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Structural insights into ABA perception and signalling: structure of ABA receptor PYR1

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By

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CERTIFICA:

Que la presente memoria titulada “Structural insights into ABA perception and signalling: structure of ABA receptor PYR1”, ha sido realizada por Julia Santiago Cuéllar 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 key role in regulation of plant development and coordination of the adaptive responses under environmental stress conditions. To obtain further insights into ABA signalling, we have characterized a new family of interacting partners of the clade A PP2C HAB1. These interacting partners belong to a 14-member subfamily of the Betv1-like superfamily and they have been named PYR/PYL/RCARs (Pyrabactin resistance/pyrabactin resistance-like/regulatory components of ABA receptor). Interaction between HAB1 and one of the PYR/PYL/RCAR members, PYL5, was confirmed by yeast-two-hybrid, BiFC and co-immunoprecipitation assays. PYL5 was localized both in nucleus and cytosol. Due to the large number of family members, an over-expression approach was carried out to study the role of these proteins in ABA signalling. PYL5 over-expression led to a globally hypersensitive response to ABA, which contrasted with the opposite phenotype reported for HAB1 over-expressing plants, indicating that this new family played a positive role in ABA signalling. Furthermore, PYL5 over-expressing plants, showed an enhanced resistance to drought. F_2 plants that over-expressed both HAB1 and PYL5 exhibited hypersensitivity to the hormone, indicating that PYL5 was antagonizing HAB1 function. Moreover, PYL5 and other members of its family were able to inhibit HAB1, ABI1 and ABI2 phosphatase activity in an ABA-dependent manner, which indicated that the PYR/PYL/RCAR members were exerting their positive role in ABA signalling by inhibiting the negative regulators of the pathway. In addition, Isothermal titration calorymetry assays revealed that PYL5 was able to bind (+)ABA, in a saturable and specific manner, with Kd values of 1.1 μM or 38 nM in the absence or presence of the PP2Ccatalytic core of HAB1, respectively. PYL5 also presented partial stereoespecificity, being able to bind the non-natural form of (-)ABA with lower affinity values (Kd=19 μM). All these data suggested that these new family might be an intracellular family of ABA receptors. To further confirm that, we carried out structural studies and as a result we obtained the crystal structure of the *Arabidopsis thaliana* PYR1 member. The PYR1 structure consisted of a dimer in which one of the subunits was bound to ABA. The monomeric subunits of this receptor protein were structurally arranged to form a hydrophobic central cavity where the hormone accommodates. Comparison of both, the free-ABA and the ABA-bound subunits, allowed studying the conformational changes induced upon binding of the hormone. In the ligand-bound subunit it could be appreciated that the loops surrounding the entry to the binding cavity fold over the ABA molecule, burying it inside, whereas in the empty subunit they were forming a passage, leaving an open access to the cavity. This was indicating that

the conformational changes in these loops had a critical role in the stabilization of the hormone-receptor complex. In addition, a mutational analysis has also revealed that these loops and their conformational change upon ABA binding also played a critical role on the interaction with the PP2C. Further characterization of this receptors family, has revealed that they can be divided in monomeric and dimeric receptors in their unactivated apo form. We have characterized a key residue in PYR1, His60, that is variable between family members and that played a critical role in determining oligomeric state. The formation of homodimers has been seen to be disfavourable for ABA and PP2C binding. This different oligomeric state confers them different properties, which suggests that the PYR/PYL/RCAR members might have differential cellular responses over the physiological range of ABA concentrations.

Moreover, we have seen that the ABA-PYR/PYL/RCAR-PP2C signalling pathway is conserved in cultivated plants, in particular in rice. This provides a new framework in ABA signalling susceptible of modification, as well as for the design or identification of new ABA activators that would allow the manipulation of ABA responses in plants, in order to improve crop yield under drought contidions.

Resumen

La hormona vegetal ácido abscísico (ABA) juega un papel fundamental en la regulación del desarrollo, así como en la coordinación de la respuesta adaptativa de las plantas bajo condiciones de estrés ambiental. Con el objetivo de profundizar en el conocimiento sobre la señalización de ABA, se ha caracterizado una nueva familia de proteínas que interacciona con la fosfatasa HAB1, perteneciente al grupo A de las PP2C. Estas nuevas proteínas han sido denominadas PYR/PYL/RCAR ("Pyrabactin resistance/pyrabactin resistance-like/regulatory components of ABA receptor") y pertenecen a una subfamilia de 14 miembros, que se engloba en la superfamilia conocida bajo el nombre BetvI. La interacción entre HAB1 y uno de los miembros de la familia PYR/PYL/RCAR, PYL5, ha sido confirmada mediante diferentes abordajes como la interacción por dos híbridos en levadura, BiFC en planta y ensayos de coinmunoprecipitación. Además se ha visto que PYL5 se localiza tanto en núcleo como en citoplasma. Debido al elevado número de miembros pertenecientes a la familia PYR/PYL/RCAR, se optó por llevar a cabo un abordaje de sobreexpresión para estudiar el papel de estas proteínas en la señalización de ABA. La caracterización fenotípica de plantas sobreexpresoras de PYL5, dio lugar a una respuesta global de hipersensibilidad al ABA, presentando un fenotipo completamente opuesto al descrito para plantas sobreexpresoras de la fosfatasa HAB1. Estos resultados indican que esta nueva familia jugaba un papel positivo en la señalización de ABA. Además, plantas sobreexpresoras de PYL5, muestran una mayor resistencia a sequía, vinculando directamente a estas proteínas en la regulación de la respuesta a estrés hídrico en plantas. La caracterización de individuos F2 que sobreexpresan tanto HAB1 como PYL5, también presentan fenotipos de hipersensibilidad a la hormona, indicando que la función de PYL5 antagoniza a la de HAB1. Además PYL5, junto con otros miembros de la familia PYR/PYL/RCAR, tienen la capacidad de inhibir la actividad fosfatasa de HAB1, ABI1 y ABI2 de manera dependiente de ABA. Esto indica que los miembros PYR/PYL/RCAR ejercen su papel positivo en la señalización de ABA, mediante la inhibición de los reguladores negativos de la ruta. Además, ensayos de calorimetría han revelado que PYL5 es capaz de unirse a (+)ABA, con valores de Kd de 1,1 M o 38 nM en función de la ausencia o presencia de HAB1, respectivamente. PYL5 también muestra una estereoespecificidad parcial, debido a que también es capaz de unir la forma no natural de (-) ABA, aunque con menor afinidad (Kd = 19 M). Todos estos datos sugieren que esta nueva familia se trata de una familia de receptores intracelulares de ABA. Con el objetivo de confirmar esta hipótesis, se han llevado a cabo estudios de cristalización y estructura por difracción de rayos X, y como resultado

se ha obtenido la estructura cristalina de la proteína PYR1 de *Arabidopsis thaliana*. La estructura de PYR1 está formada por un dímero, en el que una de las subunidades se encuentra unida a ABA. Las subunidades monoméricas de este receptor, presentan una disposición estructural para formar una cavidad hidrofóbica central que permite acomodar a la hormona en su interior. El estudio comparado entre la subunidad no unida y unida a ABA, ha permitido desvelar los cambios conformacionales inducidos por la unión de la hormona. En la subunidad unida a ABA, se puede apreciar que los bucles que se encuentran rodeando la entrada de la cavidad, se encuentran plegados sobre la molécula de ABA aislándola del solvente. Por el contrario, en la subunidad no unida a la hormona, estos bucles se encuentran desplegados, formando un pasaje hacia el interior de la cavidad. Este análisis estructural ha revelado el papel fundamental de estos bucles en la estabilización del complejo hormona-receptor. Mediante un análisis mutacional, también hemos visto que estos bucles y el cambio conformacional que sufren debido a la unión del receptor a la hormona, presentan un papel crítico en la interacción con la fosfatasa.

La caracterización bioquímica de estas proteínas PYR/PYL/RCAR, también ha revelado que se dividen en dos subgrupos cuando no están unidas a la hormona, en monómeros y dímeros. Hemos caracterizado un residuo clave en el receptor PYR1, la His60, que parece tener un papel clave en la determinación del estado de oligomerización. La formación de homodímeros resulta ser energéticamente desfavorable para la unión de ABA y de la PP2C. El diferente estado de oligomerización de estas proteínas les confiere características distintas, lo que sugiere que los distintos miembros de la familia PYR/PYL/RCAR podrían estar involucrados en diferentes respuestas celulares en función del rango de concentraciones fisiológicas de ABA en la célula.

Además, también hemos visto que la ruta de señalización ABA-PYR/PYL/RCAR-PP2C se encuentra conservada en plantas de cosecha, en particular en arroz. Esto proporciona un nuevo marco en la señalización de ABA, susceptible de ser modificado, así como utilizado para el diseño o identificación de nuevas moléculas capaces de mimetizar la acción del ABA; permitiendo así el control de la respuesta a la hormona con el objetivo de mejorar la producción de plantas de cosechas en condiciones de sequía.

