* genes. Photographs show 5-d-old seedlings that were either mock or 10 µM ABA -treated for 10 h.

PYR/PYL receptors and clade A PP2Cs mediate the root hydrotropic response

PYR/PYL receptors and clade A PP2Cs are key players for ABA signaling in root, and taking into account the important role of ABA for hydrotropism, we decided to investigate their role in the root hydrotropic response. Since PYL8 plays an important role for ABA signaling in root and interacts at least with five clade A PP2Cs, we generated an *abi1-2abi2-2hab1-1pp2ca-1* quadruple mutant, abbreviated as *Qabi2-2* (Fig. 5.3, A and B). The *Qabi2-2* mutant is impaired in four PYL8-interacting PP2Cs, and it turned out to be very hypersensitive to ABA-mediated inhibition of root and shoot growth (Fig. 5.3, A and B). Using the experimental system developed by Takahashi *et al*.

(2002), which uses split agar plates containing sorbitol in the region with low water potential, we measured the hydrotropic response of mutants showing enhanced or impaired ABA signaling (Fig. 5.3, C and D). In this assay, Murashige and Skoog medium containing 1% agar and agar containing 400 mM sorbitol are placed side by side, which generates a water potential gradient at the border between the two media (Fig. 5.3, E and F). Thus, we analyzed the hydrotropic responses of the strongly ABA-hypersensitive *Qabi2-2* mutant and the ABA-insensitive *112458* sextuple mutant, which is strongly impaired in ABA perception through PYR/PYL receptors (Gonzalez-Guzman *et al*., 2012). As a result, we found that the *Qabi2-2* mutant showed enhanced root curvature compared with the wild type when faced with a medium containing −1 MPa sorbitol (Fig. 5.3C). Conversely, the *112458* mutant showed reduced root curvature compared with the wild type (Fig. 5.3D). This response had important consequences, since seedlings of the *Qabi2-2* mutant avoided better than the wild type the entrance in medium with low water potential, whereas seedlings of the *112458* mutant were impaired in that response (Fig. 5.3, E and F).

5.4 Discussion

The expression in the root of *PYR1, PYL1, PYL2, PYL4, PYL8* and *PYL9* was predominant in the vascular tissue (Fig. 5.2; Gonzalez-Guzman *et al*., 2012), where ABA biosynthetic enzymes are localized as well (Cheng *et al*., 2002; Tan *et al*., 2003). Active ABA signaling in the root vascular tissue that carries out ABA biosynthesis might act as a positive feedback for ABA production or play a regulatory role for different transport processes (Gaymard *et al*., 1998; Barrero *et al*., 2006). Additionally, expression in columella cells could also be detected for *PYL1, PYL4*, and *PYL8*. Active pools of ABA have been detected in the columella cells by a *ProRD29B:GUS* reporter system, which suggests that even in the absence of stress, ABA signaling occurs in these cells (Christmann *et al*., 2005). Root columella cells play a key role for sensing gravity in a process governed by auxins, and the presence of ABA receptors in this region suggests that ABA signaling might somehow affect auxin signaling in this area. For instance, it has been proposed that the degradation of starch grains in amyloplasts in columella cells is required to have a hydrotropic response, since gravitropism would be inhibitory to hydrotropism (Takahashi *et al*., 2003). It has also been suggested that starch degradation in the columella cells of roots subjected to osmotic stress might be an osmoregulatory mechanism to increase osmolite concentration and to sustain Glc supply under water stress (Ponce *et al*., 2008). Since ABA signaling is required both for hydrotropism and osmoregulation of water-stressed roots (Takahashi *et al*., 2002; Sharp *et al*., 2004; this work), the presence of PYR/PYL receptors in columella cells might contribute to regulate both processes.

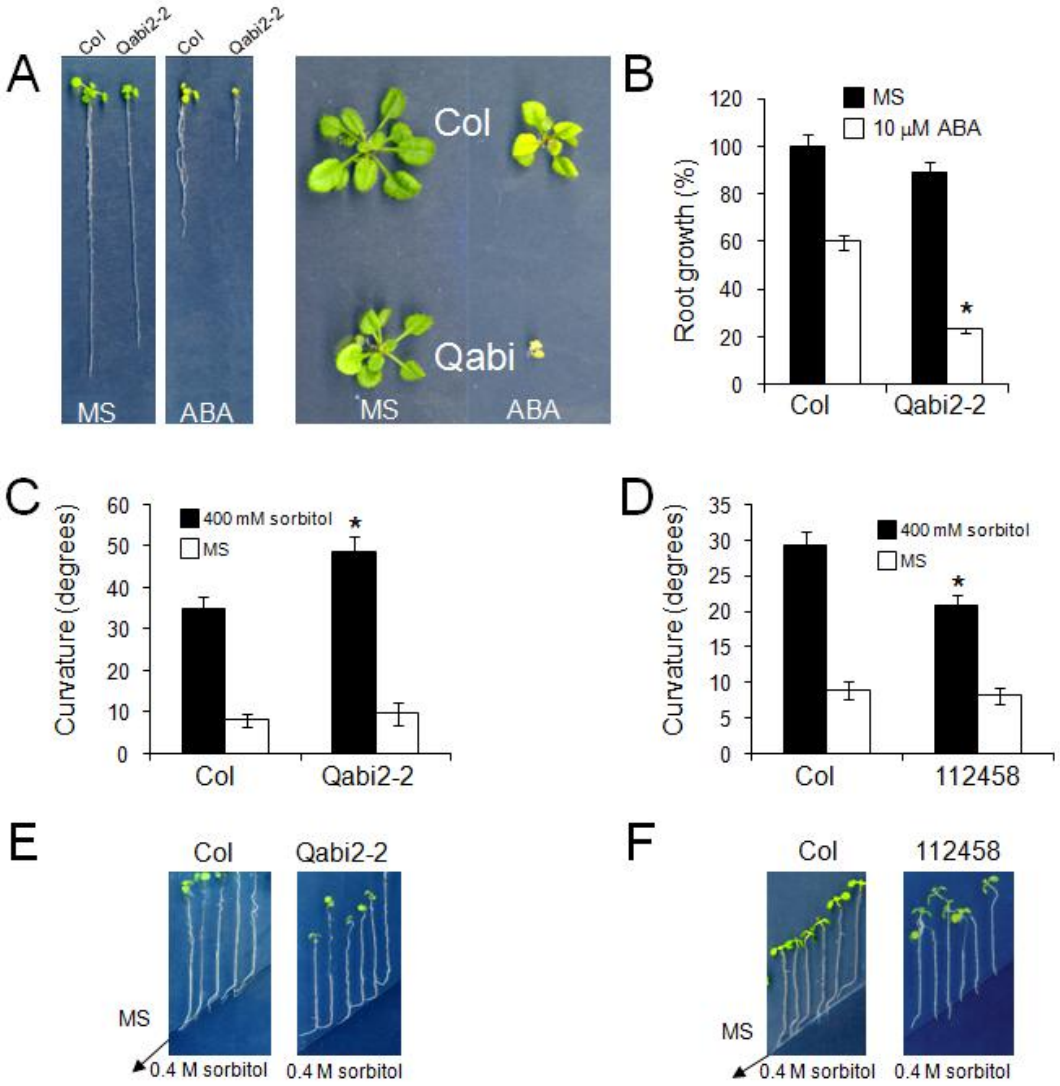


Figure 5.3.Enhanced hydrotropic response of the pp2c quadruple mutant and reduced hydrotropic response of the pyr/pyl sextuple mutant. A, ABA-hypersensitive phenotype of the hab1-1abi1-2pp2ca-1abi2-2 quadruple mutant, abbreviated as *Qabi2-2*, compared with the Col wild type. Photographs show representative seedlings 10 d (left) or 20 d (right) after the transfer of 4-d-old seedlings to Murashige and Skoog plates lacking or supplemented with 10 µM ABA. B, ABA-hypersensitive root growth inhibition of the Qabi2-2 mutant compared with the Col wild type. C, Enhanced hydrotropic response of the *Qabi2-2* mutant compared with the wild type. D, Reduced hydrotropic response of the pyr/pyl sextuple mutant compared with the wild type. C and D show hydrotropism assays with 7-d-old Arabidopsis seedlings. Data represent measures of the root curvature angle taken 14 h after the transfer of 7-d-old seedlings to split agar plates containing 0.4 M sorbitol in the region with low water potential. Values are averages from three independent experiments ± SE (n = 42 each). *P < 0.05 (Student's t test) when comparing data from each genotype and the wild type in the same assay conditions. E and F, Photographs show the experiments described in C and D, respectively, at 3 d after the transfer of 7-d-old seedlings to split agar plates containing 0.4 M sorbitol. The arrows mark the limit between Murashige and Skoog medium and medium supplemented with 0.4 M sorbitol.

Finally, the expression of *PYL8* was also documented in the root epidermis and lateral root cap. The localization of the moisture-gradient-sensing apparatus has not been precisely defined, likely because different root tissues might be required for proper hydrotropic perception and response. However, the root cap has been suggested to play a role for moisture-gradient perception (Eapen *et al*., 2003; Kobayashi *et al*., 2007). The localization of at least three ABA receptors in this area and the presence of *PYL8* in the root epidermis and the lateral root cap fit well with the requirement of ABA signaling for root hydrotropism. Moreover, we provide evidence that *pyr/pyl* mutants are impaired in hydrotropism, indicating that ABA perception by these receptors is required for a proper response. Interestingly, this response can be enhanced by multiple knocking out of the PP2Cs that represses ABA signaling under basal conditions. Thus, the enhanced hydrotropic response of *pp2c* knockouts together with their reduced water loss and enhanced transcriptional response to ABA constitute a powerful mechanism to cope with water stress (Saez *et al*., 2006; Rubio *et al*., 2009). Future studies on the role played by ABA signaling for hydrotropism should answer important questions, such as how ABA generates the asymmetric growth required to escape from low-water-potential regions of the soil or whether ABA gradients are generated in the root in an analogous manner to auxins.

5.5 Matherial and methods

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite:soil mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Then, seeds were sown on Murashige and Skoog (1962) plates composed of Murashige and Skoog basal salts, 0.1% MES, and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 μE m^{-2} s^{-1}. The *abi1-2abi2-2hab1-1pp2ca-1* quadruple mutant was generated by crossing two triple *pp2c* mutants described by Rubio *et al*. (2009).

Generation of Transgenic Lines and GUS Analyses

To construct the *ProPYL6:GUS* and *ProPYL8:GUS* genes, a fragment comprising 1.5 kb 5′ upstream of the ATG start codon plus the first 30 bp of the PYL6 or PYL8 coding sequence, respectively, was amplified by PCR and cloned into pCR8/GW/TOPO T/A. Next, it was recombined by Gateway LR reaction into pMDC163 destination vector (Curtis and Grossniklaus, 2003). To generate the *ProPYL7:GUS* gene, the upstream sequence amplified was of 0.5 kb to avoid overlapping with regulatory sequences of the At4g01023 neighboring gene. The different pMDC163-based constructs carrying *ProPYR/PYL:GUS* genes were transferred to *Agrobacterium tumefaciens* pGV2260 (Deblaere *et al*., 1985) by electroporation and used to transform Col wild-type plants by the floral dipping method. Seeds of transformed plants were harvested and plated on hygromycin (20 μg mL−1) selection medium to identify T1 transgenic plants, and T3 progeny homozygous for the selection marker were used for further studies. Imaging of GUS and GUS quantitative assays were performed as described by Jefferson *et al*. (1987). Root GUS staining was also visualized using modified PS-PI staining and confocal laser scanning microscopy as described previously (Truernit *et al*., 2008).

Hydrotropism Assay

The hydrotropic response was analyzed in 7-d-old Arabidopsis seedlings as described by Takahashi *et al*. (2002). Briefly, plastic square plates were filled with 1% agar containing Murashige and Skoog medium. After solidification of the agar, one-half of the medium was removed by cutting with a scalpel at an angle of 36° and replaced with 1% agar containing Murashige and Skoog medium supplemented with 400 mM sorbitol. Root tips were placed on the border between these two media, where a water potential gradient was generated, and plates were positioned vertically. After 14 h, the hydrotropic response was calculated by measuring the root curvature angle.

5.6 References

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6. Results: Chapter 4

A chemical genetic approach directed to isolate new agonists of abscisic acid

6.1 Introduction

Historically, both forward and reverse genetics have been used to elucidate gene function. For instance, through both physical and chemical mutagenic agents random mutations are caused in a large number of individuals. The phenotype of each mutant increases our knowledge on the affected process and finally, the identification of the gene responsible for the phenotype provides a molecular explanation. Despite the valuable contribution of this strategy some limitations may occur. For instance, in the case of some organisms as mammals the slow rate of reproduction, limitations of space or large genomes can complicate this kind of screening. In the case of mutants caused by deleterious mutations, this approach will prevent the isolation of the gene and thus, a loss of information about the genes involved in the process of interest (Stockwell, 2000). Finally, genetic redundancy will mask the role of a single gene when gene families perform similar functions (Raikhel and Pirrung, 2005; Cutler and McCourt, 2005).

One approach to bypass these limitations is chemical genetics. This method is based on the use of small molecules to modify or disrupt the functions of specific genes. Even for closely related families, small molecules can discriminate among different members of the family. Thus, a library of compounds is used for the identification of molecules that affect a certain biological process. Later on, the identification of the targets of these compounds provides new insights into the studied process (Stockwell, 2000). As the phenotype produced can be reversed by removing the compound, it is possible to identify mutations that otherwise would be lethal. Bypassing functional redundancy can be achieved through chemicals, since a single compound can bind related targets and can trigger phenotypes similar to those caused by multiple mutations (Raikhel and Pirrung, 2005). Currently, several millions of compounds are available for chemical screenings. Among them, there are synthetic compounds derived or related to known molecules or natural compounds obtained from different organisms (Stockwell, 2000). This huge amount of putative candidates makes necessary the introduction of additional filters to screen compounds with higher chances to generate phenotype. Different clues about the process of interest can be used to select these compounds. Thereby, this previous selection is a powerful tool to reduce and enrich the set of compounds that will be screened in the laboratory, which will facilitate the experimental design. One of these methods of selection is Docking, which predicts the conformation and orientation of a ligand within a tergeted binding site (Kitchen *et al*., 2004). Therefore, structural information of the target protein is necessary. Using algorithms it's possible to simulate computationally how a molecule accommodates into the binding site of a protein. These algorithms are complemented with scoring functions that, based on the shape and electrostatic properties of the molecules, evaluate the

interactions between ligand and target (Kitchen *et al*., 2004). There are several examples in plants of new protein functions discovered with chemical genetics: brassinazole, a brassinosteroid biosynthesis inhibitor (Asami *et al*., 2000); Yokonolide B, an inhibitor of auxin action (Hayashi *et al*., 2003); the identification of chemicals affecting gravitropism and vacuole morphology (Surpin *et al*., 2005).

Abscisic acid was discovered in the 1960's (Ohkuma *et al*., 1963). Several false starts claimed the identification of putative ABA receptors, later on retracted or questioned (Christmann and Grill, 2009; Cutler *et al*., 2010; Gao *et al*., 2007; Guo *et al*., 2008; Jaffé *et al*., 2012; Johnston *et al*., 2007; Liu *et al*., 2007; Muller and Hansson, 2009; Pandey *et al*., 2009; Razem *et al*., 2004; Razem *et al*., 2006; Risk *et al*., 2008; Risk *et al*., 2009; Shen *et al*.,2006; Tsuzuki *et al*., 2011; Zhang *et al*., 2002). Finally, in 2009 a chemical genetic approach allowed the isolation of a new family of proteins called PYR/PYL/RCAR that act as genuine ABA receptors (Park *et al*., 2009). From a chemical library, pyrabactin (a synthetic naphthalene sulphonamide) was selected as an inhibitor of germination (Zhao *et al*., 2007). Subsequently, a mutant resistant to pyrabactin-mediated inhibition of germination (*PYRABATIN RESISTENCE 1*, *pyr1*) was isolated and the corresponding locus cloned, leading to the identification of the 14-member PYR/PYL/RCAR family. Null mutants of PYR1 don't show any insensitive response to ABA in germination. Pyrabactin proved to be an agonist for some members of the family. Yuan *et al*. (2010) analyzed the inhibitory effect on ABI1 of all the PYR/PYL/RCAR proteins in phosphatase activity assays. While PYL1, PYL5, PYL6 and PYL9 –12 almost completely inhibited ABI1 in the presence of 10 µM pyrabactin; PYL 8 appeared more insensitive only inhibiting 80% of the phosphatase activity at 100 µM pyrabactin. Finally, PYL2–4 were the only pyrabactin insensitive receptors since, at 100 µM pyrabactin, they only inhibited 60% of the phosphatase activity of ABI1. Yeast two hybrid assays and phosphatase activity assays have shown that pyrabactin acts simultaneously on several receptors (Park *et al*., 2009; Melcher *et al*., 2010). Therefore it is difficult to explain how pyrabactin resistance maps to the PYR1 locus. One possible explanation is that PYR1 is highly expressed in seeds and likely makes a major contribution to pyrabactin-mediated inhibition of germination.