Resum

L'hormona vegetal àcid abscíssic (ABA) té un paper fonamental en la regulació del desenvolupament, així com en la coordinació de la resposta adaptativa de les plantes sota condicions d'estrès ambiental. A fi d'aprofundir en el coneixement sobre la senyalització d'ABA s'ha caracteritzat una nova família de proteïnes que interacciona amb la fosfatasa HAB1, pertanyent al grup A de les PP2C. Aquestes noves proteïnes s'han denominat PYR/PYL/RCAR ("Pyrabactin resistance/pyrabactin resistance-like/regulatory components of ABA receptor") i pertanyen a una subfamília de 14 membres, que s'engloba en la superfamília coneguda amb el nom de *BetvI*. La interacció entre HAB1 i un dels membres de la família PYR/PYL/RCAR, PYL5, ha sigut confirmada mitjançant diferents abordatges com la interacció de dos híbrids en llevat, BiFC en planta i assajos de coimmunoprecipitació. A més, s'ha vist que PYL5 es localitza tant en nucli com en citoplasma. A causa de l'elevat nombre de membres que té la família PYR/PYL/RCAR, es va optar per dur a terme un abordatge de sobreexpressió per estudiar el paper d'aquestes proteïnes en la senyalització d'ABA. La caracterització fenotípica de les plantes que sobreexpressaven PYL5 va donar lloc a una resposta global d'hipersensibilitat a l'ABA; aquestes plantes presentaven un fenotip completament oposat al descrit per a les plantes sobreexpressores de la fosfatasa HAB1. Aquests resultats indiquen que aquesta nova família tenia un paper positiu en la senyalització d'ABA. A més, les plantes sobreexpressores de PYL5 mostren una major resistència a la sequera, cosa que vincula directament aquestes proteïnes amb la regulació de la resposta a l'estrès hídric en plantes. Els individus F2 que sobreexpressen tant HAB1 com PYL5 també presenten fenotips d'hipersensibilitat a l'hormona, la qual cosa indica que la funció de PYL5 antagonitza la d'HAB1. A més, PYL5, junt amb altres membres de la família PYR/PYL/RCAR, tenen la capacitat d'inhibir l'activitat fosfatasa d'HAB1, ABI1 i ABI2 de manera dependent d'ABA. Això indica que els membres PYR/PYL/RCAR exerceixen un paper positiu en la senyalització d'ABA mitjançant la inhibició dels reguladors negatius de la ruta. A més, assajos de calorimetria han revelat que PYL5 és capaç d'unir-se a (+)ABA, amb valors de Kd d'1,1 M o 38 nM en funció de l'absència o presència d'HAB1, respectivament. PYL5 també mostra una estereoespecificitat parcial, atès que també és capaç d'unir la forma no natural de (-)ABA, encara que amb menor afinitat (Kd=19 M). Totes aquestes dades suggereixen que aquesta nova família és una família de receptors intracel·lulars d'ABA. A fi de confirmar aquesta hipòtesi, s'han dut a terme estudis de cristal·lització i estructura per difracció de raigs X, i com a resultat s'ha obtingut l'estructura cristal·lina de la proteïna PYR1 d'*Arabidopsis thaliana*. L'estructura de PYR1 està formada per un

dímer, en el qual una de les subunitats es troba unida a ABA. Les subunitats monomèriques d'aquest receptor presenten una disposició estructural per a formar una cavitat hidrofòbica central que permet acomodar-hi l'hormona a l'interior. L'estudi comparat entre la subunitat no unida i la unida a ABA ha permès desvelar els canvis conformacionals induïts per la unió de l'hormona. En la subunitat unida a ABA es pot apreciar que els bucles que envolten l'entrada de la cavitat es troben plegats sobre la molècula d'ABA i l'aïllen del solvent. Per contra, en la subunitat no unida a l'hormona, aquests bucles es troben desplegats i formen un passatge cap a l'interior de la cavitat. Aquesta anàlisi estructural ha revelat el paper fonamental d'aquests bucles en l'estabilització del complex hormona-receptor. Mitjançant una anàlisi mutacional, també hem vist que aquests bucles i el canvi conformacional que pateixen a causa de la unió del receptor a l'hormona tenen un paper crític en la interacció amb la fosfatasa.

La caracterització bioquímica d'aquestes proteïnes PYR/PYL/RCAR també ha revelat que es divideixen en dos subgrups quan no estan unides a l'hormona, en monòmers i dímers. Hem caracteritzat un residu clau en el receptor PYR1, la His60, que sembla tenir un paper clau en la determinació de l'estat d'oligomerització. La formació d'homodímers resulta ser energèticament desfavorable per a la unió d'ABA i de la PP2C. El diferent estat d'oligomerització d'aquestes proteïnes hi confereix característiques distintes, la qual cosa suggereix que els diversos membres de la família PYR/PYL/RCAR podrien estar involucrats en diferents respostes cel·lulars en funció del rang de concentracions fisiològiques d'ABA en la cèl·lula.

A més, també hem vist que la ruta de senyalització ABA-PYR/PYL/RCAR-PP2C es troba conservada en plantes de collita, en particular en arròs. Això proporciona un nou marc en la senyalització d'ABA, susceptible de ser modificat, així com utilitzat per al disseny o la identificació de noves molècules capaces de mimetitzar l'acció de l'ABA; això permet el control de la resposta a l'hormona, a fi de millorar la producció de plantes de collites en condicions de sequera.

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1. INTRODUCTION

1. INTRODUCTION

1.1 IDENTIFICATION AND DISCOVERY OF ABA

The compound that was eventually named as abscisic acid (ABA) was first discovered in the 1960s by several groups when trying to isolate endogenous plant regulators (Ohkuma et al. 1963, Cornforth et al. 1965, Cracker et al. 1969). Ohkuma et al. wanted to isolate compounds that were responsible for promoting leaf abscission in cotton. The compound isolated was named Abscissin II (Ohkuma et al. 1963). Cornforth et al. (1965) also looked for compounds that were promoting bud dormancy, thinking that these compounds might likely be general inhibitors. As a result they isolated a compound named dormin, which was a wheat embryo germination inhibitor present in sycamore leaves. Subsequent chemical analysis revealed that dormin and abscissin II were the same compound, being ultimately renamed as ABA.

Although ABA was discovered and is best understood in higher plants, it appears to be present in other organisms across kingdoms, including mosses, fern, algae, fungi (Nambara and Marion Poll, 2005) and also in metazoans, ranging from the most primitive one (the sea sponge) to mammals (Le Page-Degivry et al., 1986, Zocchi et al., 2001, Puce et al., 2004). In addition, Bruzzone et al. (2007) have reported that ABA acts as an endogenous pro-inflammatory cytokine in human granulocytes in response to high temperature. Bassaganya-Riera et al. (2011) have also demonstrated a role of ABA in modulation of inflammatory responses. More recently, ABA has been suggested to stimulate broader physiological responses in mammals, including insulin release in pancreatic islets (Scarfi et al. 2008, 2009; Bruzzone et al. 2008; Bodrato et al. 2009; Magnone et al. 2009). These evidences suggest the possibility that the origin of this hormone was prior to the great division of kingdoms.

1.2 CHEMICAL FEATURES OF ABA

The hormone ABA ($C_{15}H_{20}O_4$) is a sesquiterpenoid formed by cleavage of a carotenoid precursor derived from isopentenyl pyrophosphate. This precursor is synthesized in plastids through the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (Nambara and Marion Poll, 2005). The chemical structure of ABA owns a number of features relevant for its biological activity. The ABA molecule has one asymmetric, optically active carbon at the position C1'. The naturally active occurring form is S-(+)-ABA and the side chain is, by definition, in 2-cis, 4-trans isomeric state (Figure 1A). ABA has an amphipathic character with polar and hydrophobic modules. The polar

groups of the molecule are comprised by the hydroxyl from the chiral carbon, the carboxylic group and the oxygen from the ketone group. These polar groups have been found to be essential in the interaction with the new ABA receptor family, the PYR/PYL/RCAR members (PYRABACTIN RESISTANCE 1/PYR1-Like/REGULATORY COMPONENT OF ABA RECEPTOR), and play a critical role in anchoring and orienting the ABA molecule into the receptor's pocket. Similarly, the hydrophobic groups, comprehended by the cyclohexene ring and the isoprene moiety, have a pivotal role in helping the anchoring and inducing several structural rearrangements in the protein that would be responsible for the transmission of the ABA signal in the cascade (addressed in General discussion) (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B: Results Chapter 2 of this work; Dupeux et al., 2011; Hao et al., 2010; Peterson et al., 2010).