Since the discovery of the PYR/PYL/RCAR receptors several structural and biochemical data have been published revealing the molecular mechanism by which ABA binds to them (Melcher *et al*., 2010; Miyazono *et al*., 2009; Nishimura *et al*., 2009; Santiago *et al*., 2009;Yin *et al*., 2009;Sun *et al*., 2012). In addition to a deep understanding of the signaling pathway, biotechnological applications can be generated from this information. Drought is the major environmental limitation for plant productivity. Improvement of water use efficiency and crop yield under water stress are therefore needed for economical development and efficient water use. It's

know that ABA triggers adaptive responses to environmental conditions such as drought, salinity or cold, however abscisic acid synthesis is expensive and no commercial applications are as yet in use. Pyrabactin mimics the effect of ABA in germination but not in vegetative tissue (Park *et al*., 2009). The discovery of molecules that trigger adaptive responses to drought in mature plants would be very useful in regions of the world where water is a limited resource. Keeping in mind such possibility, we started in this work the screening of putative ABA agonists able of entering in the receptor cavity and activate the ABA signalling pathway.

6.2 Results

***In silico* screening**

In collaboration with Prof. José Antonio Márquez at the EMBL (European Molecular Biology Laboratory) a previous filtering of 6 million molecules was performed using *in silico* approaches in order to select a reasonable number of compounds for *in vivo* screenings. Although this filtering is not part of this project a brief explanation of the criteria used to select the molecules is needed. In an initial step, the binding site of the receptors was scanned for favorable interactions using crystal fingerprint of abscisic acid as template. This approach contributed to the identification of hot spots representing positions of interaction for an idealized ligand and determined potential regions providing binding specificity between different receptors. After this phase, two complementary approaches were applied for the large scale *in silico* ligand screening: a high throughput "DockCrunch" and a "Shape-Similarity" approach. In the DockCrunch approach, a collection of structures from 6 million different small molecules commercially available were systematically docked into the receptor cavities. A series of filters were applied to automatically score the resulting interactions and select those molecules and fingerprints that represented potentially meaningful interactions. The massive amount of structural information generated by this process can not be examined by means of individual user intervention steps (hundreds of thousands). In this work a data mining procedure was adopted that categorizes the docking fingerprints into families of individual binding modes. All docking fingerprints were analyses by a variation of the structural interaction fingerprint methods. Here the individual interaction points between the docked ligand and the protein are encoded in a bitstring representation (the fingerprint), where each bit represents the presence or absence of a particular interaction at a particular protein site and its chemical nature.

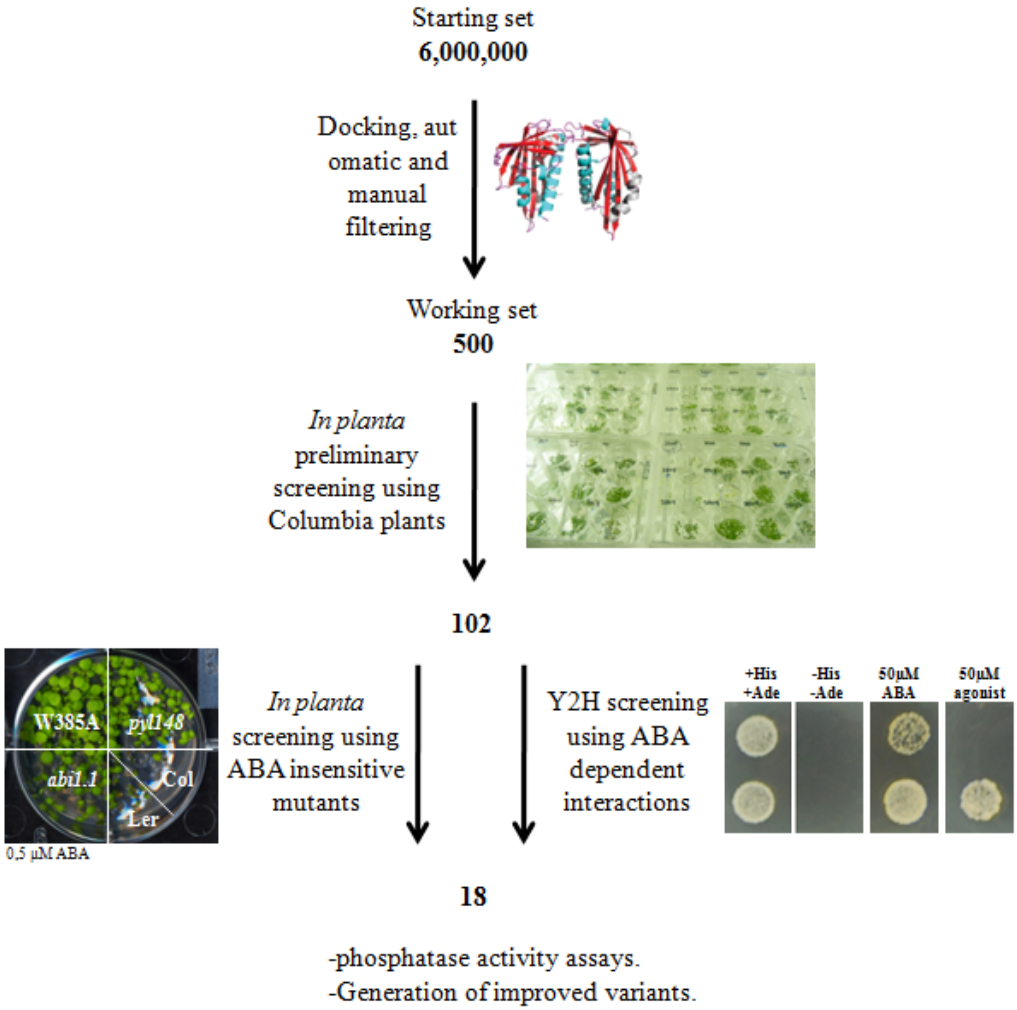

Figure 6.1. Schematic procedure of the chemical-genetic screen. 500 hundred compounds preselected by docking from a database of 6 million were screened in wild type plants. Of these, 102 compounds were selected by affecting germination or development and tested in Y2H probes using ABA-dependent PYR/PYL/RCAR-PP2C interactions and in experiments with Arabidopsis using ABA-insensitive mutants. A final number of 18 compounds have been selected as possible ABA agonists.

Fingerprints from each compound and docking pose were compared and used to group compounds into families with similar binding modes. Representative compounds within these families were manually evaluated and compared. In the Shape-Similarity approach the structure of the ABA molecules and the critical groups contacting the receptor were used to identify molecules with a similar shape and binding capacity. These molecules were docked into the receptor. After this *in silico* screening, the 1000 candidate molecules with top scores arising either from DockCrunch or the Shape-Similarity passed a manual filtering that reduced the number of compounds to a number

of 500. Figure 6.1 summarizes the different steps and procedures performed to isolate ABA agonists.

Screening in Arabidopsis

500 compounds were screened either using Arabidopsis wild type plants or ABA-insensitive mutants. Compounds able to inhibit vegetative growth (rather than seed germination) were selected. Initially, only compounds causing phenotypic differences between untreated and treated plants were selected. As shown in the figures 6.2a and 4.2b some compounds produced different degrees of growth retardation, whereas others did not show effect. This screening served to reduce the number of compounds to 102 in order to select those that exhibit an effect on plants and that would be used later on.

Further experiments were focused into select compounds that affected differentially ABA response in wild type and ABA insensitive mutants. Three ABA-insensitive mutants were selected: abi1^{G180D}(*abi1-1*) (Koornneef *et al*., 1984), a dominant mutation conferring insensitivity to ABA due to a mutation in a residue in the PP2C active site (Gly180Asp) involved in the interaction between the PP2C and the receptor; a PYR/PYL/RCAR triple mutant *pyr/pyl148* (González-Guzman *et al*., 2012) and *HAB1*^{W385A}, an overexpression line of HAB1 carrying a mutation in the Trp385, which is involved in the formation of the ternary complex trough a water-mediated hydrogen bond between the carbonyl oxygen of ABA and the side chain of Trp385 in HAB1 (Dupeux *et al*., 2011). As a result we selected 5 compounds differentially affecting ABA response (table6.I). Figure 6.2C shows the effect of one of the compounds in wild type plants compared to the ABA-insensitive mutants.

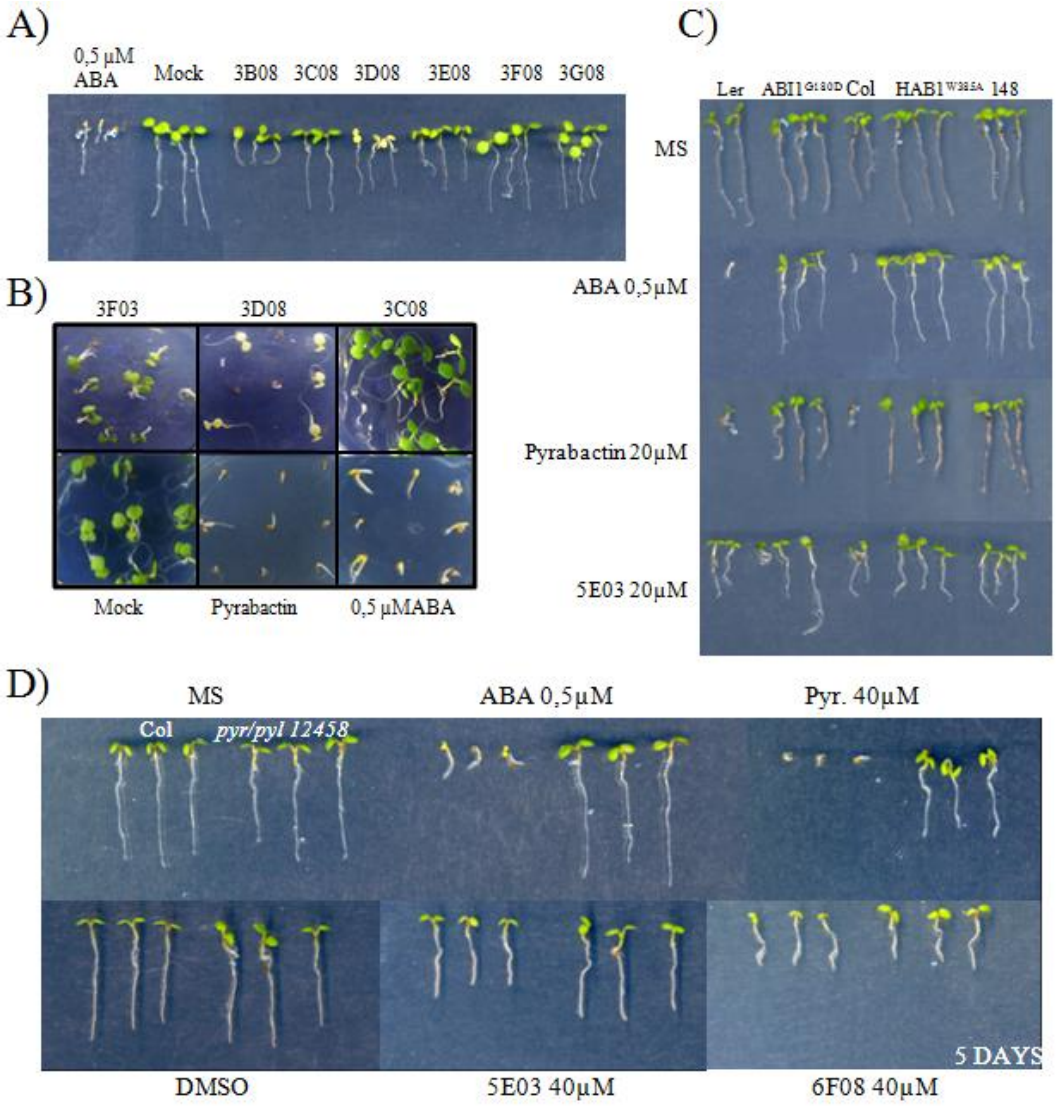

Figure 6.2. Plant growth experiments showing the effects of some compounds. Seedlings were grown in MS plates containing the corresponding compound and photographed at different times depending on the experiment. ABA and pyrabactin were taken as reference to select the candidate compounds. A) Six compounds triggering different effects on root growth of Columbia plants at 6 days. B) Seedling establishment in Columbia plants of 6 days. C) The compound 5E03 inhibits seedling growth in wild type plants and its effect is slightly reduced in ABA-insensitive plants. D) Growth experiments using the strong ABA-insensitive mutant *pyr/pyl12458*. Seedling growth inhibition produced by 5E03 and 6F08 was less pronounced in the quintuple mutant than in the wild type.

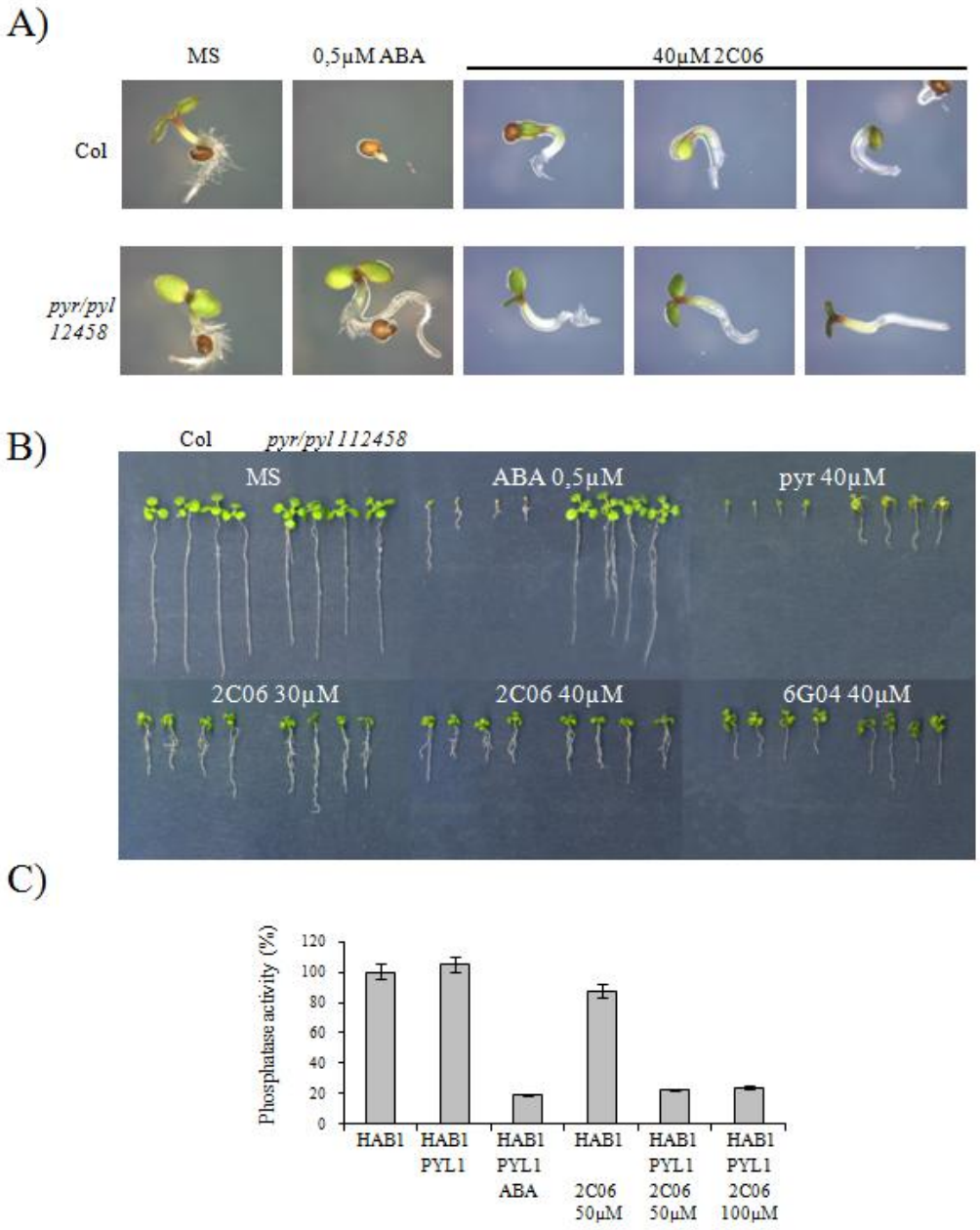

FIGURE 6.3. The compound 2C06 inhibits growth of wild type plants more strongly than of ABA-insensitive mutants and is capable of induce the inhibition of HAB1 by PYL1. A) The compound 2C06 effect in 4 days germinated plants. While Col-0 plants showed retarded germination, *pyr/pyl112458* plants were less inhibited by this compound and showed longer radicles. B) Effect of two selected compounds (2C06 and 6G04) in 10 days old seedlings**.** The sextuple mutant showed slightly reduced sensitivity to root growth inhibition by both compounds compared to the wild type. C) The effect of 2C06 on the phosphatase activity of HAB1. The compound decreased the phosphatase activity only in the presence of PYL1 indicating a specific inhibition of HAB1 through the PYR/PYL/RCAR receptors.

While this project was in process, quintuple and sextuple mutants of the PYR/PYL/RCAR receptors were generated (Gonzalez-Guzman *et al*., 2012) and they were used in these experiments (Figure 6.2D). Between the 5 compounds, the 2C06 was the more effective and thus the chosen for further assays. Germination of the quintuple mutant was less affected by the 2C06 compared to the wild type, which suggests this compound acts through some of these receptors (Figure 6.3A). Even though the quintuple and the sextuple mutants show lower root growth than the wild type in MS medium (Gonzalez-Guzman *et al*., 2012), in 10-days-old seedlings treated with 2C06 longer roots were observed in the sextuple mutant compared to the wild type (figure 6.3B).

Yeast Two Hybrid (Y2H) screening

At the same time a yeast two hybrid screen was performed with the 102 compounds previously selected in the preliminary screen performed with wild type plants. ABA binding by PYR/PYL/RCAR receptors leads to a conformational change that allows interaction with clade A PP2Cs. This property can be reproduced in yeast two hybrid screens. Thus, yeast growth can be used to monitor ligand binding to ABA receptors. The ability of the selected compounds to mimic the effect of ABA was tested using different ABA-dependent PYR/PYL/RCAR-PP2C interactions. This strategy served to confirm the effectivity of the compounds to promote PYR/PYL/RCAR interactions as well as the putative specificity of the ligands for some PYR/PYL/RCAR proteins ultimately leading to the identification of the receptors affected by the compound. PP2C phosphatases were used as prey (fused to the Gal4activation domain, GAD) and PYR/PYL/RCAR receptors were used as baits (fused to the Gal4 DNA-binding domain, GBD). Pyrabactin and ABA were used as controls. In addition to Arabidopsis receptors, we used some tomato orthologous receptors in order to check the validity of selected compounds in receptors of this species. After screening 102 compounds in Y2H assays, some of the compounds were able to trigger the interactions of HAI1 with PYL5 and of HAB1 with PYL1 as ABA and pyrabactin did (Figure 6.4, A and table 6.I). Tomato receptors were more sensitive to restore ABA-dependent interactions by the addition of some compound. In particular, the compound 2C06 was able to trigger the interaction of Solyc06g061180 with HAB1, PP2CA and HAI1 or of Solyc09g015380 with HAB1. Results are summarized in figure 6.4, B.

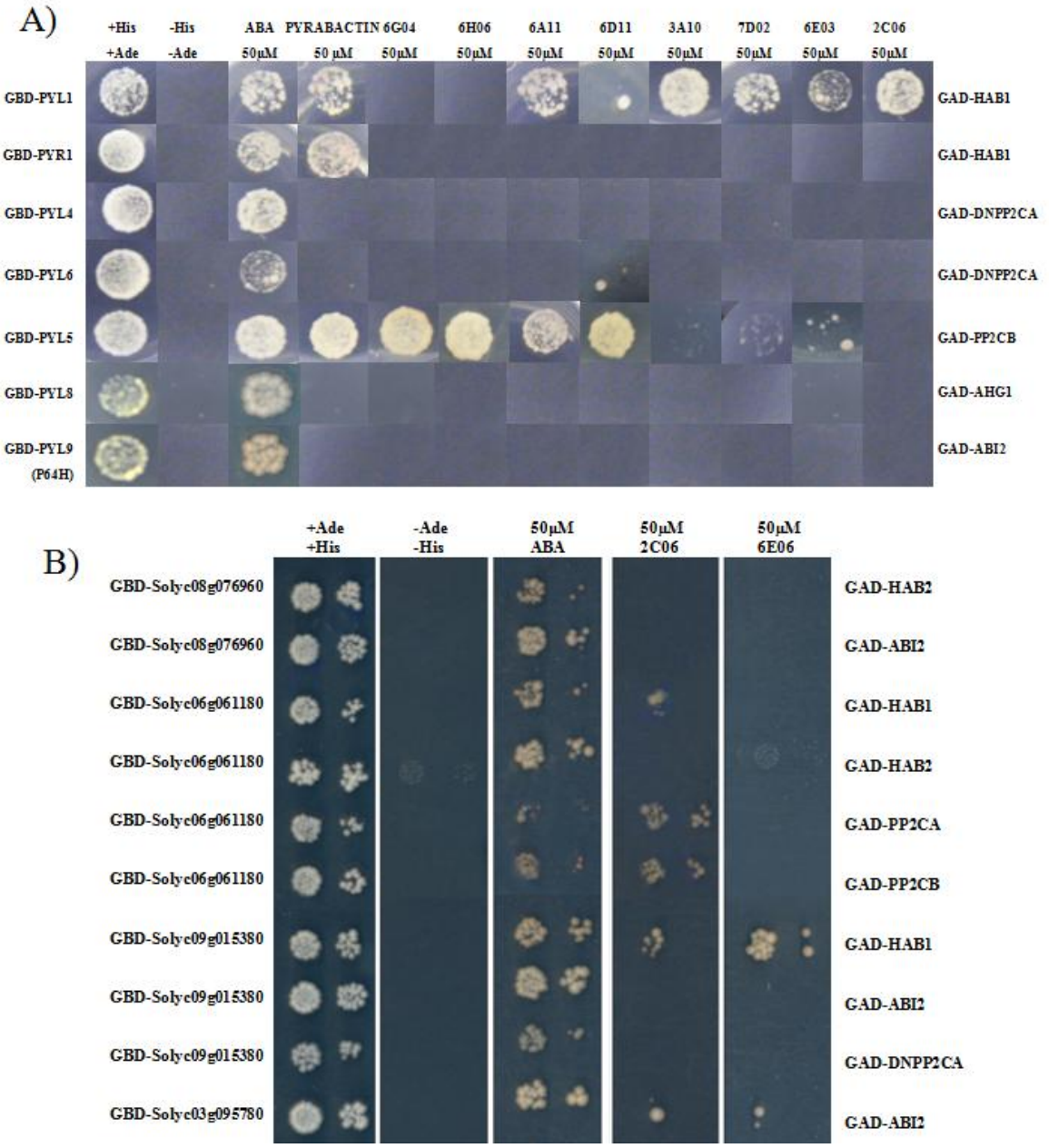

Figure 6.4. Yeast two hybrid experiments using ABA-dependent interactions. Interaction was determined by growth assay on medium lacking His and Ade. When indicated, the medium was supplemented with 10 µM racemic ABA or the corresponding compound. Dilutions 10^{-2} and 10^{-3} of saturated cultures were spotted onto the plates, and photographs were taken after 6 days. A) Interaction assay using Arabidopsis PYR/PYL/RCAR receptors as bait (fused to the Gal4 DNA-binding domain, GBD) and PP2C phosphatases as prey (fused to the Gal4 activation domain, GAD). B) Tomato PYR/PYL/RCAR receptors as bait (fused to GBD) and PP2C phosphatases as prey (fused to GAD).

The compound 2C06 triggers the inactivation of the PP2C in PP2C *in vitro* activity assays.

After performing the screenings described above the compound 2C06 was selected as one of the best candidates to mimic the effect of ABA. In addition to affect plant growth this compound was able to reproduce some ABA-dependent interactions in Y2H hybrid experiments. To confirm these results, *in vitro* PP2C activity assays were performed. As shown in figure 6.3B, while PP2C activity was not affected by PYL1 in the absence of ABA, the presence of ABA or 2C06 reduced the activity of HAB1 to less than 20%. The activity of the phosphatase was not affected by increasing the concentration of 2C06 to 100µM most probably because 50 µM is already a high concentration enough to inhibit 80% of the PP2C activity. The 87% of phosphatase activity in the control performed with the PP2C and the compound alone confirmed that inhibition occurred through the PYR/PYL/RCAR receptors and not through direct inhibition of the PP2Cs.

COMPOUND NUMBER	Y2H INTERACTION			NO EFFECT IN Arabidopsis INSENTIVE MUTANTS
	HAB1-PYL1	PP2CB-PYL5	DNPP2CA-PYL6	
1C03	X	X		
1D03	X	X		
2C06	X			X
3A10	X			
3B10	X			
4G10		X		
5C06			X	
5D03				X
5E03				X
6A11	X	X		
6D11		X		
6E03	X	X		
6E04				
6F08				X
6G04	X	X		X
6H06		X		
6H08		X		
7D02	X	X		

Table 6.I. Summary of the compounds selected after the *in planta* and the yeast two hybrid screens. Compounds differentially affecting wild type and ABA-insensitive mutants or by triggering ABA-dependent interactions in yeast two hybrid experiments are indicated with an X character.

6.3 Discussion

The objective of this work was to isolate ABA agonists, which could be used as promising chemicals to generate molecules able to activate ABA signaling for instance under drought stress. Pyrabactin has been described as an ABA agonist in germination assays but, in seedling responses, ABA and pyrabactin-regulated genes differ and few ABA-responsive genes respond to pyrabactin in vegetative tissue (Park *et al*., 2009). Indeed, ABA-insensitive mutants showed impaired growth after 10 days in medium supplemented with pyrabactin (figure 6.3B). Therefore, we focused on compounds that could affect seedling growth more that inhibit germination.

Water availability is one of the most important factors limiting crop production. One strategy to avoid this stress would be to apply ABA directly in the field but ABA synthesis is expensive. Moreover, UV light can convert S-(+)-ABA into its inactive form R-(-)-ABA, which has comparable activity to the natural S-(+)-ABA in some ABA-regulated responses but in others, such as stomatal closure, has a weaker effect. Chemical genetics can bypass this problem by isolating chemical compounds able to mimic the effect of the hormone in mature plants. To achieve this goal I took advantage of the recent advances in the understanding of ABA perception. In particular, structural data of PYR1 and ABA were used to select compounds with better chances to be accommodated in the receptor cavity. This first selection was done *in silico* on 6 million compounds and allowed us to perform screenings with a final number of 500 compounds. After assaying them in wild type plants, 102 compounds were selected for further experiments with ABA-insensitive mutants. This second screen rendered 5 compounds, selected by their capacity to inhibit root and seedling growth in wild type plants more that ABA-insensitive mutants.

We were also interested in elucidating which receptors were activated by the selected compounds in plants. This was possible through a yeast two hybrid approach using ABA-dependent interactions of ABA receptors and PP2C phosphatases. Two of the compounds selected in the plant screen were able to trigger ABA-dependent interactions in this assay. In addition we found other compounds without any remarkable effect in plants but able to reproduce some ABA-dependent PP2C-PYR/PYL/RCAR interactions. A possible explanation for these results could be that perhaps in plants, they are metabolized to inactive forms or they are not able to cross the plasma membrane. The compound 6G04 presented solubility problems and for this reason, we have followed further experiments with the 2C06 compound. 2C06 shows a clear effect in plants and in Y2H experiments and also induces PP2C inhibition in a ligand dependent manner. Although ABA-insensitive mutants were less affected than the wild type by 2C06, some impairment was observed, which

suggest that the compound is affecting other processes or acting through additional receptors. However this molecule could serve in the future as a template to generate new variants that could have more chances to mimic more specifically the effect of ABA in plant growth.

6.4 Materials and methods

Compound solutions

The 500 compounds were ordered from Chembridge (http://www.chembridge.com/index.php). Compounds were diluted in 100% DMSO to final concentration of 100mM. Work solutions were prepared at 50mM in 50% DMSO-H_2O. The final compound concentration for all the assays varied between 20-50 µM. DMSO final concentration varied between 0.1% and 0,25% which didn't affect plant or yeast growth and neither PP2C activity in *in vitro* assays in the corresponding controls performed (data not showed).

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds were surface sterilized by treatment with 70% ethanol for 10 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% Triton X-100 for 5 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Then, seeds were sowed on 12 well plates composed of Murashige and Skoog basal salts (Murashige and Skoog, 1962), 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar and 1% sucrose . The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Each well was filled with media supplemented with the corresponding compound. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h light, 8-h dark photoperiod at 80 to 100 µE m−2 s−1.

Seedling establishment and Root growth assays

To measure compound sensitivity, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% sucrose, and the corresponding compound. Selection of compounds in the preliminary screen was based on the existence of affected root growth or seedling establishment compared to mock treatment in Col-0 plants. Selection of compounds affecting specifically the ABA response was performed by selecting those compounds affecting root growth or seedling establishment in Col-0 plants but with absent or minor effects in the *abi1*^{D180D}, *hab1*^{W385A}, *pyr/pyl148* ABA insensitive mutants. In the preliminary screening performed with Col-0 plants and in the screening performed with the ABA-insensitive mutants the compound concentration was 20µM. In subsequent experiments with the selected compounds dose-response assays were

performed using compound concentrations between 20-50µM. Strong ABA-insensitive mutants such as *pyr/pyl112458* or *pyr/pyl11258* were used to discriminate between the efficiency of the different compounds.

PP2C *in vitro* activity assays

The coding sequence of HAB1 and PYL1 were excised from pCR/GW/TOPO vector by NcoI/EcoRI digestion and cloned into pETM11 to encode N-terminally 6X His-tagged recombinant proteins. Phosphatase activity was measured using the Ser/Thr phosphataseassay system (Promega) using the RRA(phosphoT)VA peptide as substrate. Assays were performed in a 100-µL reaction volume containing 25 mMTris-HCl pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 25 µM peptide substrate, and the PP2C. When indicated, PYR-PYL recombinant proteins and ABA were included in the PP2C activity assay. After incubation for 60 min at 30ºC, the reaction was stopped by addition of 30 mL molybdate dye (Baykov *et al*., 1988) and the absorbance was read at 630 nm with a 96-wellplate reader.

Yeast two hybrid assays

Constructs of some of the PYR/PYL/RCAR homologues of tomato to perform yeast two hybrid experiments were generated in Gateway-compatible vectors. To this end, the coding sequences (Solyc08g076960, Solyc06g061180, Solyc09g015380 and Solyc03g095780) were PCR-amplified using cDNA from leaves or mature fruits. The PCR products were cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pGBKT7 destination vector to generate in-frame fusions with the GBD. The coding sequences of the PP2C phosphatases were amplified by PCR and cloned in pCR8/GW/TOPO, excised with EcoRI, cloned into pGADT7 and fused to the GAL4 activation domain (GAD). PYR/PYL/RCAR from Arabidopsis were amplified by PCR and cloned in pCR8/GW/TOPO, excised with NcoI-EcoRI or NcoI-BamHI, cloned into pGADT7 and fused to the GAL4 activation domain (GAD).