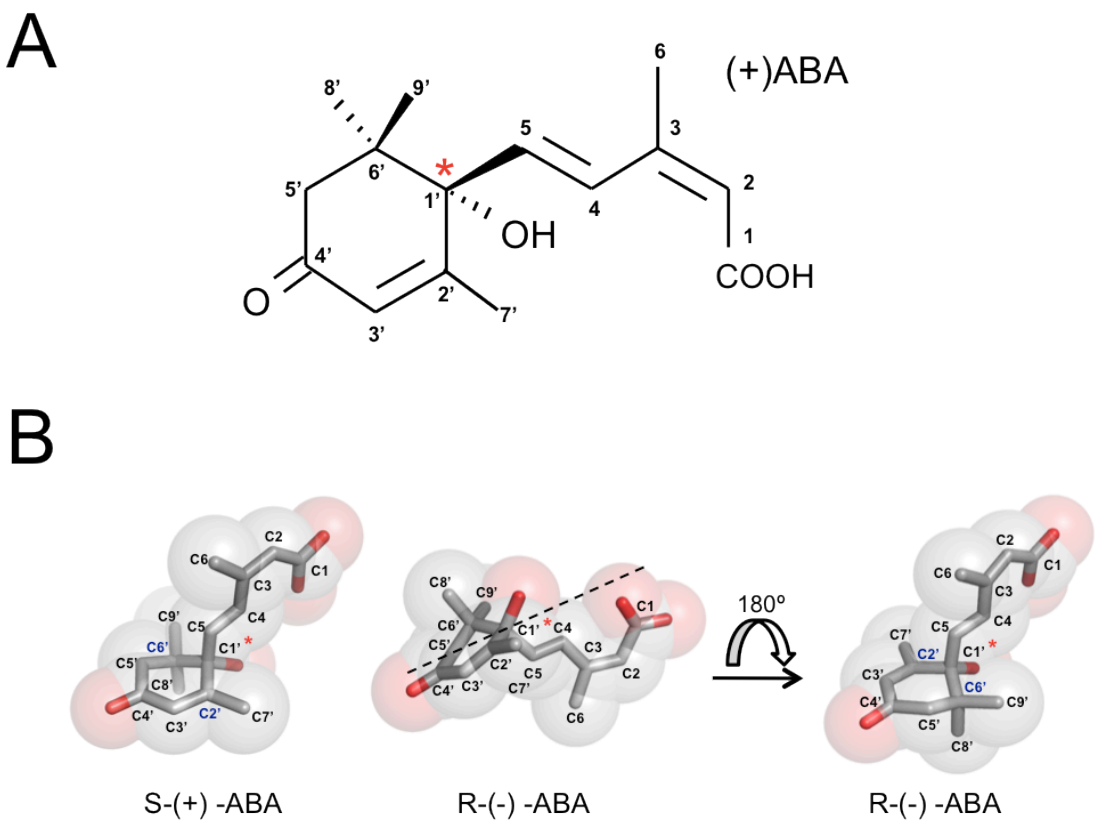

Figure 1. Chemical structure of the phytohormone ABA. A, 2D structure of the natural S-(+) form of ABA. The red asterisk points out the C1' asymmetric carbon of the molecule. **B,** 3D structure of the two ABA enantiomers. The (-) enantiomer has been rotated 180º, to illustrate the structural difference of the ring methyl groups with respect to the (+) enantiomer in position C'2 and C'6. The plane of symmetry is indicated by a dotted line.

Besides the natural form, the unnatural enantiomer R-(-)-ABA has also been reported to be biologically active in different well known responses to the hormone (Walker-Simmons et al,. 1994; Nambara et al., 2002; Lin et al., 2005; Huang et al., 2007). In support to these evidences, it has been recently reported that (-)-ABA is able to bind to some PYR/PYL/RCAR receptors, although with lower affinity than (+)-ABA (Santiago et al., 2009A: Results Chapter 1 of this work), as well as to induce the binding and inhibitory effect of some of these ABA receptors over the negative regulators of the pathway, the protein phosphatases 2C (Park et al., 2009; Santiago et al., 2009A). An explanation for the bioactivity of the non-natural form can be explained if the chemical structure of both enantiomers is analyzed. In principle both enantiomers present a different structure, but in the ABA molecule a plane of symmetry can be drawn along the chiral carbon (Figure 1B). Thus, if the (-)-ABA form is rotated 180º, the structural differences between the two enantiomers are in the methyl groups located in positions C'2 and C'6. Due to this plane of symmetry, the rest of the functional groups in the (-)-ABA remain intact in position as compared to the (+)-ABA form. This provides an explanation why the (-)-ABA can also be biologically active.

1.3 PHYSIOLOGICAL ROLES OF ABA IN THE PLANT

The phytohormone abscisic acid is involved in the regulation of numerous physiological processes in the plant. Particularly, it plays a critical role in coordinating the plant response to conditions of low water potential, such as drought and salinity (Zhu, 2002; Verslues et al., 2006).

1.3.1 ROLE OF ABA UNDER DROUGHT AND SALT STRESS CONDITIONS

Drought and high salinity provoke a strong increase of ABA levels in the plant, resulting in the triggering of major changes in gene expression and physiological adaptive responses. The role of ABA in drought and salt stress is at least twofold: water balance and cellular dehydration tolerance (Zhu, 2002).

Salt stress induces both osmotic and ion stress. The ion stress branch seems to be regulated through the SALT OVERLY SENSITIVE (SOS) pathway. Briefly, this pathway is activated by an increase in cytosolic Ca^{2+}, which is sensed by SOS3. SOS3 interacts and activates SOS2, a serine/threonine kinase (Halfter et al., 2000; Liu et al., 2000). Thus, SOS3 and SOS2 regulate the expression level of *SOS1*, which is a salt tolerance effector encoding a plasma membrane Na^+/H^+ antiporter (Shi et al., 2002), leading to ion homeostasis. ABA might also contribute to the activation of this pathway through the generation of second messengers such as increase in cytosolic Ca^{2+}, via

the generation of 1,4,5-thriphosphate (IP_3) (Lee et al., 1996; Xiong et al., 2001A), or reactive oxygen species (ROS) production (Pei et al., 2000). Additionally, a protein phosphatase type 2C (PP2C) implicated in ABA signalling, and described as a negative regulator of the pathway (ABI2), has also been reported to interact with SOS2. Characterization of *abi1-1* and *abi2-1* mutants, whose mutations were seen to disrupt the interaction with SOS2, presented enhanced osmotic tolerance (Ohta et al., 2003).

As a first line of action, plants launch different responses in order to avoid water stress. These responses have the purpose of increasing water uptake or limiting water loss, so that water balance is maintained. Such a balance is achieved in the short term by control of stomatal closure, leading to a reduced transpiration rate, and in the longer term by inducing root growth to maximize water uptake (Verslues et al., 2006). The perception of ABA in guard cells promotes stomatal closing. Induction of stomatal closure is a way to rapidly prevent from loss of water through transpiration and it is induced by a decrease in turgor pressure and volume. This change in turgor is mainly caused by efflux of anions and potassium ions from the cell, which leads to water exit, resulting in the pore closing (Israelsson et al., 2006; Kim et al., 2010).

Abscisic acid also changes the growing root/shoot ratio, promoting primary root elongation while inhibiting shoot growth as an adaptive feature, in order to counterbalance water shortage (van der Weele et al., 2000; Sharp, 2002; Sharp et al., 2004). Sharp et al. have seen that high ABA concentrations in the root tip are required for the maintenance of maize primary root elongation under water stress conditions (Sharp, 2002; Sharp et al., 2004). ABA maintains root growth during water deficits by regulating (directly or indirectly) different mechanisms such osmotic adjustment, promotion of antioxidant systems keeping reactive oxygen species at-non damaging levels, restricting ethylene production and stimulating cell wall loosening (Ober and Sharp, 1994; Sharp et al., 2000; Guan et al., 2000; Spollen et al., 2000; LeNoble et al., 2004; Sharp, 2002; Sharp et al., 2004).

On the other hand, ABA has been implicated in inhibiting the development of lateral roots under water stress conditions (De Smet et al., 2003, 2006; Xiong et al., 2006). This might represent an adaptive response from the plant by restricting root horizontal proliferation and benefiting primary root growth, having this way, better chances to search for new water resources deep down in the soil (Xiong et al., 2006). However, there is one type of specialized lateral roots, the so-called “short roots”, whose developmental program seems to be promoted by ABA. These short roots, once initiated, switch to a dormant mode if sub-lethal drought conditions persist. Upon

rehydration, these short roots restart growth replacing eventually the dehydrated old lateral roots (Vartanian et al., 1994; Wasilewska et al., 2008).