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7. GENERAL DISCUSSION

7. DISCUSSION

7.1 MOLECULAR FEATURES OF THE INTERACTION BETWEEN PP2Cs AND PYR/PYL/RCARs

The crystallization and X-ray analyses of ABA receptors and PP2Cs have generated structural data describing the inhibition of clade A PP2Cs after ABA perception. When ABA enters into the cavity of the PYR/PYL/RCAR receptors, it establishes interactions that induce conformational changes in the receptors. ABA promotes a closed conformation where the gating loops of the receptor cover the entry site of the cavity and avoid ABA exit. This closed conformation offers a surface favorable for the interaction with the PP2Cs and explains why ABA is required to inhibit the phosphatases. As shown in Figure 7.1, two major zones of interaction between the PP2C and the receptor are observed. The first zone of interaction comprises the active site of the phosphatase. The Ser-85 located in the β3-β4 loop of PYR1 establishes hydrogen bonds with Gly-G246 and Glu-203 and blocks the access to the phosphatase catalytic site. Although Ma *et al.* (2009) suggested a noncompetitive mechanism for the inhibition of the PP2Cs by the PYR/PYL/RCAR proteins, different structural and biochemical data have suggested a competitive mechanism (Dupeux *et al*., 2011a; Melcher *et al*., 2009; Miyazono *et al*., 2009; Yin *et al*., 2009). Later on, Soon *et al*. (2012), showed that the interaction of the phosphatases with one of its substrates (SnRK2s) physically mimics the interaction with the receptors, which confirms that ABA-bound receptors and kinases compete for the interaction with PP2Cs, turning on and off, respectively, the ABA signaling pathway. Previously, Melcher *et al*. (2009) had showed that the inhibition of the phosphatase by the receptor can be reduced by increasing the concentration of OST1. The second zone of interaction between the PP2C and the receptor comprises the flap subdomain of the phosphatase. This domain contains the Trp-385 that in the ternary complex is inserted between the gating loops and establishes a hydrogen bond with a water molecule. This water molecule establishes simultaneously hydrogen bonds with amino acid residues of the gating loops of the receptor and the ketone group of ABA. This set of interactions participates in the stabilization of the ternary complex and provides a mechanism by which the PP2C can detect the presence of the hormone in the cavity of the receptor. The Trp-385 is only present in the plant clade A PP2Cs and it is a highly conserved residue that is only missed in AHG1. The transgenic plants generated in this work carrying the overexpression of *hab1*^{W385A} showed a strong ABA-insensitive phenotype in ABA-mediated inhibition of seed germination and seedling establishment, enhanced water loss and reduced expression of ABA-responsive genes. The phenotype of these lines is stronger than the overexpression

of the wild type *HAB1*, which points out the crucial role of this residue in the generation of a functional ternary complex. In addition the activities of HAB1, hab1^{W385A} and hab1^{G246D} were analyzed *in vitro*, either through phosphatase assays or OST1 dephosphorylation. Both mutations were refractory to ABA-dependent inhibition by the PYR/PYL/RCAR proteins. Additionally, in the absence of ABA and PYR/PYL/RCAR, while hab1^{W385A} showed similar activity to HAB1, hab1^{G246D} had reduced phosphatase activity, likely because the Gly-246 residue is located at the active site. These mutations have been described previously as dominant and this work explains that this effect is due to their capacity to avoid the ABA-dependent inhibition by the PYR/PYL/RCAR proteins. Therefore, in the presence of ABA and PYR/PYL/RCARs these mutant proteins, in contrast to wild type, are able to maintain the dephosphorylation of their substrates and they can be classified as dominant hypermorphic mutations (Santiago *et al*., 2012). The same reasoning applies to the original *abi1-1* and *abi2-1* mutations.

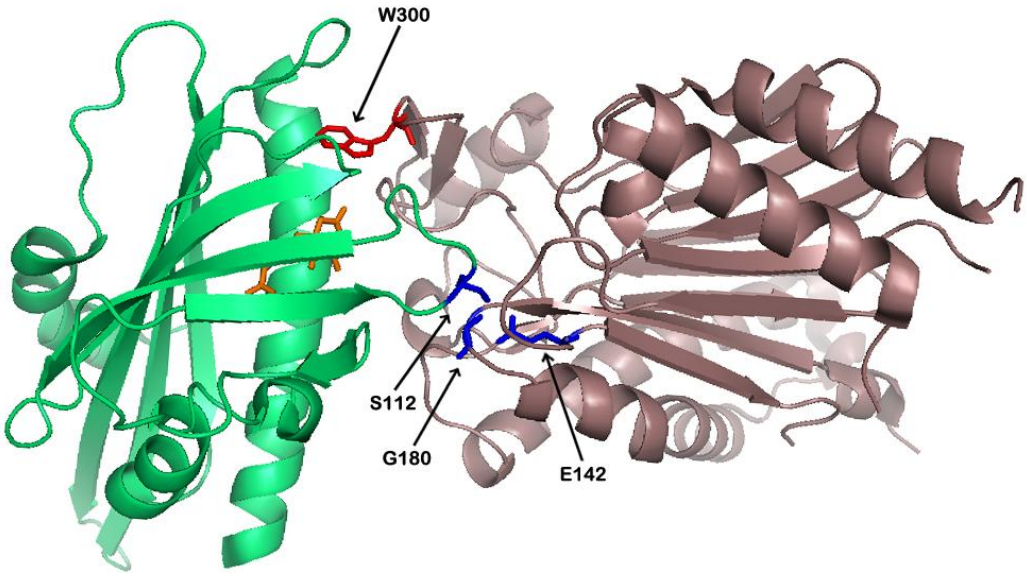

Figure 7.1. Structural details of the ABA-PYL1-ABI1 complex. Two zones of interaction between PP2Cs and PYR/PYL/RCAR can be observed. The W300 at the flap subdomain of ABI1 interacts with the hydrophobic pocket of ABA-bound PYL1 and contacts ABA. The second zone of interaction comprises two residues at the active site of ABI1 (E142 and G180). E142 and G180 interact by direct and water-mediated hydrogen bonds with the S112 of PYL1. In addition, other residues of PYL1 are located near the active-site of ABI1, reducing the accessible surface area of the active-site and preventing the interaction of ABI1 with its substrates. Based on Miyazono *et al*. (2009).

7.2 CHARACTERIZATION OF A SUBRANCH OF THE CLADE A PP2Cs

According to amino acid similarity clade A PP2Cs can be divided in the ABI1/HAB1 branch (comprising ABI1, ABI2, HAB1 and HAB2) and in the PP2CA branch (comprising PP2CA, AHG1, HAI1, HAI2 and HAI3) (Figure 1.4). In this work I have focused in the characterization of PP2Cs that belong to the less studied PP2CA branch, specifically I have focused on AHG1, PP2CA and HAI1 (At5g59220). The analysis of the phosphatase activity of these three phosphatases reveals that, in contrast to the ABI1/HAB1 branch, they show differences in their susceptibility to be inhibited by the receptors. PP2CA is inhibited by all the receptors assayed, HAI1 is resistant to inhibition by PYL4 and PYL6 and, interestingly, the activity of AHG1 is not affected by any of the PYR/PYL/RCAR used in the assays. Hao *et al*. (2011) have suggested the existence of a subclass of PYR/PYL/RCARs represented by PYL10 that would be able to fully inhibit the PP2Cs in the absence of the hormone. Thus, PYL10 was able to inhibit in the absence of ABA almost completely the PP2C activity of ABI1, HAB1 and HAB2 but showed a 50% reduction in the case of PP2CA using a 100:1 ratio of receptor:PP2C. However other receptors are not as efficient as PYL10 in the ABA-independent inhibition of PP2Cs and PYL10 expression levels are very low to undetectable in different whole-genome microarrays (Laubinger *et al*., 2008; Yamada *et al*., 2003; Chekanova *et al*., 2007). In our work we have also tested this possibility but using 4:1 or 10:1 ratios of receptor:PP2C and only PYL8 was able to reduce by 15% PP2CA activity. Additionally, Antoni *et al.* (2013) have showed that the formation of PYL8:PP2C complexes *in vivo* required ABA supplementation.

AHG1 is a clade A PP2C that is expressed during seed development and early postgermination. Two peaks of *AHG1* expression can be observed at 8 and 16 DAF (Days After Flowering). The knock out mutant is very hypersensitive to the ABA-dependent inhibition of germination but shows wild type phenotype in other ABA responses. AHG1 is the only PP2C of the clade A missing the Trp-385, which suggests a particular role for this protein. In seeds ABA levels are high enough to inhibit significantly the rest of clade A PP2Cs. AHG1 might escape from the receptors by the failure to form functional ternary complexes, which would maintain basal level of phosphatase activity even at high ABA concentrations. Thus, this phosphatase could help to maintain a basal negative regulation of the ABA signaling pathway during seed development while other PP2Cs are inhibited. Thereby it could avoid overactivation of the ABA cascade, in order to maintain a correct balance with other hormonal responses.

The characterization of knock out mutants and overexpression lines of HAI1 are consistent with a role as negative regulator of the ABA pathway. *hai1* null mutants did not show any remarkable

phenotype but the generation and characterization of a double mutant with *pp2ca-1* allowed a confirmation of its role in root growth regulation, of expression of ABA-responsive genes, control of water loss and regulation of seedling establishment in response to mannitol or glucose. Since *pp2ca-1* shows a strong phenotype in ABA-mediated inhibition of germination and seedling establishment no additive effect was found in the *pp2ca-1hai1-1* double mutant. However the ABA hypersensitive phenotype of the double mutant in other responses reveals functional redundancy with PP2CA. Other authors have also studied the role of this branch of clade A PP2Cs. Bhaskara *et al*. (2012) have generated a triple mutant of the Highly ABA Induced PP2Cs (HAI) (*hai1-2hai2-1hai3-1*). This work points out the role of these three proteins in osmotic adjustment as the single mutants showed higher proline concentration than wild type at low water potential. Significant differences in ABA sensitivity could be appreciated with the generation of double and triple mutants of these proteins. The triple mutant is insensitive for ABA-mediated inhibition of radical emergence while it is hypersensitive to ABA in other ABA-related responses such as ABA-mediated inhibition of seedling establishment or induction of ABA responsive genes. This would mean that these phosphatases positively regulate germination in contrast to the role as negative regulators of other clade A PP2Cs in this particular process (Rubio *et al*., 2009). In contrast, I could not adress this positive role of HAI1 as the double mutant that I generated with *pp2ca-1* was strongly affected in this developmental stage, probably due to the strong contribution of PP2CA to inhibit germination. Guo *et al*. (2010) and Lim *et al*. (2012) have also described a positive role of HAI1 and HAI2 respectively in germination although compared to the wild type the differences are very subtle. Thus, according to the work of Bhaskara *et al*. (2012), the HAI PP2Cs could have a positive role in ABA signaling during germination; however, they are negative regulators after germination. This paradox might be explained taking into account that the apparent "ABA-resistant" phenotype for radical emergence might reflect the role of these PP2Cs in osmotic adjustment.

In this work, the subcellular localization of PP2CA and HAI1 was analysed. As it could be expected by the localization of their interacting partners, biochemical fractionation showed that the proteins are present in both nucleus and cytosol. Interestingly both proteins were present in the nuclear insoluble fraction (chromatin associated) were chromatin-remodeling complexes SWI/SNF are localized. Indeed, the interaction of SWI3B, a component of the SWI/SNF complex, with PP2CA has been previously described (Saez *et al*., 2008). On the other hand, these PP2Cs are also present in the microsomal fraction. Therefore, PP2CA is localized together with two of its interacting partners in the plasma membrane, AKT2 (a weakly rectifying K^+ channel) and SLAC1 (a slow anion channel) respectively. SLAC1 participates in ABA-mediated stomatal closure and AKT2 is involved in the

regulation of K^+ homeostasis (Cherel *et al*., 2002; Lee *et al*., 2009). HAI1 presents a characteristic N-terminal region with two nuclear localization patterns and I was interested in probing the role of these signals in the localization of the proteins. In agreement with Fujita *et al*. (2009) and Bhaskara *et al*. (2012) using GFP fusion proteins of HAI1 and PP2CA I found a predominant nuclear localization of both proteins. By deleting the N-terminal region the protein became more cytoplasmic, which confirmed that the N-terminus is responsible of targeting the protein to the nucleus.

7.3 ANALYSIS OF THE EXPRESSION PATTERNS REVEALS IMPORTANT DIFFERENCES BETWEEN MEMBERS OF THE PYR/PYL/RCAR FAMILY.

Genetic redundancy precluded the identification of PYR/PYL/RCARs by classical genetic approaches. The discovery of these receptors took more time than expected and it was only possible to isolate them through alternative approaches. However different evidences indicate that the function of these receptors is not completely overlapping. Transcriptomic data as well as GUS reporter analysis have shown that the PYR/PYL/RCAR receptors present different expression levels and different expression patterns in Arabidopsis. For instance *PYL3* and *PYL10* to *PYL13* show low to undetectable expression levels in different microarray analyses (Kilian *et al*., 2007; Winter *et al*, 2007). In addition, GUS reporter analyses in plant tissues show important differences between these proteins. Gonzalez-Guzman *et al*. (2012) analyzed the expression pattern of *PYR1, PYL1, PYL2, PYL4, PYL5*, and *PYL8* in different tissues. They have reported that all of them are present in guard cells of mature leaves, in the peripheral layer of the embryo (embryo epidermal layer) as well as in the provascular cells within the cotyledons and hypocotyls. In contrast, in the endosperm of 24h germinated seeds only *PYL8* and *PYR1* showed high expression levels. In this work I have analyzed the expression patterns of these receptors and also of *PYL6, PYL7* and *PYL9*. I have focused particularly in root due to the important role that ABA plays to control root growth depending on the environmental conditions. In this tissue I found interesting differences in the expression pattern among the different PYR/PYL/RCAR proteins. Thus, whereas *PYR1, PYL1, PYL2, PYL8* and *PYL9* are present in the vascular tissue of the root cap, *PYL5, PYL6* and *PYL7* expression levels are undetectable in this part of the root. In addition, *PYL1, PYL4* and *PYL8* are expressed in columella cells and specifically *PYL8* is also expressed in the lateral root cap. The particular expression pattern of *PYL8* together with its biochemical features might explain why *pyl8-1* showed insensitivity to ABA-mediated inhibition of root growth even at 20µM ABA. The generation of combined *pyr/pyl/rcar* mutants resulted in an increased insensitivity to ABA

in root growth especially for the quintuple and sextuple the mutants in 50µM ABA compared to *pyl8-1*. These data suggest that although PYL8 plays an important role in root growth this process is also controlled by other PYR/PYL/RCARs receptors in an additive manner.

7.4 THE ROLE OF ABSCISIC ACID AS A GROWTH PROMOTER

Although the role of ABA as growth inhibitor is well known, the phenotypes displayed by ABA-deficient and ABA-insensitive mutants also suggest a role as growth promoter. For instance, a HAB1 overexpression line shows reduced root growth in MS plates compared to plates supplemented with ABA. And this defect is more dramatic in extreme ABA insensitive mutants. A sextuple mutant of the receptors (*pyr/pyl112458)* shows less growth and less seed production compared to the wild type (Gonzalez-Guzman *et al*., 2012). Moreover, the *snrk2.2/2.3/2.6* triple mutant is severely impaired in growth and reproduction. Only in high humidity conditions the mutant was able to produce seeds and to produce leaves with 60% of the length of compared with those of the wild type (Fujii and Zhu, 2009). The ABA-deficient mutant *aba1* shows growth delay even under high-humidity non stress conditions and the application of small concentrations of ABA restores growth (Barrero *et al*., 2005). Since in *aba1* the ABA perception machinery is intact, the application of small amounts of ABA is enough to restore normal growth. In contrast, mutants severely impaired in ABA perception require high concentrations of ABA to activate residual perception and improve growth.

Endogenous ABA has an important role in the control of ethylene production required for the maintenance of root elongation at low water potentials (Sharp *et al*., 2004). Indeed, ABA increases the expression of genes for antioxidant enzymes (Guan *et al*. 2000). These enzymes contribute to reduce ROS levels that can increase ethylene synthesis (Overmyer *et al*. 2000).

Cell growth caused by expansion is regulated primarily by turgor pressure and is maintained by osmotic regulation via osmotically active substances, such as potassium ions (K^+), sugars, and amino acids. Proline concentration increases dramatically in water-stressed roots, and contributes up to 50% of the osmotic adjustment (Sharp *et al*., 2004). Additional work showed that accumulation of ABA is required for the increase in proline deposition at low water potential (Ober and Sharp, 1994). The role of ABA as a regulator of potassium homeostasis in plants is supported by several data, among which is worth highlighting the ABA-dependent regulation of several potassium transporters. OST1 inhibits KAT1 activity by phosphorylation (Sato *et al*., 2009) and SLAC1 is directly activated by SnRK2s. Recently Osakabe *et al*. (2013) have shown that the activity of the KUP potassium transporter family is

also regulated by ABA. Indeed, they have observed that OST1 interacts with KUP6 in yeast and plant cells and the conserved sequence motif in the KUP6 C-terminus is phosphorylated by SnRK2s in *in-gel* kinase assays.