In cases where the water stress persists, the plant launches additional mechanisms focused on avoiding and tolerating dehydration, in order to maintain its functions. Avoidance mechanisms are base on osmotic adjustment, while dehydration tolerance is focused on preventing or repairing cellular damage (Verslues et al., 2006). The plant avoids dehydration by accumulation of osmocompatible solutes such as sucrose, proline and glycine betaine (Ingram and Bartels, 1996; Verslues et al., 2006). Moreover, ions sequestration in the vacuole also plays an important role in retaining water in the cell (Zhu, 2002; Yokoi et al., 2002; Shi and Zhu, 2002; Cheong and Yun, 2007). In the case of dehydration tolerance, most of the mechanisms studied are based on protecting the cellular structure from the effects of dehydration. ABA also regulates the synthesis of dehydrins and late-embryogenesis-abundant (LEA) proteins, which are known to act as chaperons protecting proteins and membranes under stress conditions (Ingram and Bartels, 1996; Hara et el., 2001; Verslues et al., 2006). In addition, as mentioned before, ABA has also been implicated in promoting antioxidant systems, keeping reactive oxygen species at-non damaging levels (Guan et al., 2000).

1.3.2 ROLE OF ABA UNDER BIOTIC STRESS CONDITIONS

Besides its role as a key regulator in the plant adaptive response to abiotic stresses, ABA, among other hormones, has also been involved in pathogen response. However, the role of ABA in disease resistance is not straightforward (Ton et al., 2009).

Stomatal closure has been reported to be part of the plant innate immune response to restrict microbe invasion (Melotto et al., 2006). The ABA signalling pathway in guard cells has been connected with the rapid stomatal closure upon bacterial perception. Mutants impaired either in ABA biosynthesis (*aba3-1*) or insensitive to ABA (*ost1-2*) cannot induce stomatal closure upon infection (Melotto et al., 2006). Thus, ABA exerts a positive role in pre-invasive defense against bacteria. Another early stage response is the pathogen-induced callose deposition in which ABA seems to also have a role. That role seems to be conditioned by the nature of the pathogen. Upon infection with *Pseudomonas syringae*, ABA hypersensitive *abi2* mutants have shown to deposit less callose, whereas the opposite phenotype has been observed in ABA-insensitive *abi2-1* mutants (de Torres-Zabala et al., 2007). In agreement with this, ABA has also been found to suppress callose deposition in *Arabidopsis* cotyledons upon treatment with the bacterial pathogen-associated molecular pattern (PAMP) flagellin (Clay et al., 2009). Hence, ABA seems to play a

negative role in bacteria-induced callose deposition in *Arabidopsis*. Conversely, ABA has been seen to play a positive role in callose deposition against infection of the necrotroph *Leptosphaeria maculans*. When exposed to this necrotroph, both ABA biosynthetic mutant *aba1-3* and the ABA-insensitive mutant *abi1-1* displayed enhanced disease susceptibility that was linked to reduced callose deposition (Kaliff et al., 2007).

In late disease resistance, ABA also exerts different effects by either suppressing resistance (Mohr et al., 2007; Yasuda et al., 2008) or promoting susceptibility (Anderson et al., 2004). ABA has been reported to suppress the salicylic acid (SA)-dependent disease resistance (Yasuda et al., 2008). These results confirmed previous evidence in which it had been seen that application of ABA was suppressing SA-inducible defense activation by *Pseudomonas syringae* DC3000 (Mohr et al., 2007). These evidences suggest that ABA functions as an inhibitor of SA-dependent defenses. On the other hand, ABA has been reported to promote susceptibility to *Fusarium oxysporum*, and suppress jasmonic acid (JA)- and ethylene (ET)-dependent induction of defense-related genes, such as *PDF1.2* (*PLANT DEFENSIN 1.2*) and *PR4* (*PATHOGENESIS RELATED PROTEIN 4*), among others (Anderson et al., 2004).

There is also evidence that ABA has a positive effect on resistance against insects. It has been reported that ABA-deficient mutants in tomato and *Arabidopsis thaliana* are more susceptible to infestation by insects (Thaler and Bosctock, 2004; Bodenhausen and Reynol, 2007).

1.3.3 GROWTH AND DEVELOPMENT

ABA is not only involved in plant abiotic and biotic stress responses but it is also required to fine-tune control of growth and development under non-stress conditions. Its effect depends on tissue, concentration and environmental conditions.

The fundamental role of endogenous ABA in promoting growth and development, can be appreciated when examining the strong dwarfed phenotype and defects on seed production of severe ABA-deficient (*aba1* or *aba2*) or ABA-insensitive (*snkr2.2/2.3/2.6*) mutants, even under well-watered conditions (Cheng et al., 2002; Barrero et al., 2005; Fujii and Zhu, 2009; Nakashima et al., 2009).

It is well known that ABA also regulates germination and it is crucial is seed development (Finkelstein et al., 2002). It has been described that in early embryogenesis, ABA prevents seed abortion and promotes embryo growth (Cheng et al., 2002; Frey et al., 2004). On the other hand, in late embryogenesis, ABA is absolutely required to induce and maintain seed dormancy and to promote seed

desiccation tolerance (Karssen et al., 1983; revised in Kucera et al., 2005). In addition, ABA has also been reported to promote inhibition of germination and seedling arrest under osmotic stress conditions (González-Guzmán et al., 2002).

1.4 REGULATION OF ABA LEVELS IN THE PLANT IN RELATION TO ITS PHYSIOLOGICAL ROLES: METABOLISM, STORAGE AND TRANSPORT

As we have previously described, ABA plays multiple roles in numerous and different physiological processes during the plant lifecycle, acting not only as a stress signal but also as a growth regulator. The regulation of all these processes is dependent on the endogenous ABA levels in the cell. The endogenous ABA content, which ranges from nanomolar to micromolar (McCourt and Creelman, 2008), is determined by the balance among biosynthesis, release from inactive conjugates, catabolism and transportation rates.

Genetic approaches together with biochemical studies, have greatly contributed to depict the molecular basis of ABA metabolism.

The novo ABA synthesis starts with the production in plastids of the early C5 precursor of ABA -isopentenyl pyrophosphate (IPP)- from glyceraldehydes 3-phosphate and pyruvate via the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway. IPP is then converted to a C_{20} product, geranylgeranyl pyrophosphate (GGPP). This leads to the successive synthesis of C_{40} phytoene, which is the common precursor of all plant carotenoids, by condensation of two molecules of GGPP. Desaturation and isomerization reactions convert this into the all-*trans*-lycopene, which will be subsequently cyclized and hydroxylated to finally yield zeaxanthin via β,β-carotene. Conversion of zeaxanthin to violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP) (ABA1) via the intermediate antheraxanthin (Bouvier et al., 1996; Marin et al., 1996). Then *trans*-violaxanthin is transformed into *trans*-neoxanthin. This step seems to be carried out by a predicted neoxanthin synthase codified by the *ABA4* gene from *Arabidopsis*. ABA4 is involved is neoxanthin synthesis, however, in vitro assays of neoxanthin synthase activity using recombinant ABA4 protein, haven not been successful. Nevertheless, the overexpression of ABA4 in transgenic plants results in increased levels of trans-neoxanthin, which suggests that ABA4 is directly involved in enzyme activity (North et al., 2007). *Cis*-isomers of violaxanthin and neoxanthin are cleaved by nice-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes into a C_{15} product, which is xanthoxin, and a C_{25} metabolite (Schwartz et al., 1997). All of these catalytic

steps take place in the plastids; however, ABA is produced in the cytosol. Thus, xanthoxin is presumed to migrate to the cytosol. The biologically active form of ABA is produced from xanthoxin by two enzymatic steps, via the intermediate abscisic aldehyde. The conversion of xanthoxin to abscisic aldehyde is catalyzed by ABA2, which is a short-chain dehydrogenase/reductase (Cheng et al., 2002; González-Gúzman et al., 2002). Finally, the aldehyde is oxidated to give the carboxylic acid, as the final step in ABA biosynthesis. This step is catalyzed by the aldehyde oxidase AAO3 (Seo et al., 2000). A molybdenum cofactor (MoCo) is crucial for the aldehyde oxidase to catalyze the reaction. Therefore, mutations in the genes that biosynthesize the MoCo cofactor, such as *ABA3* in *Arabidopsis*, are ABA deficient (Bittner et al., 2001; Xiong et al., 2001B; reviewed in Nambara and Marion Poll, 2005 and Wasilewska et al., 2008) (Figure 2).

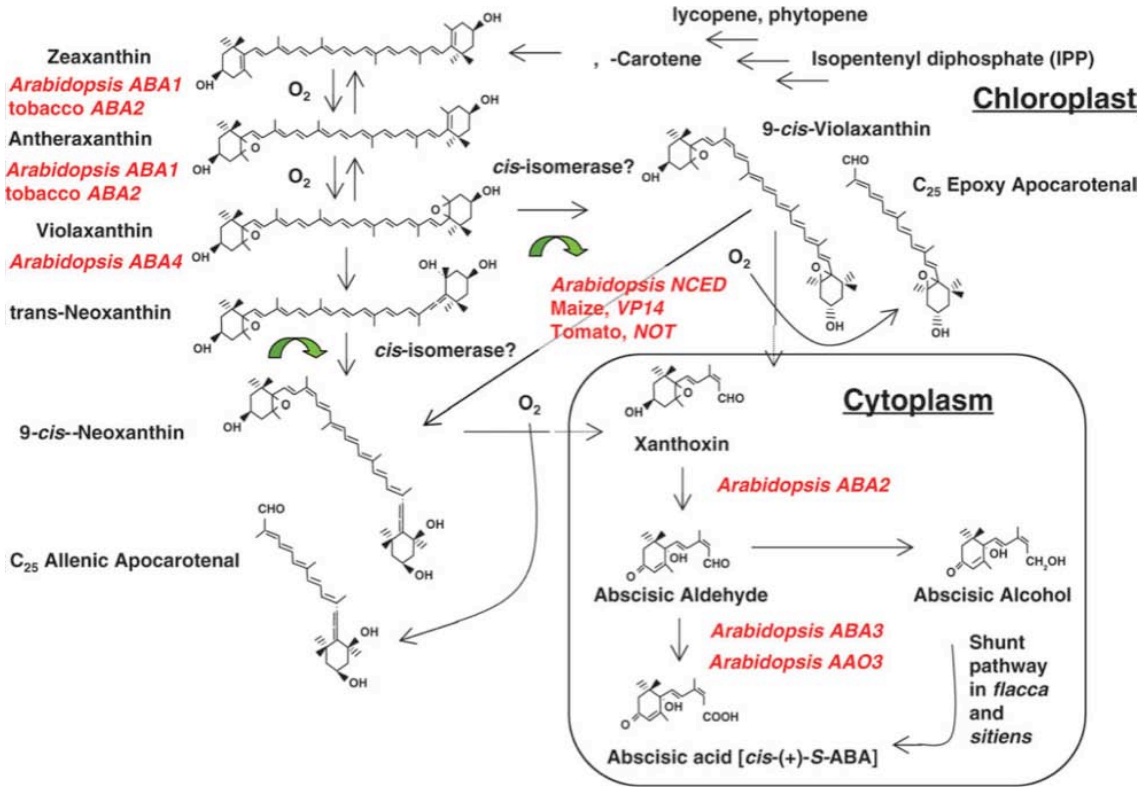

Figure 2. Summary of the biosynthetic pathway of ABA. Figure from Wasilewska et al., 2008.

Regulation of ABA biosynthesis is responsive to stress conditions. The ABA levels increase dramatically during dehydration, correlating with the upregulation of biosynthetic genes, both in roots and in leaves (Barrero et al., 2006). Quantitative analyses, to examine the expression of ABA biosynthetic genes under osmotic stress conditions, have revealed that expression of *NCD3*, *AAO3*, *ABA1* and *ABA3* is up-

regulated. Even though expression of these genes can be found both in roots and rosettes separately under stress conditions, higher induction ratios are registered in rosettes than in roots. Moreover, among them, *NCED3* and *AAO3* seem to show the highest ratios of induction in *Arabidopsis* (Barrero et al., 2006). The fact that expression of biosynthetic genes is found in root seems to be in contrast with the new evidence that ABA biosynthesis in concentrated in the aerial part (Endo et al., 2008; Ikegami et al., 2009). In addition, ABA biosynthesis seems to present a positive regulation feedback. According to Barrero et al. (2006), the osmotic stress signal would be sensed by the different biosynthetic genes in a different extent (mainly by *NCED3*, and also by *AAO3* and, to a lesser extent by *ABA1* and *ABA3*). The increased transcription of these genes would lead to an increase in ABA levels, which in turn, would induce a positive feedback regulation over them (Xiong et al., 2002; Barrero et al., 2006). This model contrast with the one previously proposed by Xiong et al. (2002), in which the stress induction of ABA biosynthesis presents a more linear cascade of events. In their model, NCED3 plays a role as the main sensor of the stress signal, also stating that the ability of ABA to induce its expression is limited.

Catabolism also plays an important role in determining the ABA levels in response to environmental conditions. Inactivation of ABA occurs in a stepwise manner. The major ABA catabolic pathway in higher plants is the one involving ABA 8'-hydroxylation (Cutler and Krochko, 1999). ABA 8'-hydroxylation is catalyzed by a cytochrome P450 monooxygenase, to form 8'-hydroxy ABA, which will spontaneously isomerize to phaseic acid (PA) (Krochko et al., 1998). PA is further reduced to dihydrophaseic acid (DPA), by a soluble reductase (Gillard and Walton, 1976). The genes encoding ABA 8'-hydrolase have been identified to be members of the CYP707A (CYTOCHROME P450, family 707, subfamily A) subfamily in *Arabidopsis thaliana* (Kushiro et al., 2004; Saito et al., 2004). Some of these CYP707As are expressed mainly in seeds, such as CYP707A1 and CYP707A2, and they are involved in regulating ABA levels during seed maturation and germination (Okamoto et al., 2006). Furthermore, the transcript levels of all four *CYP707A* members are induced by dehydration and exogenous ABA treatment (Kushiro et al., 2004; Saito et al., 2004). Some of these CYP707As are also expressed in vegetative tissue, such as CYP707A3 and CYP707A1, and play an important role in the regulation of transpiration. It has been reported that CYP707A3 and CYP707A1 reduce the amount of mobile ABA in vascular tissues and inactivate local ABA pools inside guard cells, respectively (Okamoto et al., 2009).

Another source of ABA is storage of the hormone as an inactive conjugate in the form of ABA glucosyl ester (ABA-GE) in vacuoles (Boyer and Zeevaart, 1982; Dietz et al., 2000). In response to dehydration, this storage of ABA is hydrolyzed by β-glucosidases, contributing to increase the concentration of active ABA in the cell (Lee et al., 2006). The β-glucosidase AtBG1 has been identified from an *Arabidopis* subtraction library enriched for NaCl-stress-inducible cDNAs. Loss of AtBG1 causes defective stomatal movement, early germination, abiotic stress-sensitive phenotypes and lower ABA levels. Conversely, *35S:AtBG1* plants display higher levels of ABA and exhibit enhanced tolerance to dehydration. These results indicate that AtBG1 is implicated in ABA-mediated responses, including the drought response. AtBG1 is localized in the endoplasmic reticulum and exhibits ABA-GE hydrolyzing activity, which seems to be enhanced by dehydration stress. Thus, mobilization of inactive ABA conjugates by AtBG1 has been proposed as a mechanism by which plants would rapidly adjust the ABA levels, in order to quickly respond to changing environmental conditions (Lee et al., 2006).

Different reports indicate that, under stress conditions, ABA is mainly synthesized in vascular tissues and then transported to target cells (Cheng et al., 2002; Endo et al., 2008). Previous studies had suggested that ABA was produced in the roots and from there transported to the aerial part of the plant, acting as an alarm of water deficit and triggering subsequent physiological responses (Wilkinson and Davis, 2002). However, more recent works indicate that the major tissues providing dehydration-induced ABA pools are the vascular tissues localized in the shoot (Endo et al., 2008, Ikegami et al., 2009). Immunohistochemical analysis has revealed that, under water stressed conditions, AtNCED3 is detected exclusively in the vascular parenchyma cells of leaves, together with AtABA2 and AAO3. These results highlight the relevance of this cell type in drought stress-induced ABA synthesis. Moreover, another approach by *in situ* hybridization has also showed that drought-induced expression of *AtNCED3* is restricted to vascular tissues (Endo et al., 2008). This suggests that ABA biosynthesis would be spatially constrained there. Moreover, vascular tissues might also play a role in perceiving drought stress. It has been observed that early induction of other drought-responsive genes, within an hour of dehydration, also occurs there (Endo et al., 2008). This suggest that ABA is not biosynthesized but translocated to the roots (Christmann et al., 2007; Ikegami et al.,2009). However, this ABA transport seems to be induced when drought stress is mainly localized in the roots. In such case, water stress signal might also be perceived by the roots and then transferred to shoots, by an unknown

messenger or hydraulic signal, in order to stimulate ABA translocation from leaves to roots (Ikegami et al., 2009).

In relation to the developmental processes regulated by ABA, modulation of its levels is also crucial. We have described its relevant role in embryogenesis and seed maturation, as well as in establishment and maintenance of dormancy. These processes elicit the activation of the biosynthetic machinery. On the other hand, germination, which entails a decrease in ABA levels, involves the blockage of the novo synthesis and the activation of catabolic enzymes such as CYP707A2, which is responsible for the rapid decrease in ABA levels during seed imbibition (Kushiro et al., 2004).