7.5 THE ROLE OF ABA IN THE HYDROTROPIC RESPONSE

The role of ABA in drought stress has been extensively described. Among the responses controlled by ABA under water shortage, the mechanisms and regulation for ABA control of stomatal closure and regulation of gene expression are well known. However, there are other adaptive traits directed to avoid water deficit that have been less studied. One of these processes is hydrotropism, a mechanism by which roots direct growth towards zones of the soil with higher moisture. ABA has been involved in this response (Moriwaki *et al*., 2012; Takahashi *et al*., 2002). Columella cells have been associated to the perception of environmental stimuli that modify root growth like gravity or water stress (Perrin *et al*. 2005; Jaffe *et al*., 1985). After a gravitropic stimulus, a change of the localization of the amyloplasts present in columella cells is the cause of the modification in the direction of root growth (Strohm *et al*., 2012). Some evidence indicates that the root cap is the site of perception for moisture gradients in the root. Takahashi *et al*. (2003) have observed a reduction of the amount of amyloplasts in the columella cells after a hydrotropic stimulus. In addition, the abnormal morphogenesis of the root apical meristem and root cap showed by the non hydrotropic mutant *nhr1* points out the importance of these tissues for a normal hydrotropic response (Eapen *et al*., 2003). The expression pattern of PYL8 in the columella and in the lateral root cap suggests a possible role of the receptor in this response and for this reason I have analysed the hydrotropic response of multiple mutants of the PYR/PYL/RCAR family and of the PP2Cs. In this assay, Murashige and Skoog medium containing 1% agar and agar containing 400 mM sorbitol are placed side by side, which generates a water potential gradient at the border between the two media. I also analysed the hydrotropic response of *pyl8-1* but no difference was observed compared to the wild type probably due to the effect of genetic redundancy. Moreover, the sextuple mutant of the receptors (*pyr/pyl112458)* presents less curvature than the wild type reflecting reduced hydrotropism as other mutants affected in this response. In contrast a quadruple mutant of the phosphatases impaired in *HAB1, ABI1, ABI2* and *PP2CA* showed enhanced root curvature in comparison to the wild type. These data confirm the involvement of the ABA core pathway in the hydrotropic response and offer new elements to better understand how this environmental signal is transmitted.

7.6 ISOLATION OF NEW ABA AGONISTS THROUGH CHEMICAL GENETICS.

Drought in some parts of the world has hurt crop production and contributed to food scarcity and increase in food prices, highlighting the need to improve water use efficiency. In this direction, the development of new strategies directed to reduce water consumption in agriculture could be crucial to ensure food production in areas where this stress is more severe. Abscisic acid is the principal phytohormone that triggers adaptive changes to bypass the damage caused by water stress, such as stomatal closure, promotion of root growth, accumulation of compatible osmolites, etc. Therefore ABA application in crops could be an interesting strategy to render crops more resistant under water deficits. However the hormone itself has not been used for this purpose because industrial-scale production of abscisic acid would be very expensive and the ABA molecule is photolabile. Chemical synthesis is expensive because it produces the two enantiomers, i.e. R-(-)-ABA and S-(+)-ABA, and a chiral procedure is finally required to isolate the natural S-(+)-ABA. Although R-(-)-ABA is not completely inactive, it shows important differences in the responses elicited compared to the natural form (S-(+)-ABA) as for example low ABA-activity in stomatal closure assays (Zaharia *et al*., 2005). One possible alternative to these constraints would be to design chemicals to mimic the action of abscisic acid that could be sprayed on crops to protect them against drought. Pyrabactin effectiveness as an ABA agonist in seeds has been widely tested. However its effect as ABA agonist is restricted to germination as in seedlings it elicits a different response than ABA. Thus, in germination both ABA and pyrabactin elicit a similar pattern of gene expression; however, in seedlings a clear divergence in the regulated genes is observed (Park *et al*., 2009) (Figure 1.7, D). For these reasons, different research groups are interested in isolating new compounds acting as ABA agonists in adult plants, with the ultimate purpose of applying them in crop plants. Structural information about PYR1 and ABA has been used to select those chemical compounds with more chances to accommodate in the receptor cavity. I have tested these compounds in Arabidopsis plants and I have also used other complementary approaches such as yeast two hybrid or phosphatase activity assays. As a result I have selected a compound that is: i) able to promote ABA-dependent PYR/PYL/RCAR-PP2C interactions in yeast two hybrid, ii) produces a decrease in the PP2C activity in the presence of PYL1 and iii) reduces root length in wild type plats more than in strong ABA-insensitive mutants. However, as it can be observed also for pyrabactin, this compound is likely affecting other processes in the plant, since some growth impairment also occurs in

these ABA-insensitive mutants. However, it might be possible in the future to produce new variants of this compound with improved properties.

8. CONCLUSIONS

1. CONCLUSIONS

The Trp-385 of HAB1 plays a pivotal role in the formation of the ternary complex ABA-phosphatase-receptor and it represents an elegant PP2C-dependent mechanism to check the occupancy of the ABA-binding site in the PYR/PYL/RCAR receptor. *hab1*^{W385A} is a hypermorphic mutation that generates a phosphatase refractory to inhibition by PYR/PYL/RCARs. As a result, transgenic lines harboring the *hab1*^{W385A} mutation show strong ABA insensitivity, which provides *in vivo* evidence on the biological significance of crystal structure of ternary complexes.

HAI1, a previously uncharacterized member of clade A PP2Cs, is a negative regulator of osmotic stress and ABA signaling as revealed by the analyses of the *pp2ca-1hai1-1* loss-of-function mutant.

Biochemical fractionation of HA-tagged versions of HAI1 and PP2CA reveals a nuclear, cytosolic and microsomal subcelular localization. The N-terminus of HAI1 is responsible of the nuclear localization.

HAI1, PP2CA and AHG1 show different sensitivity to be inhibited by the PYR/PYL/RCAR ABA receptors. Biochemical analyses of AHG1, the only PP2C lacking the conserved Trp residue, indicate that this PP2C can remain active even in the presence of ABA and PYR/PYL/RCAR receptors and remark the importance of this residue in the formation of functional ternary complexes. This differential sensitivity reveals a functional specialization of PYR/PYL/RCAR receptors to inhibit certain PP2Cs.

GUS reporter analyses of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5*, *PYL6*, *PYL7*, *PYL8* and *PYL9* promoters reveal that most of them are expressed at high levels in the root, which reveals its importance in root ABA signaling. PYL8 plays a nonredundant role for the regulation of root ABA sensitivity.

The core elements of the ABA signaling pathway are involved in the hydrotropic response. An ABA-hypersensitive *pp2c* quadruple mutant shows enhanced hydrotropism, whereas an ABA-insensitive sextuple *pyr/pyl/rcar* mutant shows reduced hydrotropic response.

A chemical genetic approach allowed to isolate some candidate ABA agonists. The compound 2C06 was able to inhibit root and seedling growth in wild type plants more than in mutants lacking ABA receptors. 2C06 induced PP2C inhibition in a ligand-dependent manner and promoted the ligand-dependent interaction between PP2Cs and PYR/PYL/RCARs in Y2H assays.

9. REFERENCES

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10. APPENDIX 1

In vitro reconstitution of an abscisic acid signalling pathway

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Abstract

The phytohormone abscisic acid (ABA) regulates the expression of many genes in plants and plays critical roles in stress resistance, and 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 signaling[3,4,14]. However, the identities of ABA receptors remain controversial and the mechanism of signaling from perception to downstream gene expression is unclear[15,16]. Here we show that by combining the recently identified ABA receptor PYR1, with the protein phosphatase 2C 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. The 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 receptor proteins can disrupt the interaction between the SnRK2s and PP2Cs, thus preventing the PP2Cs-mediated dephosphorylation of the SnRK2s and resulting in the activation of the SnRK2 kinases. Our results reveal new insights into ABA signaling mechanisms and define a minimal set of core components of a complete major ABA signaling pathway.

Introduction and results

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 (pyrabactin resistance1/PYR1-likes, also known as RCARs), were identified as ABA receptors. Several of the PYR/PYLs were shown to interact with and inhibit clade-A PP2Cs (type 2C protein phosphatases)[11-13]. The PP2Cs (ABI1, ABI2, HAB1, PP2CA/AHG3) negatively regulate ABA responses[13]. On the contrary, a subfamily of ABA-activated SnRK2s are positive regulators of ABA signaling[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, signaling and finally ABA-responsive gene expression. ABA-dependent phosphorylation of ABF2 at amino acid residues S26, S86, S94 and T135 was suggested to be 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* expression even in the presence of ABA, but co-transfection of ABF2 with SnRK2.6 resulted in 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 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). Substitution of lysine 50, a conserved residue critical for ATP-binding and kinase activity, with asparagine (K50N) inactivates SnRK2.6 in phosphorylation assays *in vitro* (our unpublished data). 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 signaling 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 ABA-induction of *RD29B-LUC* expression.

We next tested the effect of ABI1 and PYR1 on ABA-induction of *RD29B-LUC* expression. Transfection of ABI1 together with ABF2 and SnRK2.6 resulted in inhibition of *RD29B-LUC* expression (Fig. 1b, Supplementary Fig. 1a). This shows that ABI1 negatively regulates SnRK2.6- and ABF2-dependent activation of *RD29B-LUC* expression. Addition of PYR1 together with ABI1, SnRK2.6 and ABF2 enabled ABA-dependent induction of *RD29B-LUC* expression (Fig. 1b,Supplementary Fig. 1a). However, addition of $PYR1^{P88S}$ that is defective in interaction with and inhibition of PP2Cs[12] did not enable ABA-dependent induction of *RD29B-LUC* expression. The dominant *abi1-1* mutation (G180D) disrupts the interaction between ABI1 and PYR1[12]. Like the wild type ABI1, ABI^{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,Supplementary Fig. 1a). This suggests that the ABI^{G180D} mutant protein retains the inhibitory activity but can no longer be regulated. Thus reconstitution with PYR1, ABI1, SnRK2.6 and ABF2 is sufficient to enable ABA-mediated gene expression in protoplasts, providing *in vivo* evidence to our previously proposed model of ABA signaling[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 question, we reconstituted the ABA signaling pathway with different members of the PYR/PYL family. Our results show that all of the tested PYR1/PYLs could antagonize the ability of ABI1 to inhibit 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 signaling 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 PYR1 or PYL2 can antagonize this inhibition. The inhibitory effect of ABI1 was stronger than that of HAB1 in the reconstituted ABA signaling system in protoplasts (Fig. 1b-d, Supplementary Fig. 1). The three clade A PP2Cs (ABI1, ABI2 and HAB1) were each 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 those of ABI2 and HAB1 (Supplementary Fig. 2a), which correlate with the level of inhibitory effect of ABI1 and HAB1 in the protoplast assay (Fig. 1d, Supplementary Fig. 1b). A C-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 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,Supplementary Fig. 1). We hypothesized that the PYR/PYLs may prevent the inhibitory effect of the PP2Cs by disrupting the interaction between the PP2Cs and the SnRK2s. We tested whether co-expression of PYLs might disrupt the interaction between PP2Cs and SnRK2s by yeast triple-hybrid assays. 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 into the SnRK2.6-pBridge construct the PYL5 and PYL8, which 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 together with GBD-SnRK2.6 abrogated or reduced (depending on the dilution of the yeast culture) the interaction with GAD-ABI1 (Supplementary Fig. 3). Similar results were obtained when GBD-SnRK2.6 and GAD-ABI2 or GAD-HAB1 was 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 signaling pathway for stress responsive gene expression by co-expression of the PYR/PYLs, PP2Cs, SnRK2s and ABF2 in Arabidopsis protoplasts (Fig. 1b-d, Supplementary Fig. 1). To verify whether these are the minimal signaling components that are both necessary and sufficient for ABA signaling in the absence of other cellular components, we attempted to reconstitute the pathway *in vitro*. We constructed recombinant MBP-SnRK2.6, and found that it is capable of phosphorylating an ABF2 fragment as well as autophosphorylation (Fig. 2a and b). Incubation of GST-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 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 reducing 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 Ser175 is essential for the kinase activity of SnRK2.6 *in vitro*[24]. We tested and found that ABI1 can dephosphorylate a synthetic phosphopeptide corresponding to amino acids His170-Pro180 of SnRK2.6, which is phosphorylated at Ser175 (HSQPKpSTVGTP; Fig. 2e). These results suggest that ABI1 may deactivate SnRK2.6 by dephosphorylating Ser175.

When His-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 and 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[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 show that it is possible to reconstitute ABA activation of ABF2 phosphorylation *in vitro*. Importantly, ABF2 phosphorylation status in this reconstituted *in vitro* system responds to ABA in a concentration-dependent manner (Fig. 3c). The apparent IC50 of this response is 0.8 μM, which is similar to the IC50 value for ABA inhibition of seed germination[11] and falls within the physiological range of ABA concentrations in plants. Similar ABA responses 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, we showed previously that the SnRK2s are substantially less activated by ABA in the *pyr1pyl1pyl2pyl4* mutant compared with the wild type[12]. The model also predicts that the SnRK2s may be constitutively activated in mutant plants that are deficient in the PP2Cs. Indeed, the PP2C triple mutant *abi1-2hab1-1pp2ca-1* shows a constitutive activation of 42 and 45 kD 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 and c), as reported previously[25]. In contrast, the PP2C triple mutant *abi1hab1abi2* does not have a constitutive ABA response as strong as in *abi1-2hab1-1pp2ca-1* (Fig. 4b and c), and does not show a strong constitutive activation of the SnRK2s (Fig. 4a).

To our knowledge, this is the first report of *in vitro* reconstitution of a phytohormone signal transduction pathway using recombinant proteins. The *in vitro* reconstitution results are supported by the reconstitution assays in 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 suggest that all in 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 may connect to form a signaling pathway. Our study has revealed significant new 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 upon binding to ABA, the PYR/PYL receptor protein can disrupt or reduce the interaction between the SnRK2s and PP2Cs, and prevent the PP2Cs-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 signaling 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. Since 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 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 signaling, RNA metabolism and protein degradation are known to have important roles 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 impact 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] may also use components of the PYR/PYLs-

PP2C-SnRK2 regulatory module and whether additional receptors and core signaling components are involved.

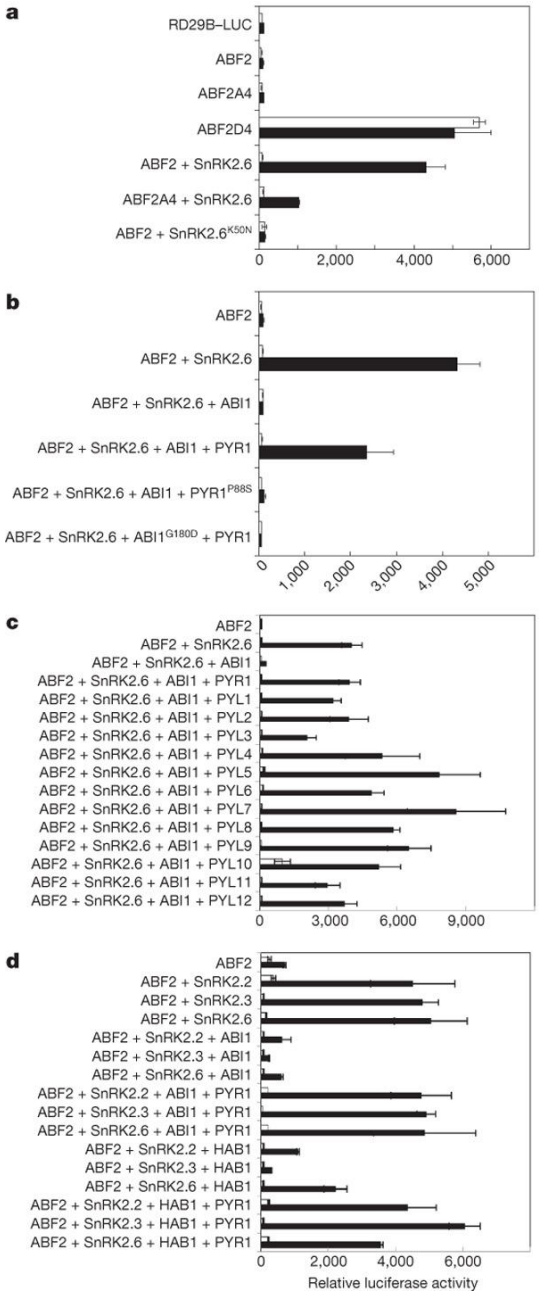

FIGURE 1. Reconstitution of the ABA signalling pathway for stress-responsive gene expression in Arabidopsis protoplasts. a, SnRK2-mediated phosphorylation of ABF2 is sufficient for ABA-responsive gene expression. **b**, Reconstitution of ABA signalling pathway by co-expression of PYR1, ABI1, SnRK2.6 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).