It is well known, that despite ABA being synthesized and metabolized in vascular tissues, it acts in stomatal responses of distant guard cells (Cheng et al., 2002, Koiwai et al., 2004, Okamoto et al., 2009). Moreover, it has also been proved that not all cells express all of the biosynthetic and modification enzymes for complete ABA synthesis (reviewed in Wasilewska et al., 2008). All this implies intercellular transportation of the hormone. Up to now, it was assumed that ABA was only transported by passive diffusion into target cells. ABA is a weak acid (pKa= 4.8), and thus, it can exist in either protonated, uncharged form (AH) or in anionic form (A-), depending on the pH relative to its pKa. Due to the capacity of the uncharged form to diffuse through the membrane, the requirement of specific uptake transporters was somehow diminished. Recently, this panorama has drastically changed with the identification in *Arabidopsis thaliana* of two plasma membrane-type ABC transporters, who have shed light on the active control of cell-to-cell transport of ABA (Kuromori et al., 2010, Kang et al., 2010). The AtABCG25 exporter was originally identified in a genetic screening looking for ABA-hypersensitive mutants (Kuromori et al., 2010). *atabcg25-1* and *atabc25-2* loss-of-function mutants presented hypersensitivity to ABA in germination and seedling establishment. Gene expression analysis in plant organs and subcellular localization experiments, revealed that AtABCG25 was a plasma membrane-localized protein and that it was mainly expressed in vascular tissues, which is the main area where ABA biosynthesis takes place. To assess whether AtABCG25 could transport ABA through the membrane, Kuromori et al. (2010) performed a vesicle transport assay. As a result they observed that AtABCG25 had the capacity of transporting ABA molecules. Additionally, AtABCG25 overexpressing plants presented higher leaf temperature and a slower rate of water loss by transpiration. These data suggested that overexpression of AtABCG25 might induce the

accumulation of ABA in guard cells, promoting stomatal closure. Taken together, the experimental evidence indicated that AtABCG25 was a functional factor of ABA transport and that its role was probably the export of ABA from plant cells. This would correlate and explain the ABA hypersensitive phenotypes observed for the *atabcg25* mutants, which had lost the capacity to remove ABA from the cells (Kuromori et al., 2010). In the case of the AtABCG40 transporter, it was identified among 13 PDR homozygous knockout-mutants tested for seed germination and stomatal movement phenotypes (Kang et al., 2010). *atabcg40* plants showed ABA insensitive phenotypes in seed germination, root development, expression of ABA-responsive genes and a strong delay in ABA-induced stomatal closure, being prone to wilt under drought-stress conditions. To assess whether or not AtABCG40 was an ABA transporter, Kang et al. (2010) expressed the *AtABCG40* cDNA in a heterologous system, which carried loss-of-funtion mutations in several ABC transporters. Yeast expressing *AtABCG40* were able to take up ABA consistently faster than the control empty vector, indicating that AtABCG40 was able to transport the hormone. To further assess the role of AtABCG40 as an ABA transporter, they also measured the ABA uptake in protoplasts from *atabcg40* loss-of-function mutants. Indeed, ABA was taken up more slowly into protoplast isolated from *atabcg40* than from wild-type leaves. Moreover, AtABCG40 was also described as a membrane protein and it was reported to be mainly expressed in leaves, with its expression being highest in guard cells. In addition, no difference was observed for stomatal closure in response to Ca^{2+}. Altogether, the results suggested that AtABCG40 was in charge of importing ABA into de cell (Kang et al., 2010).

These findings give evidence that ABA is actively transported across the membranes and not only by passive diffusion, providing insights into the intercellular regulation of ABA transport. The presence of ABA transporters indicates that both ABA extrusion from ABA-producing cells and ABA uptake into cells for triggering ABA responses, are regulated processes. The fact that ABA can be delivered into the cell in a regulated manner, is in accordance with the recent identification of soluble intracellular receptors, the PYR/PYL/RCAR family (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A). This suggests an intracellular model of hormone perception, in which a rapid delivery of the hormone into de cell would be crucial to elicit a timely response. In contrast, the passive diffusion mechanism might not fit with rapid delivery requirements during stress conditions. Stress conditions are known to elevate the extracellular pH; this pH increase would favour the charged form of ABA, which cannot passively diffuse across the lipid bilayer. This would be in contradiction with the need to

rapidly deliver ABA into de cell in order to trigger a stress response (Kang et al., 2010), which in turn, highlights the relevance of an ABA transporter.

1.5 ABA SIGNALLING CASCADE

The ABA signalling pathway has always been considered to be a very complex network, due to the numerous other proteins that have been involved (Hiroyama Shinozaki and, 2007). However, recent works have simplified the cascade by identifying the "core elements" of the pathway, PYR/PYL/RCAR-PP2Cs-SnRK2s (Fujii et al., 2009; Umezawa et al., 2009; Vlad et al, 2009). The identification of this core pathway has simplified the ABA signalling cascade to four steps that go from hormone perception to gene expression. The newly identified family of intracellular receptors named as PYR/PYL/RCAR (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A; Nishimura et al., 2010), whose characterization is the aim of this work, have been described to be at the apex of the pathway. This receptor family has the ability to perceive the hormone and start signal transduction.

As a general overview, in this simplified model, type 2C protein phosphatases (PP2C) negatively regulate sucrose non-fermenting1 (SNF1)-related protein kinase 2 family (SnRK2), by direct dephosphorylation of different serine residues in their activation loop (Umezawa et al., 2009; Vlad et al., 2009). ABA regulates the activity of these PP2C through the PYR/PYL/RCAR proteins. This new receptor family inhibits PP2Cs phosphatase activity (Park et al., 2009; Santiago et al., 2009A; Ma et al., 2009), releasing protein kinases from inhibition and allowing their activation, being able, this way, to phosphorylate downstream ABF/AREB (ABA-RESPONSE ELEMENT BINDING FACTOR) basic leucine zipper (b-ZIP) transcription factors (TFs). These transcriptions factors activate by phosphorylation and act as positive regulators, inducing the transcription of ABA-responsive genes (Fujii et al., 2009; Umeawa et al., 2009). SnRK2 kinases, and particularly OST1 (OPEN STOMATA 1), also have other key targets of the ABA signalling pathway in guard cells, which are upstream of transcription, such as NADPH oxidases activated by phosphorylation (Sirichandra et al., 2009A), or the ion channels SLAC1 (SLOW-ASSOCIATED ANION CHANNEL 1) and KAT1 (*Arabidopsis thaliana* K^+ channel), which are activated and inhibited, respectively, being all of them implicated in ABA-regulated stomatal closure (Kwak et al., 2003; Geiger et al., 2009; Lee et al., 2009; Sato et al., 2009). Moreover, calcium regulated kinases implicated in ABA signalling, CDPKs, seem to converge with OST1 in SLAC1 regulation and they have also been reported to be under the control of ABI1/ABA-receptor complex (Geiger et al., 2010). Description of these core and key elements, and their molecular way of action, will be developed in the following points.

1.5.1 TYPE 2C PROTEIN PHOSPHATASES (PP2C): NEGATIVE REGULATORS IN THE ABA SIGNALLING CASCADE

PP2Cs are serine/threonine phosphatases, monomeric enzymes, dependent on Mg^{2+} or Mn^{2+} for their activity (Rodríguez, 1998A). PP2Cs comprehend the largest class of protein phosphatases in *Arabidopsis*, comprising 76 genes classified in ten groups (Scheweighofer et al, 2004). A similar number of putative PP2Cs can be found in other higher plants such as poplar or rice when consulting public available genome databases. Surprisingly, plants have a significant higher number of PP2Cs in comparison with other organisms (15 in humans, 10 in yeast and 8 in worm), suggesting that PP2Cs in plants might have a broader functional diversity than in other eucaryotes (Scheweighofer et al, 2004). Among the ten groups, clade A, which comprises 9 members, has an important role in ABA signalling.

Clade A PP2Cs are composed by a N-terminal of 100-180 amino acids and a C-terminal of around 300 residues. They present a highly conserved catalytic core, with 11 specific conserved motifs typical of PP2Cs (Rodríguez, 1998A; Scheweighofer et al, 2004). When comparing the catalytic core among the different *Arabidopsis thaliana* clade A PP2Cs and with other plant species orthologs such as PP2C1 from *Fagus silvatica* or *Oryza sativa*, and even with a more distant one such as human PP2C, we can observe that critical active-site residues are highly conserved among all of them (Figure 3). This similarity has also been proved by the elucidation of the catalytic core structure of two plant PP2Cs, ABI1 and HAB1 (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011) and their comparison with the previous existing structure of human PP2C (Das et al., 1996). The two plant PP2Cs share a similar folding pattern with human PP2C, formed by a central sandwich of two 5-stranded β-sheets enclosed by two α-helices at each side of the catalytic site. The catalytic site contains 3 Mn^{2+} or Mg^{2+} ions, located at the top part of the β-sandwich.

Due to the similitude of the active site with the human PP2C, the proposed catalytic mechanism involving metal-bound water molecules acting as nucleophiles and general acids (Das et al., 1996) could be extrapolated. Dephosphorylation of the substrate happens through a S_N2 reaction (nucleophilic substitution type 2) whose most general reaction form is described as follows:

Nuc**:** + R-LG → R-Nuc + LG**:**

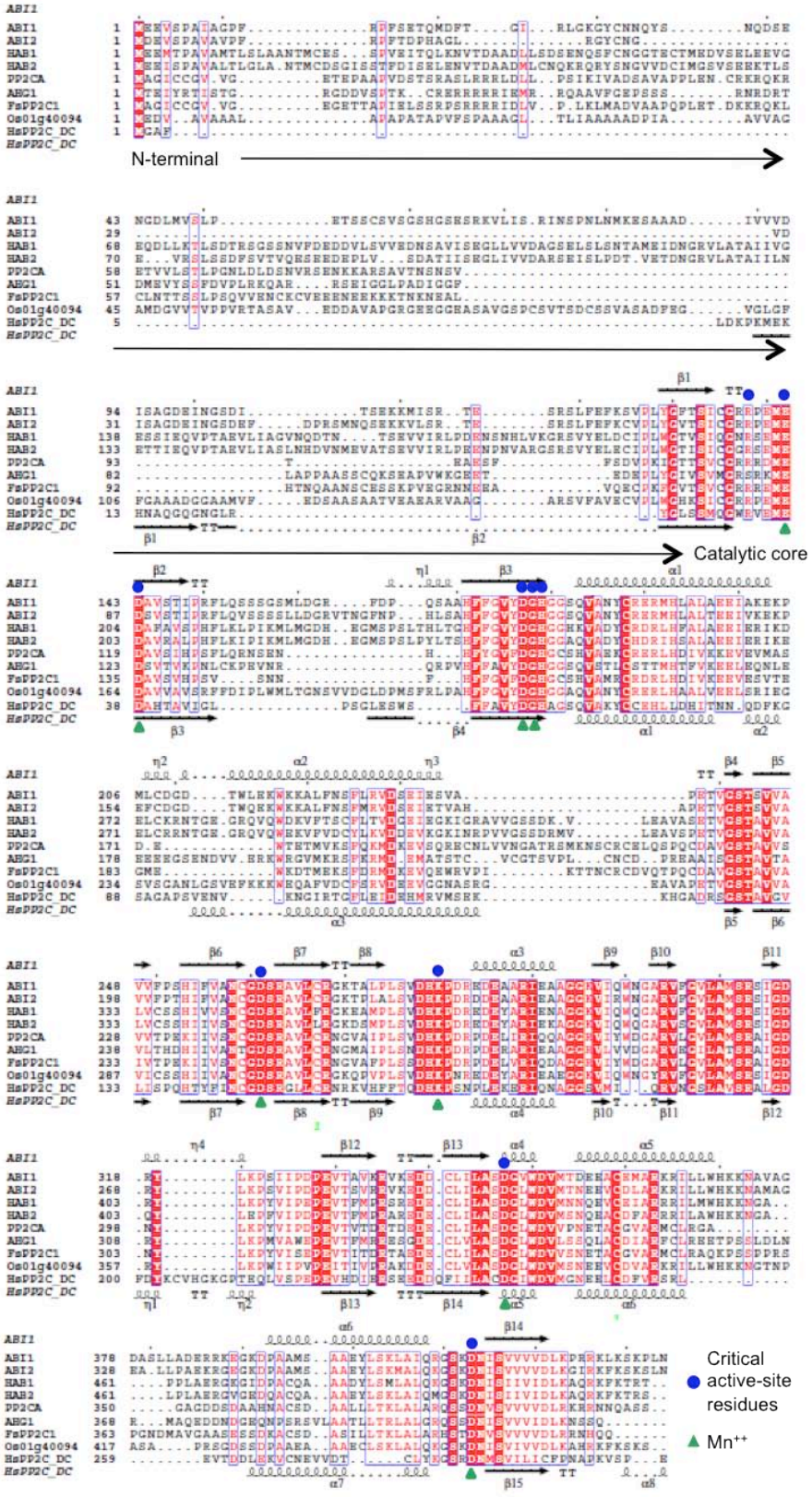

Figure 3. Amino acid sequence and secondary structure alignment of plant clade A PP2Cs with the catalytic core of human PP2C (residues 1-300). Figure generated with ESPript 2.2. The N-terminal and catalytic core are indicated. Colour codes indicate conserved critical active-site residues and the amino acid residues involved in the interaction with the metal ions. Figure generated with ESPript 2.2 (Gouet et al., 1999).

In this sort of reaction the electron pair (**:**) from the nucleophile (Nuc) attacks the substrate (R-LG) forming a new bond, while the leaving group (LG) departs with an electron pair. Typically, the nucleophile may be electrically neutral or negatively charged, whereas the substrate is neutral or positively charged. The active site is rich in Asp and Glu amino acids, conferring it a negative electrostatic potential. So the presence of the Mn^{2+} or Mg^{2+} ions, together with the Arg199 in HAB1 (Arg138 in ABI1 and Arg33 in HsPP2C), create, in the first place, a local positive charge recognizing and attracting the phosphate group of the substrate. The phosphate group is then coordinated in the active site through hydrogen bonds to metal-bound water molecules. Dephosphorylation of the substrate takes place by an S_N2 reaction in which a metal-bridging water molecule, acting as nucleophile, would attack the phosphorus atom, while a second metal-bound water molecule, also bridging a Glu residue, protonates the oxygen atom of the seryl/threonyl leaving group (Figure 4) (Das et al., 1996).

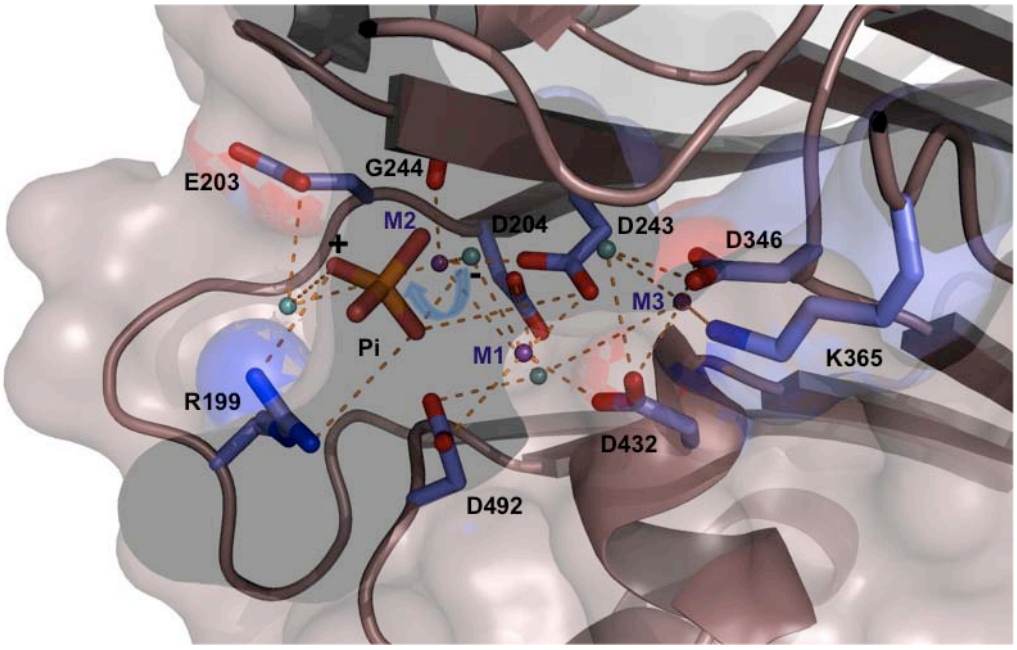

Figure 4. Details of PP2C HAB1 catalytic site and a proposed dephosphorylation mechanism catalysed through metal ions. The structures of HAB1 and the human PP2Cα (not shown) have been superposed, and the phosphate ion found at the PP2Cα catalytic site is depicted as stick model. Residues coordinating the three metal ions at the PP2C active site are shown in blue sticks. The Mn^{2+} ions are depicted as purple spheres and the water molecules implicated in metal coordination are represented as blue spheres. The blue arrow represents the S_N2 reaction by a metal-bound water molecule, negatively charged, acting as a nucleophile over the phosphorus atom. A second water-bridging molecule, also bonded to the side chain of Glu203, eases the dephosphorylation reaction by protonating the oxygen atom of the seryl/threonyl leaving group.

On the other hand, the N-terminal of these proteins, whose structure has not yet been elucidated, presents low sequence similarity and extension, suggesting that this part of the protein could establish specific interactions with cellular substrates, regulatory proteins or secondary messengers (Rodríguez, 1998A). For instance, it has been described that phosphatidic acid (PA), produced by Phospholipase Dα-1 (PLDα-1), who interacts and inhibits ABA INSENSITIVE1 (ABI1) PP2C promoting the closure of open stomata (Zhang et al., 2004), binds to the PP2C in its N-terminal region (Zhang et al., 2004; Mishra et al., 2006). Site-specific mutation showed that arginine 73 in ABI1 was critical for PA-ABI1 binding *in vitro* and *in vivo* (Zhang et al. 2004; Mishra et al. 2006). Also, it has been reported that the N-terminal part of ABI1 (1-93 amino acids) is equally efficient than ABI1 wild type in docking to the mitogen-activated protein (MAP) kinase 6 (MAPK6), both *in vitro* and in yeast two hybrid assays (Leung et al., 2006).