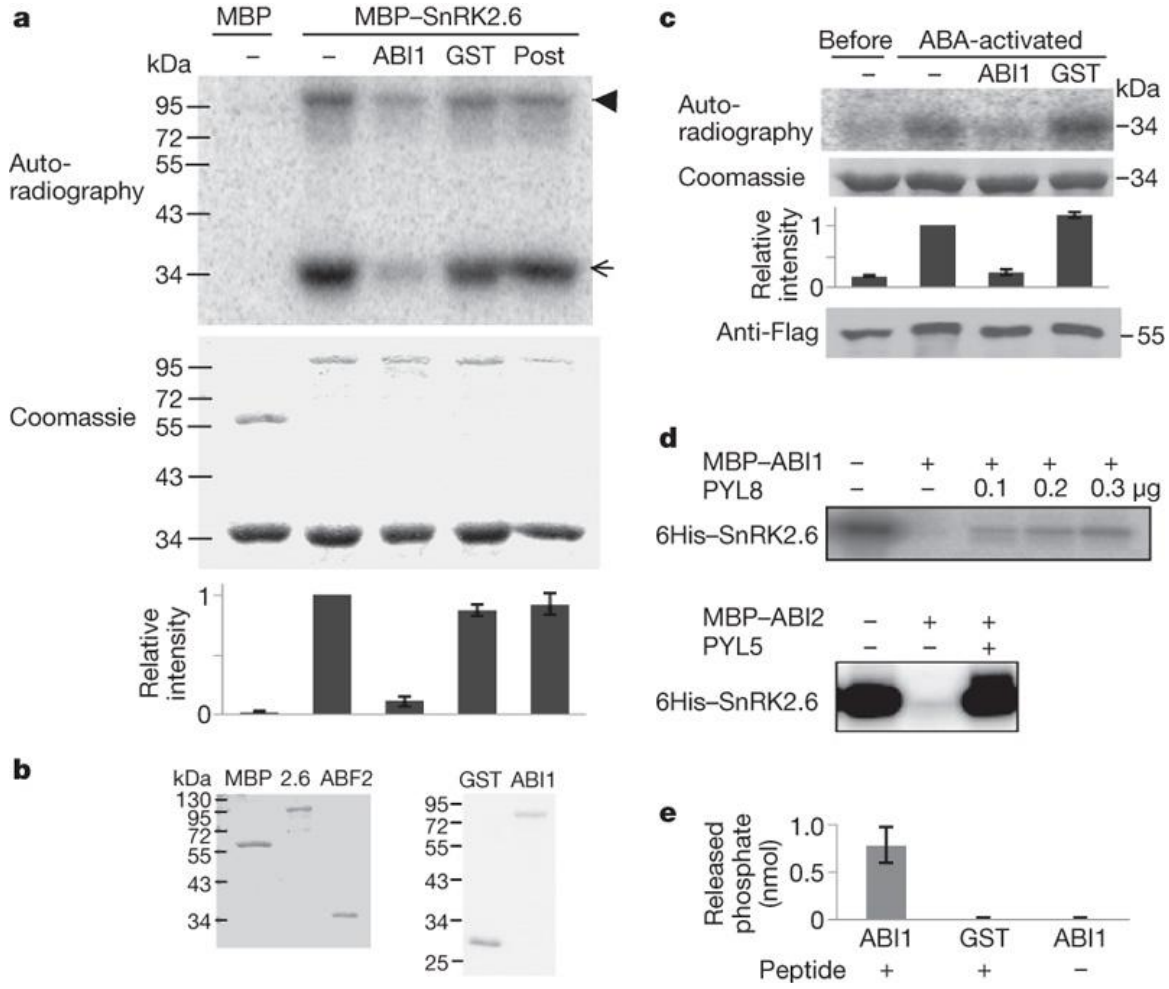

FIGURE 2. ABI1 and ABI2 inhibit SnRK2.6 by dephosphorylation. a, SnRK2.6 is deactivated by ABI1. MBP or MBP–SnRK2.6 treated without (-) or with GST–ABI1 or GST was incubated with a GST–ABF2 fragment (Gly 73 to Gln 119) in the presence of [γ-^{32}P]ATP. In the rightmost lane (Post), GST–ABI1 was added 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).

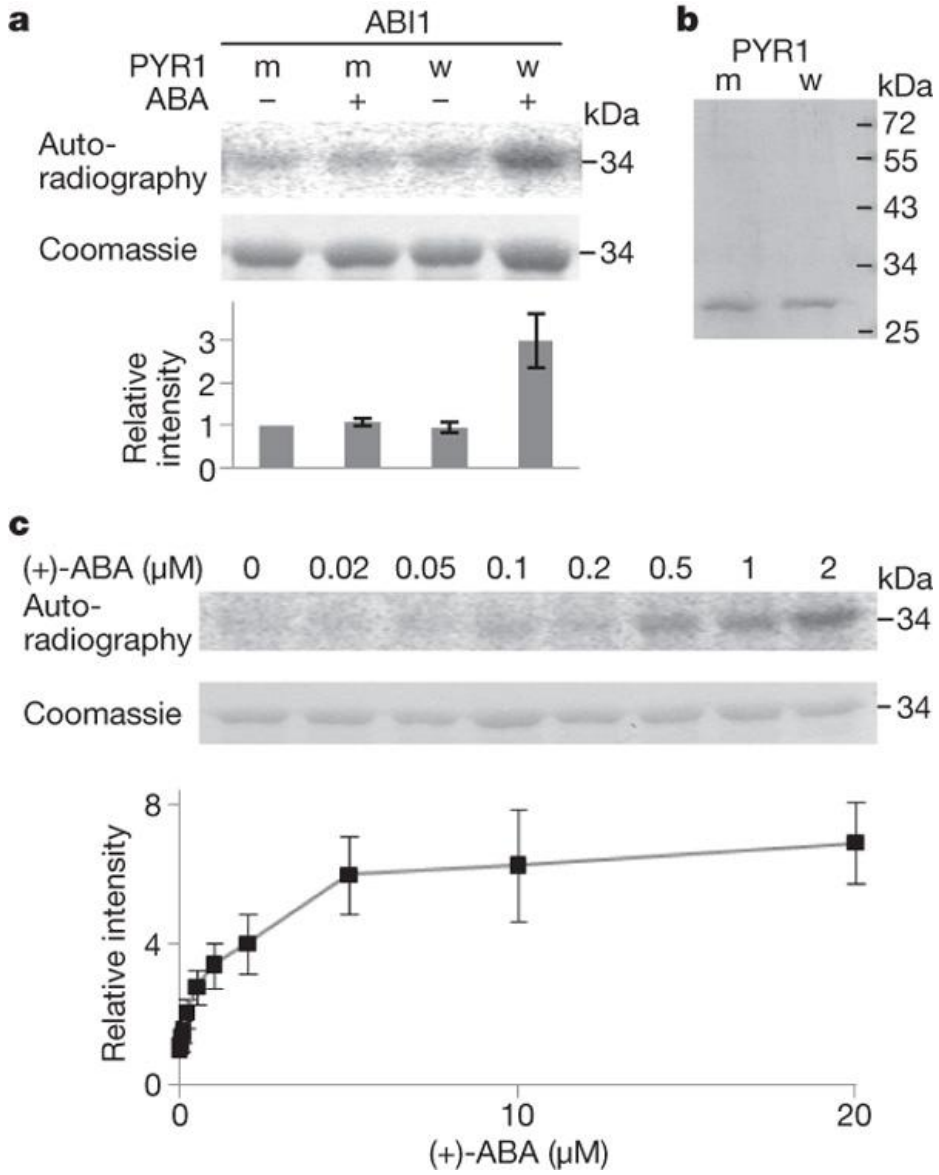

FIGURE 3. The combined effect of ABA, PYR1 and ABI1 on the phosphorylation of the GST–ABF2 fragment by SnRK2.6 in vitro. a, Reconstitution of ABA regulation of ABF2 phosphorylation. MBP–SnRK2.6 treated with GST–ABI1 and His-tagged wild-type PYR1 (w) or mutated PYR1^{P88S} (m) in the absence (-) or presence (+) 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).

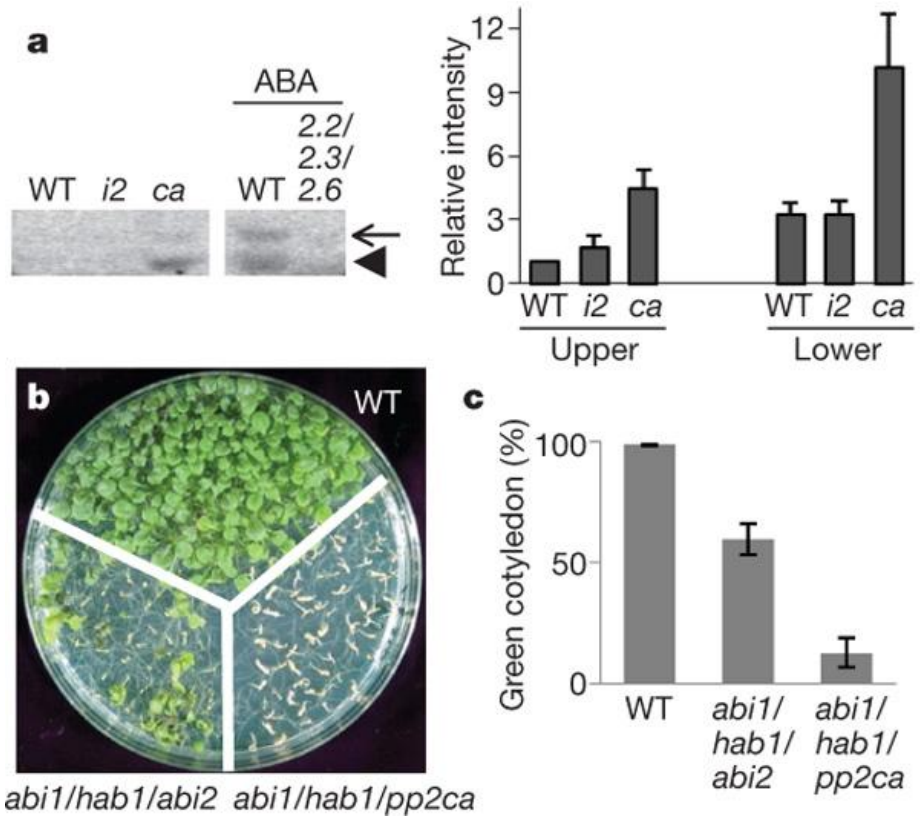

FIGURE 4. Effect of PP2C mutations on ABA response phenotypes and kinase activities of SnRK2s. a, In-gel kinase assay showing the activities of SnRK2s in the *abi1/hab1/abi2* (*i2*) and *abi1/hab1/pp2ca* (*ca*) triple mutants. *snrk2.2/2.3/2.6*was used as a control. A GST-fused ABF2 fragment (Gly 73 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).

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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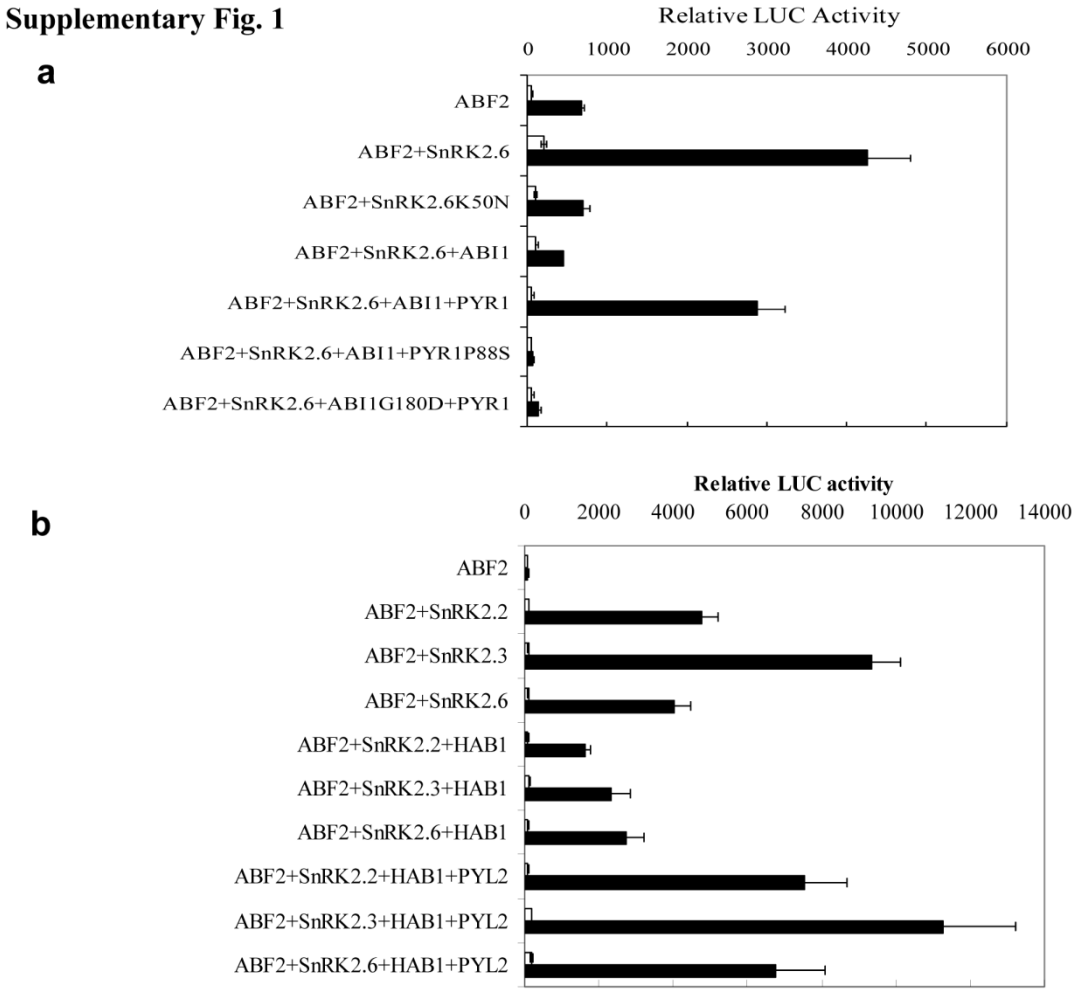
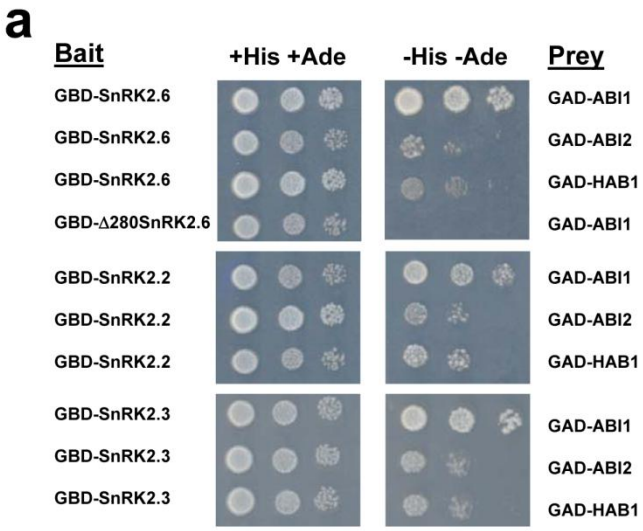
Supplemental material

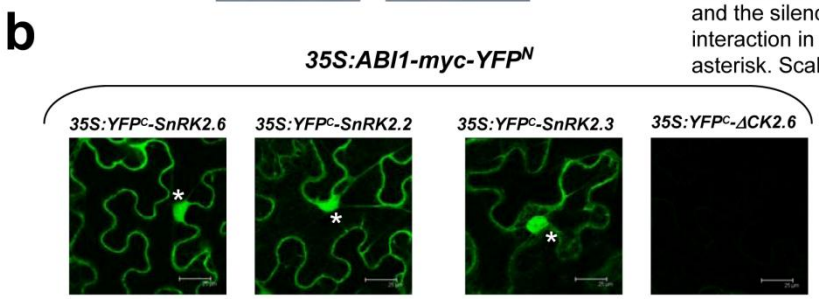
Supplementary Fig. 1

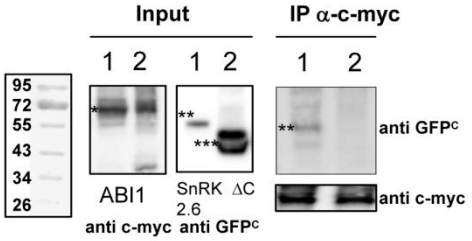


Reconstitution of ABA signaling pathway for stress responsive gene expression in *Arabidopsis* protoplasts. (a) Reconstitution of ABA signaling pathway using PYR1, ABI1, SnRK2.6 and ABF2 in wild type protoplasts. (b) Reconstitution using PYL2, HAB1, different SnRK2s and ABF2 in protoplasts from the *snrk2.2/3/6* triple mutant. The *RD29B* promoter fused with *LUC* coding sequence was used as an ABA-responsive reporter (7 µg per transfection). *ZmUBQ*::*GUS* was included in each sample as an internal control (3 µg per transfection). ABF2-HA, SnRK2s-Flag, His-PYR1/PYL2 and HAB1-myc plasmid constructs were used at 3 µg per transfection, while ABI1 was used at 2 µg per transfection. After transfection, protoplasts were incubated for 5 h under light and in the presence of 0 (open bars) or 5 µM (solid bar) ABA. Data shown are mean ± s.e.m. (n=3).

Supplementary Fig. 2



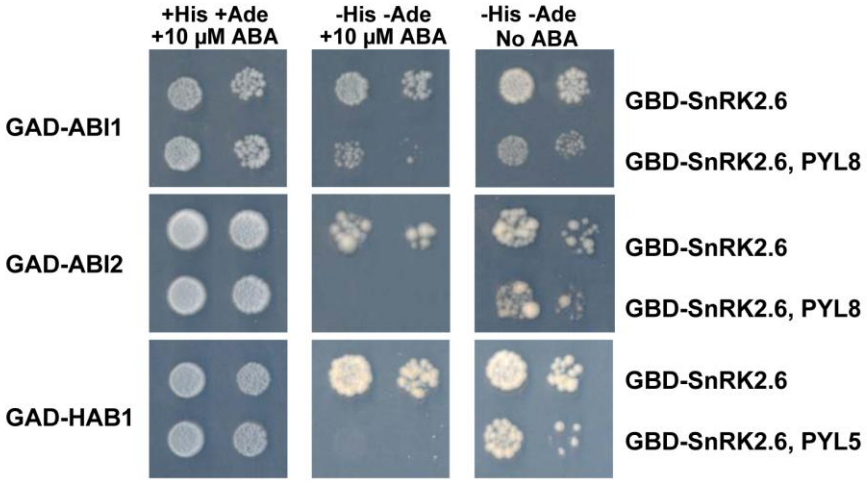




Interaction of clade A PP2Cs with SnRK2s in yeast and plants.
(a) Clade A PP2Cs interact with SnRK2s in a yeast two hybrid assay. SnRK2.2, 2.3 and 2.6 fused to the GAL4-DNA binding domain (GBD) were used as baits and PP2Cs fused to the GAL4-activating domain (GAD) were used as preys. A C-terminal deletion of SnRK2.6 lacking amino acids 280-362 (GBD-Δ280SnRK2.6) did not interact with ABI1. Interaction was determined by growth assay on media lacking His and Ade. Dilutions (10-1, 10-2, 10-3) of saturated cultures were spotted onto the plates and photographs were taken after 5 days. (b) BiFC analysis shows that ABI1 interacts with SnRK2.6, SnRK2.2 and SnRK2.3 in both the nucleus and cytosol of tobacco epidermal cells. Interaction of ABI1 and the three SnRK2s in *Agrobacterium*-infiltrated tobacco (*N. benthamiana*) leaves is shown in epifluorescence images of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring the indicated constructs and the silencing suppressor p19. The interaction in the nucleus is marked with an asterisk. Scale bar corresponds to 25 µm.

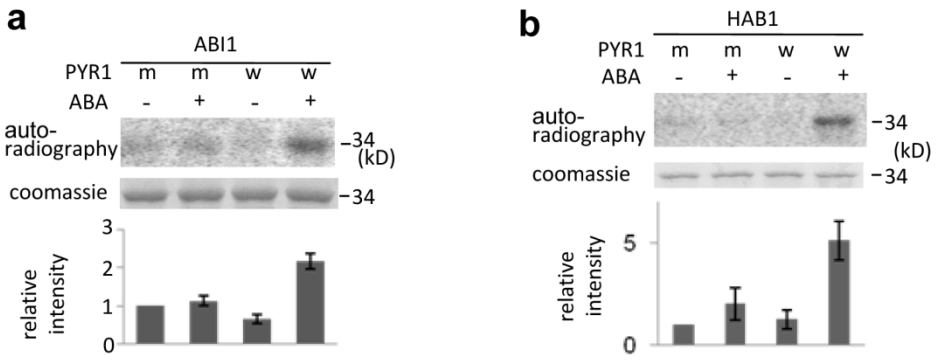
(c) Expression of ABI1 and SnRK2.6 in *Agrobacterium*-infiltrated tobacco (*N. benthamiana*) leaves and co-immunoprecipitation analysis. Protein gel blot analysis demonstrates the expression of the different fusion proteins containing ABI1, the SnRK2.6 and the C-terminal deletion (ΔC) of SnRK2.6 (Input). Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* harboring the construct 35S:ABI1-myc-YFPN/35S:YFPC-SnRK2.6 (lane 1) or 35S:ABI1-myc-YFPN/ 35S:YFPC-Δ280SnRK2.6 (lane 2), were analyzed by immunoblots using anti-c-myc or anti-GFPC antibodies. Anti-myc immunoprecipitates (IP α-c-myc) were probed with anti-GFPC antibodies. Fusion proteins are marked with asterisks.

Supplementary Fig. 3



PYLs disrupt the interaction between the PP2Cs and SnRK2.6 in an ABA-dependent manner. Co-expression of ABA receptors abrogates or reduces the interaction of ABI1, ABI2 and HAB1 with SnRK2.6. Interaction was determined by yeast growth assay on media lacking His and Ade. Dilutions (10-2, 10-3) of saturated cultures were spotted onto the plates and photographs were taken after 4 days (ABI1) or six days (ABI2 and HAB1). Full length PP2Cs were fused to the GAD, whereas SnRK2.6 was fused to the GBD in the pBridge three-hybrid vector. When indicated, the ABA-receptors PYL8 and PYL5 were cloned in the SnRK2.6-pBridge construct and co-expressed with SnRK2.6.

Supplementary Fig. 4



Reconstitution of ABA signalling. (a) Reconstitution of ABA signalling. with ABA-activated SnRK2.6 from plant extracts. FLAG-SnRK2.6, which is pulled down from extracts of transgenic plants after ABA treatment and treated with GST-ABI1 and His-tagged wild type PYR1 (w) or mutated PYR1P88S (m) in the absence (-) or presence (+) of 2 μM (+)-ABA, was incubated with GST-ABF2 fragment in the presence of [γ32P]-ATP. Coomassie staining, autoradiography and relative radioactivities of GST-ABF2 fragment are shown. Radioactivities of GST-ABF2 fragment were normalized, taking the radioactivity of the band with PYR1P88S in the absence of ABA as 1 (mean ± s.e.m., n = 7). (b) Reconstitution of ABA signalling with HAB1 instead of ABI1. MBP-SnRK2.6 treated with GST-HAB1 and His-tagged wild type PYR1 (w) or mutated PYR1P88S (m) in the absence (-) or presence (+) of 2 μM (+)-ABA was incubated with the GST-ABF2 fragment in the presence of [γ32P]-ATP. Coomassie staining, autoradiography and relative radioactivities ofGST-ABF2 fragment are shown. Radioactivities of GST-ABF2 fragment were normalized, taking the radioactivity of the band with PYR1P88S in the absence of ABA as 1 (mean ± s.e.m., n = 3).

11. APPENDIX 2

News on ABA transport, protein degradation and ABFs/WRKYs in ABA signaling

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Abstract

The recent identification of abscisic acid (ABA) transporters provides an important insight into the delivery of ABA from the vascular system and its uptake by target cells. A putative connection with PYR/PYL receptors is envisaged, linking ABA uptake and intracellular perception by a fast and efficient mechanism. Downstream signaling of the core pathway involves regulation of ABA-responsive element binding factors (ABFs/AREBs) through phosphorylation, ubiquitination and sumoylation in the case of ABI5. Several E3 ligases appear to regulate ABA signaling either positively or negatively, although relatively few targets are known yet. ABFs/AREBs are themselves subjected to transcriptional regulation, and some transcription factors harboring the WRKY domain (WRKYs) appear to regulate their expression through W-box sequences present in the promoters of ABFs/AREBs.

Introduction

The phytohormone abscisic acid (ABA) represents a key signal to regulate plant growth and development as well as plant response to abiotic and biotic stress [1]. In the plant field, the pivotal role played by ABA to coordinate the plant adaptive response under drought stress and hence potential applications in agriculture have led to numerous studies focused on the elucidation of ABA perception and downstream signaling. Challenging our perspective as plant biologists, the discovery of ABA in humans and its prophylactic and therapeutic efficacy in mouse models of diabetes and atherosclerosis have further extended the interest in this animal/plant molecule [2•,3•]. In 2009, the plant family of PYR/PYL/RCAR ABA receptors was discovered and its connection with key elements of the pathway, i.e. PP2Cs and SnRK2s, was established (Figure 1). The module receptor-ABA-phosphatase controls phosphorylation signaling cascades in a ligand-dependent manner through regulation of ABA-activated SnRK2s and in concert with other kinases, e.g. calcium-dependent kinases (CPKs/CDPKs) (Figure 1). These findings have been extensively reviewed recently and they will not be the main topic of this review [1,4–10]. Instead, we will focus on other emerging aspects of the ABA pathway, such as the identification of ABA transporters, an update on the effect of protein degradation/stability in ABA signaling, the connection between ABFs/WRKYs transcription factors (TFs) as well as new reports on Mg-chelatase function.

Efflux and uptake of ABA

Since ABA biosynthesis occurs predominantly in vascular parenchyma cells and ABA has systemic effects, a requirement for efficient intercellular transport of ABA, beyond that of passive diffusion, had been envisaged [11–13]. For instance, ABA2, AAO3 and NCED3, key enzymes of the ABA biosynthetic pathway, are expressed in specific areas of vascular tissues, which suggested the existence of a transport system to deliver ABA to target tissues and cells [11–13]. In 2010, two ABA transporters were identified by genetic screenings [14••,15••]. A search for Arabidopsis ABA-hypersensitive mutants in germination and seedling growth led to the identification of the *abcg25* mutant [14••]. The *ABCG25* gene, which encodes an ATP-binding cassette (ABC) transporter, is expressed mainly in vascular tissues and the protein is localized at the plasma membrane (Figure 1). A transport assay with vesicle membranes obtained in transfected insect cells indicated that ABCG25 might have ATP-dependent ABA-efflux activity in plant cells. Indeed, overexpression of *ABCG25* in Arabidopsis led to reduced sensitivity to ABA-mediated inhibition of growth, probably because the cells remove ABA by active transport, and reduced water loss, probably because this transporter facilitates the delivery of ABA to guard cells.

ABA delivery from vascular tissues to the apoplast of guard cells might be connected with ABA uptake from the apoplast to the cytosol through another plasma membrane-localized transporter, ABCG40/PDR12 (Figure 1). ABCG40 was identified by direct screening for potential ABA transporters in the PDR-type subfamily of ABC transporters [15••]. To this end, seed germination and stomatal response were analyzed in 13 out of 15 knockout mutants (*abcg29-41*), and as a result, the mutant *abcg40* was identified as having marked differences with respect to wild type (wt). Stomata of *abcg40* showed reduced stomatal closure and lower inhibition of stomatal opening in the presence of ABA, and therefore, *abcg40* plants showed enhanced wilting under drought stress and reduced increase in leaf temperature in response to ABA. ABCG40 function is also required in cell types other than guard cells, although gene expression in guard cells was higher than in mesophyll cells. Thus, experiments conducted in rosette tissue also showed delayed and reduced expression of three ABA-responsive genes in *abcg40*. Results obtained with *abcg40* seeds are more difficult to interpret, because although these seeds were less-sensitive to inhibition of germination mediated by exogenous ABA, they also showed faster germination on medium lacking ABA. Finally, biochemical experiments in the yeast heterologous system and tobacco cell suspensions showed that *ABCG40* is a high-affinity (K_m = 1 μM) and specific uptake ABA transporter.

Although both transporters belong to the large ABC subfamily G, they are grouped in different branches because of an important structural difference, i.e. ABCG25 belongs to the branch of half-size transporters (AtABCG1–28) and ABCG40 to that of full-size transporters (AtABCG29–43) [16]. Since *ABCG25* belongs to a large gene family, functional redundancy might explain why the *abcg25* mutant does not show aerial phenotypes. However, *ABCG40* also belongs to a gene family and, nevertheless, the stomatal response of *abcg40* was notably affected. Since *abcg40* also affects ABA-response of mesophyll cells, the authors could assess the contribution of *ABCG40* to ABA uptake in Arabidopsis protoplasts, concluding that this gene product is the major ABA importer in leaf-cell protoplasts. Moreover, an apparent paradox is now solved. The pH-dependent diffusion of undissociated ABA is a component of ABA uptake, which would be markedly reduced under drought stress that increases the pH of xylem sap [17]. The discovery of *ABCG40* offers a reasonable alternative, under drought-stress less ABA would be nonspecifically trapped by passive diffusion in nontarget tissue and more ABA would be available for pH-independent uptake [15••].

Protein degradation and transcriptional regulation

The ubiquitin/26S proteasome pathway plays a key role in the perception and transmission of environmental and hormonal signals [18]. For instance, perception of auxins, jasmonates and

gibberellins are closely linked to this pathway, and ethylene and ABA signaling also involve components of this protein degradation pathway [19]. Either negative or positive transcriptional regulators of these hormonal pathways are targets of the 26S proteasome, and therefore, inactivation of transcriptional repressors or ceasing degradation of activators, respectively, leads to hormone signaling. ABA signaling is affected in different mutants that show lesions either in a regulatory subunit of the 26S proteasome [20], different E3 ligases [21–24••,25•] or substrate receptors of E3 ligases [26••] (Figure 2). Additionally, sumoylation, which can act competitively on targets regulated by ubiquitination to regulate protein stability, also affects ABA signaling through negative regulation of ABI5 activity [27,28••]. Indeed, pioneering work on the regulation of ABI5 protein stability was crucial to link the 26S proteasome and ABA signaling [29].

Mutants of some proteolysis-related components have a pleiotropic effect including impaired ABA signaling. For instance, the *rpn10* mutant, which is impaired in a subunit of the 19S regulatory particle of the 26S proteasome, is affected in a number of processes and it shows hypersensitivity to ABA in seed germination and root growth assays as well as stabilization of the short-lived ABI5 transcription factor [20]. Pleiotropic effects, including ABA hypersensitivity, were also found in the *siz1* mutant, which was impaired in a SUMO E3 ligase. SIZ1 negatively regulates ABA signaling through sumoylation of ABI5, which inactivates the protein and prevents its proteasome-mediated degradation [28••]. ABI5 transcript accumulation, protein stability and protein phosphorylation are highly regulated by ABA [29]. In the absence of ABA, ABI5 is degraded to allow germination and postgerminative growth, whereas ABA induces ABI5 stabilization, when applied between 48 and 60 h poststratification, to prevent early growth under osmotic stress conditions [29]. The RING E3 ligase KEG is required for ABI5 degradation under normal growth conditions and ABA causes ABI5 accumulation by promoting KEG degradation [22,26••]. Phosphorylation of KEG is required for its ABA-induced degradation, which opens a possible link with the SnRK2s of the core ABA signaling pathway.

ABI5 seems to be a highly courted TF, since also CUL4-based E3 ligases regulate its stability through the proteins DWA1 and DWA2, which are the components of the ligase that mediate substrate recognition [30••]. Finally, another element that regulates ABI5 protein levels is ABI five binding protein (AFP); however, its mechanism of action is not yet clear. AFP belongs to a small family of proteins, AFP1–4, that are able to interact with ABI5 [31,32]. Initially, it was proposed that AFP might promote ABI5 degradation by the 26S proteasome [31]; however, AFP is not an E3 ligase. Instead, a characteristic feature of AFP1–4 proteins is the presence of an ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif at the N-terminus. The EAR motif is a hallmark of transcriptional repressors such as AUX/IAA and NINJA

proteins, which function as adaptor proteins to recruit the Groucho/Tup1-type co-repressor TOPLESS (TPL) [33•]. Interaction of AFP2 and AFP3 with TPL has been observed by yeast two-hybrid assays, which suggests the tempting hypothesis that some AFP proteins and TPL (or TPL-related proteins) form a high-molecular mass complex, acting as transcriptional repressors of ABA signaling by blockade of ABI5 function [33•].

ABI3 is another target of the 26S proteasome and the RING E3 ligase AIP2 is a negative regulator of ABA signaling that promotes ABI3 degradation [21,34]. Thus, during vegetative growth, ABA promotes ABI3 degradation through enhancement of AIP2 function [21]. Conversely, ABA promotes the accumulation of ABI3 during seed maturation and the time period when post-germination growth arrest occurs, via transcriptional and post-translational mechanisms. PRT6 (Proteolysis6) is another type of E3 ligase that negatively regulates seed sensitivity to ABA [24••]. PRT6 is an N-recognin E3 ligase that recognizes amino-terminal destabilizing residues of proteins, targeting them for degradation at the 26S proteasome. Mutant *prt6* seeds are very hypersensitive to ABA-mediated inhibition of seed germination and according to genetic interactions with various *abi* mutants, it has been hypothesized that PRT6 might degrade a positive regulator of ABA signaling during seed after-ripening. The E3 ligases described up to now are genetically defined as negative regulators of ABA signaling. However, other E3 ligases, such as the RING finger E3 ligases SDIR1 (salt- and drought-induced ring finger1) and RHA2a (ring-H2), are genetically characterized as positive regulators because *sdir1* and *rha2a* mutants show reduced sensitivity to ABA in seed germination and early seedling growth assays, and in the case of *sdir1*, also reduced stomatal closure by ABA [23,25•]. Therefore, these ligases might be involved in the degradation of transcriptional repressors or negative regulators of ABA signaling.

ABFs, WRKYs and Mg-chelatase in ABA signaling

Different families of transcription factors regulate ABA signaling in a positive or negative manner [1]. Among the best known positive regulators of ABA signaling and key targets of SnRK2s are the bZIP-type ABFs/AREBs, which recognize the ABA-responsive elements in the promoters of ABA-inducible genes. A comprehensive analysis of the AREB1/ABF2, AREB2/ABF4 and ABF3 TFs has been performed through the generation of multiple combinations of mutants [35•]. During seed germination, none of the mutants showed different sensitivity to ABA compared to wt. However, vegetative responses to ABA were particularly impaired in the triple mutant *areb1areb2abf3*, as illustrated by its resistance to ABA-mediated inhibition of root growth and diminished expression of stress-responsive genes. Compared to this, the triple mutant only shows a

modest increase in water-loss rate compared to wt, indicating that other targets of ABA-activated SnRK2s, different than bZIP-type AREB/ABFs, are mostly responsible for the regulation of stomatal aperture.

Different rice and Arabidopsis WRKY TFs have been implicated in ABA signaling [36–38•,39•,40•]. Usually, WRKYs have been described as TFs inducible by pathogen infection or salicylic acid treatment, and indeed, a large number of pathogen-inducible genes contain W-box sequences that are recognized by WRKY proteins. Interestingly, ABA signaling genes as *ABF2*, *ABF4*, *ABI4* or *ABI5* contain W-box sequences in their promoter regions [38•, 40•]. Thus, WRKY63 positively regulates expression of *ABF2* through binding to W-boxes of its promoter (Figure 3), but intriguingly, *wrky63* shows enhanced sensitivity to ABA during seed germination and seedling growth, whereas it is ABA-hyposensitive for stomatal closure [38•]. Using ChIP analysis, Shang *et al*. [40•] have shown that WRKY40 binds the promoters of *ABF2*, *ABF4*, *ABI4* and *ABI5*, and for instance, represses *ABI5* expression (Figure 3). Accordingly, the *wrky40* mutant shows enhanced sensitivity to ABA-mediated inhibition of germination and early seedling growth. In agreement, Chen *et al.* [39•] obtained similar results during the characterization of *wrky40*. In contrast, conflicting results were obtained with respect to ABA sensitivity of *wrky18* and *wrky60* mutants, which are defined as positive regulators of ABA signaling [39•], whereas Shang *et al.* [40•] catalogued them as repressors. Finally, this article poses a model for Mg-chelatase H subunit (CHLH/ABAR)-mediated ABA signaling that involves recruitment of WRKY40 at the cytosol upon ABA perception by the cytosolic tail of CHLH [40•]. This model faces important criticisms since two groups have failed to show ABA binding by barley or Arabidopsis CHLH [41•, 42•], apparently the carboxylate group of ABA, which is required for bioactivity, is not required for ABA binding by CHLH [43, 44•] and finally, no alteration in regulation of stomatal aperture was reported in any of the single or combined *wrky* mutants [40•]. In spite of this controversy, it seems well supported that CHLH affects ABA signaling in stomatal guard cells, since impairment of its function in RNAi lines [45, 46••] or the missense mutants *cch* (encoding $chlh^{P642L}$) [45] and *rtl1*(encoding $chlh^{L690F}$) [42•] led to enhanced water-loss and lack of ABA-induced stomatal closing. Since another mutant impaired in a different subunit of Mg-chelatase, CHLI, shows impaired stomatal closure, it has been suggested that the Mg-chelatase complex as a whole plays an indirect role in ABA signaling, likely through regulation of Ca^{++} mobilization from chloroplastic stores [42•]. Structural evidence supporting ABA-binding by CHLH would be a definitive answer to the above described controversy.

Conclusions

The recent identification of PYR/PYL intracellular ABA-receptors nicely matches with the discovery of an active transport system for ABA-uptake, which allows fast delivery of ABA to target cells for efficient inactivation of clade A PP2Cs through PYR/PYL receptors. It somehow seemed ABA signaling was inefficiently designed, spending so much investment on the core pathway, i.e. receptors-phosphatases-kinases, and depending exclusively on passive diffusion for intracellular ABA delivery. In addition to protein phosphorylation, regulation of protein stability by the 26S proteasome is an important mechanism for ABA signaling, particularly during germination and early seedling growth. Several E3 ligases are involved in this process, acting either positively or negatively. Additionally, a few E3 ligase mutants, e.g. *sdir1* and *dwa1 dwa2*, are also known to be affected in the regulation of stomatal aperture, and this phenotype can´t be explained with the reduced number of targets identified so far. Therefore, an important question for the future is the identification of additional targets of E3 ligases beyond of ABI3 and ABI5. Finally, transcriptional regulation of ABFs/AREBs by WRKYs is a novel finding in the complex regulation of gene expression in response to ABA.

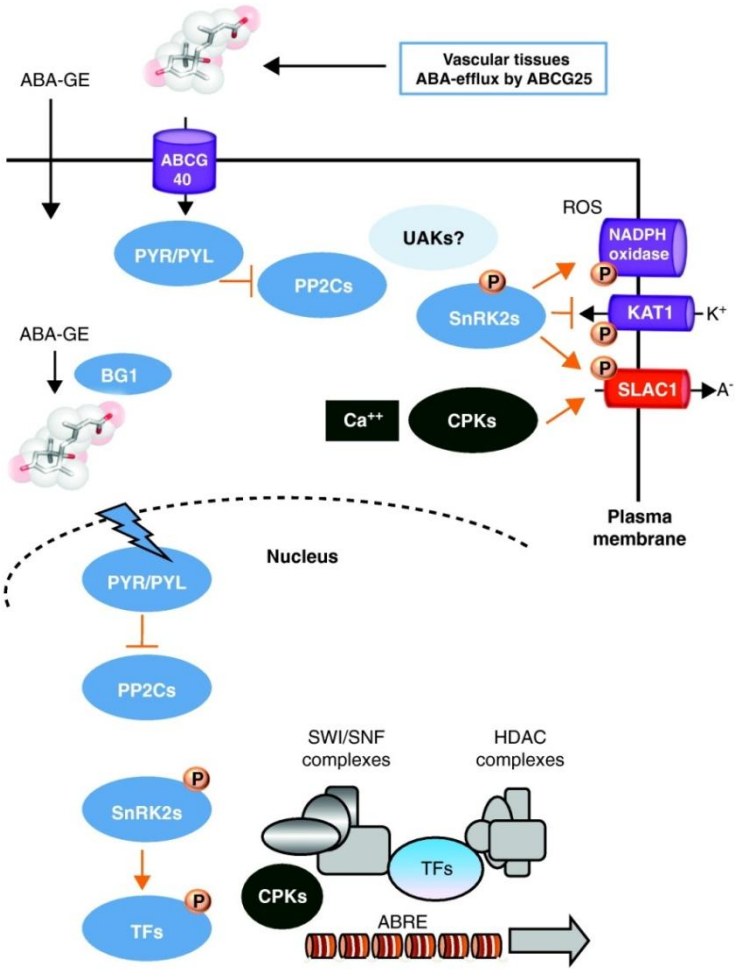

Figure 1. A simplified model of the ABA pathway that integrates ABA transport and signaling. PYR/PYL/RCAR receptors perceive ABA intracellularly, either at cytosol or nucleus, and form stable ternary complexes with clade A PP2Cs. Thus, phosphatases are inactivated, which allows the activation of downstream targets of the PP2Cs, for instance SnRK2.2, 2.3 and 2.6/OST1. These kinases are either autophosphorylated or activated by putative upstream activating kinases (UAKs), leading to ABA-induced regulation of plasma membrane and nuclear targets, such as NADPH oxidase, KAT1, SLAC1 and ABFs/AREBs (reviewed in 1, 4-10). In addition to SnRK2s, the calcium-dependent protein kinases (CPKs) also regulate ion fluxes and transcriptional response to ABA, and for instance, the CPK and SnRK2 branches converge on the anion channel SLAC1. TFs are supposed to act in the context of chromatin and components of chromatin remodeling complexes, e.g. type SWI/SNF and histone deacetylases (HDAC), have been shown to regulate ABA signaling [47–51]. ABA and its glucose ester (ABA–GE) are subjected to intercellular and likely intracellular transport. The role of ABC transporters, ABCG25 and ABCG40, in ABA transport is highlighted and putatively connected with ABA perception. BG1 is an intracellular b-glucosidase localized to ER that releases ABA from ABA–GE stored in the vacuole or imported from the vascular system [52].

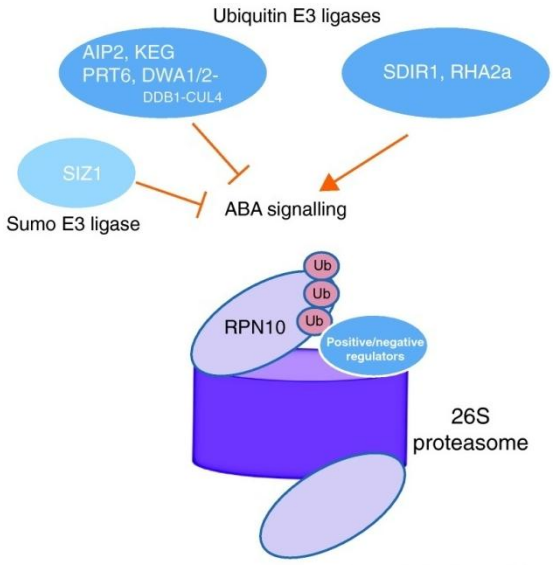

Figure 2. Ubiquitin and SUMO E3 ligases as regulators of ABA signaling. Whereas ubiquitin-modified proteins are targeted for degradation by the 26S proteasome, the fate of sumoylated proteins depends on the target. In the case of ABI5, sumoylation by SIZ1 protects it from proteasome degradation and maintains the TF in an inactive form. AIP2, KEG, PRT6 and DWA1/2-DDB1-CUL4 promote degradation of positive regulators of ABA signaling (ABI3 by AIP2, ABI5 by both KEG and DWA complex). Conversely, SDIR1 and RH2a are supposed to promote degradation of unidentified negative regulators. RPN10 is a regulatory subunit of the proteasome that mediates degradation of ABI5.

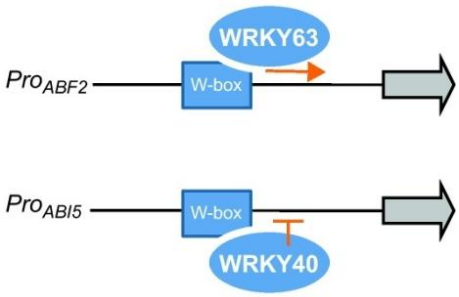

Figure 3. Transcriptional regulation of *ABF2* and *ABI5* expression by WRKY TFs. Several WRKYs have been involved in ABA signaling, namely WRKY2, WRKY18, WRKY40, WRKY60 and WRKY63. Binding to W-box sequences of *ABF2* promoter by WRKY63 or *ABF4*, *ABI4* and *ABI5* promoters by WRKY40 has been demonstrated. WRKY63 activates expression of *ABF2*, whereas WRKY40 represses expression of *ABI5*.

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●● of outstanding interest

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●A genetic screening for mutants showing enhanced water-loss led to the identification of a rapid-transpiration mutant, named *rtl1*, which is ABA insensitive both for ABA-induced stomatal closure and inhibition of light-induced stomatal opening. Map-based cloning revealed a mutation in CHLH,

L690F, as being responsible of the phenotype. The authors confirmed that both CHLH RNAi lines and the cch mutant (a missense mutation P642L) did not display ABA-induced stomatal closure. These results, together with [44•, 45], confirm CHLH affects ABA signaling in stomatal guard cells. Unfortunately, recombinant CHLH did not bind ^{3}H-labeled ABA using similar conditions to those described in [44•], whereas PYR1 in the presence of ABI1 bound ABA. PYR1 alone did not bind ABA in this assay, likely because its Kd for ABA is >50 □ M in the absence of the PP2C. Finally, 5 mM extracellular Ca^{++} restored ABA-induced stomatal closure of *rtl1*, which led the authors to suggest a role for CHLH in Ca^{++} mobilization from chloroplastic stores.

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●The authors use ABA-affinity chromatography to detect ABA binding to Arabidopsis, barley and rice CHLH/ABAR. Additionally, they use ^{3}H-labeled ABA to further confirm that the C-terminal part of ABAR (amino acid residues 631-999) binds ABA. Such portion of the protein might be an excellent starting point for co-crystallization with ABA and structural studies that would provide a definitive answer to the controversy with [41•, 42•] results. Finally, the authors identified two TILING *abar* alleles, *abar-2* (encoding chlh^{L348F}) and *abar-3* (chlh^{S183F}), which show opposite phenotypes in seed germination assays (the first one ABA-insensitive, the second one ABA-hypersensitive) but lack stomatal phenotypes.

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Professional CV

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Date of birth: 24 March, 1983
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Languages:
Spanish (primary), Italian, English, Catalan.

Academic Achievements

Education and work Experience

Ph.D.	Lab. of Dr.Pedro Luís Rodríguea Egea. Instituto de Biología Molecular y Celular de Plantas (IBMCP). Universidad Politécnica de Valencia. (August 2008-).
Student collaborator	Lab. of Dr.Pedro Luís Rodríguea Egea. Instituto de Biología Molecular y Celular de Plantas (IBMCP). Universidad Politécnica de Valencia. (01/01/2008-31/08/2008).
Ph.D. courses	Associated to the "Máster en Biología Molecular y Celular de plantas" (2007 and 2008).
Degree	Licenciatura en Biología. Universidad de Valencia.(18/7/2007).

Academic Awards

Beca de Colaboración del Ministerio (Undergraduate fellowship)	September 2006

Other relevant information

Erasmus fellowship. Università degli studi di Roma "La Sapienza". (2005-2006).

Certificado de Aptitud Pedagógica. Universidad Politécnica de Valencia. (2007).

Research results

Publications

Antoni R, Gonzalez-Guzman M, Rodriguez L, Peirats-Llobet M, Pizzio GA, Fernandez MA, De Winne N, De Jaeger G, Dietrich D, Bennett MJ, Rodriguez PL. (2013). PYRABACTIN RESISTANCE1-LIKE8 Plays an Important Role for the Regulation of Abscisic Acid Signaling in Root. **Plant Physiology** 161: 931-941

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Poster presentations

M. González-Guzman, L. Rodríguez, **R. Antoni**, J. Santiago and P.L. Rodriguez. ABAreceptors regulate the interaction between PP2Cs and SnRKs. 20th IPGSA Conference (International Conference on Plant Growth Substances). Tarragona, Spain, (2009).

J. Santiago, F. Dupeux, **R. Antoni**, K. Betz, M. González-Guzman, L. Rodríguez, S. Rubio, J.A. Márquez and P.L. Rodriguez. ABA-receptors regulate the interaction between PP2Cs and SnRK2s. 1st PCUBE Workshop and Users meeting. Grenoble, France (2010).

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