The first clade A PP2Cs identified and implicated in ABA signalling were ABI1 and ABI2 (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodríguez et al., 1998B) after the identification, in *Arabidopsis*, of the ABA-insensitive abi1-1 and abi2-1 mutants (Koonneerf et al., 1984), characterized as dominant mutations (*abi1-1D* and *abi2-1D*) (Koonneerf et al., 1982; Leung et al., 1997). Both mutants revealed pleitropic alterations in their response towards ABA, suggesting that PP2Cs had a role as regulators of ABA signalling. ABI1 was cloned by chromosome walking (Leung et al., 1994; Meyer et al., 1994) and the ABI2 gene was identified by a combination of positional mapping and homology to ABI1 (Leung et al., 1997; Rodríguez et al., 1998B). The first evidence of their role as negative regulators, from loss-of-function alleles, came from isolated intragenic revertants mutants of the *abi1-1D* and *abi2-1D*, named *abi1-1R1* to *R7* (Gosti et al., 1999) and *abi2-1R1* (Merlot et al., 2001). Currently, at least 6, out of the 9 clade A PP2Cs, are known to be involved in ABA signalling acting as negative regulators. They are named ABI1, ABI2, HYPERSENSITIVE TO ABA1 (HAB1), HYPERSENSITIVE TO ABA2 (HAB2), ABA HYPERSENSITIVE GERMINATION1 (AHG1) and AHG3/AtPP2CA (Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004 Saez et al., 2004; Kuhn et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007). HAB1, previously named AtP2CA-HA, was first identified by homology to ABI1/ABI2 and cloned from an EST (Rodríguez et al., 1998C). In the case of the loci AHG1 and AHG3, they were first identified through a genetic screening looking for mutants with enhanced ABA response in germination and post-germination growth and subsequently cloned by map-based gene cloning (Nishimura et al., 2004; Yoshida et al., 2006; Nishimura et al., 2007). In parallel, another group looking for new loci in ABA signalling by screening for

ABA-insensitive mutants, identified AtPP2CA (Kuhn et al., 2006) which resulted to be identical to AHG3.

The isolation and characterization of direct knockout alleles, either single or multiple, has been crucial to elucidate their role in ABA signalling (Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007; Rubio et al., 2009). Characterization of loss-of-function mutants from ABI1, HAB1 and PP2CA, reveal hypersensitive phenotypes to the hormone in germination, growth, stomatal closure and expression of ABA-responsive genes. This suggests that they are key regulators of ABA signalling both in seeds and vegetative tissues (Leonhardt et al., 2004; Saez et al., 2004; Kunh et al., 2006; Saez et al., 2006; Yoshida et al., 2006). In the case of AHG1, analysis of the hypersensitive mutant *ahg1-1*, exhibits a strong phenotype in germination and post-germination growth but no evident phenotype in adult plants, suggesting that AHG1 has a specific function in seed and germination (Nishimura et al., 2007). Conversely, analysis of 35S:PP2CA and 35S:HAB1 overexpression lines show ABA insensitivity in seed germination and growth as well as increased transpiration rates (Saez et al., 2004; Kuhn et al., 2006) mainly due to reduced stomatal closure (Kuhn et al., 2006). These data correlates with gene expression data extracted from public databases in which all of them, except from AHG1, are expressed in seeds, seedlings and guard cells, and they are up-regulated by ABA (Rubio et al., 2009). Both public and Nishimura et al., 2007 expression data, confirms that AHG1 shows the strongest expression in developing and dry seeds.

The analysis of mutants with combined inactivation of several of these PP2Cs, has made possible to unravel overlapping and additive functions as well as to establish certain hierarchy in different responses to the hormone (Saez et al., 2006; Nishimura et al., 2007; Rubio et al., 2009). For instance, inactivation of two major PP2Cs such as HAB1/ABI1 leads to a stronger response to ABA than that found in the monogenic mutants *hab1-1* and *abi1-2*. This indicates partial overlapping functions for HAB1 and ABI1, although ABI1 seems to play a predominant role in growth control, when comparing *abi1-2* and *abi1-3 versus hab1-1* mutant (Saez et al., 2006). Additionally, analysis of double mutant *ahg1-1 ahg3-1* also exhibits a stronger phenotype than the parental mutant lines in germination and post-germination growth, but functional differences, such as germination efficiency or seed dormancy, can be observed when analyzing the singles mutants separately (Nishimura et al., 2007).

Moreover, the work of Saez et al. (2006) and Rubio et al, (2009) have established the generation of drought resistant plants by the inactivation of two major

PP2Cs in *Arabidopsis* (HAB1/ABI1, ABI1/ABI2, ABI1/PP2CA and HAB1/PP2CA), which suggests that combined inactivation of these PP2Cs could be a potential biotechnological approach to apply in crops. However, the work from Rubio et al. (2009) also revealed that inactivation of three of these PP2Cs such as in *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants, besides exhibiting an extreme response to exogenous ABA, were also impaired in growth, showing a constitutive response to endogenous ABA. Transcriptomical analysis comparing the triple mutant plants untreated *versus* wild type Col plants treated with 10 μM ABA, revealed a constitutive up-regulation and down-regulation of ABA-responsive genes in the triple knockout mutants. These data suggest that clade A PP2Cs have a major role in modulating the ABA response in plants and that a fine tuning control of these proteins would be required in order to avoid a constitutive response to endogenous ABA. In the absence of environmental stress, this constitutive response has been shown to lead to a detrimental effect in plant growth and development (Rubio et al., 2009).

Different PP2C targets have been identified in plants. Among the proteins described, we find protein kinases that belong to the SNF1-related protein kinase 2 (SnRK2) family, which have been described as positive regulators of the ABA signalling pathway (Fujii et al., 2009; Fujita et al., 2009). Yoshida et al. (2006) reported the *in vivo* (in yeast-two-hybrid) and *in vitro* interaction between ABI1 and OPEN STOMATA (OST)1/SnRK2.6, a kinase that plays a crucial role in control of stomata closure. In line with these results, more recent works have extended the analysis to other clade A PP2Cs and SnRK2s, demonstrating their physical interaction and direct dephosphorylation of SnRK2s by the PP2Cs (Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). The physical interaction between PP2Cs and SnRK2s seems to happen both in cytosol and nucleus (Fujita et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). These observations qualify clade A PP2Cs as main regulators of the ABA-activated SnRK2s, which will be further described in the following point.

1.5.2 PROTEIN KINASES INVOLDED IN THE ABA SIGNALLING CASCADE

In line with the implication of PP2Cs as key elements of the pathway, several protein kinases have also been isolated and characterized as crucial ABA signalling factors (Mustilli et al., 2002, Yoshida et al., 2002, Fujii et al., 2007; Fujita et al., 2009; Fujii and Zhu, 2009). Among the kinases implicated in ABA signalling, both calcium-independent (SnRK2s) and calcium-regulated (CPKs and SnRK3s/CIPKs) proteins have emerged as important factors. Moreover, other protein kinases from other families have also been implicated in ABA signalling. For instance, the receptor-like-kinase 1

(RPK1) has been reported as a positive regulator of the pathway, due to the insensitive phenotypes exhibited by loss-of-function mutants in different ABA resposes such as germination, plant growth, stomatal closure and ABA-induced gene expression (Osakabe et al., 2005). This ABA-inducible receptor kinase has been recently described as a positive regulator of senescence in *Arabidopsis* leaves. Apparently, RPK1 mediates the ABA-induced leaf senescence as well as age-induced leaf senescence (Lee et al., 2011). Similarly, MAPK proteins have also been implicated in ABA signalling (Jammes et al., 2009). 2 MPK genes, *MPK9* and *MPK12* are highly and preferentially expressed in guard cells and they positively regulate ABA signalling acting downstream of reactive oxygen species (ROS). The double mutant *mpk9-1mkp12-1* show reduced ABA-induced stomatal closure, being impaired in the ABA and calcium activation of anion channels. Moreover, ABA and H_2O_2 induced the activation of the MPK12 kinase (Jammes et al., 2009).

1.5.2.1 Ca^{2+}-INDEPENDENT KINASES

1.5.2.1.1 SNF1-RELATED PROTEIN KINASE 2 FAMILY (SnRK2)

Arabidopsis contains 38 protein kinases that are related to SNF1 from yeast. The SnRKs are divided in three subgroups: SnRK1, SnRK2 and SnRK3. This classification is based on sequence similarity and domain structure (Hrabak et al., 2003). The group of calcium-independent kinases, SnRK2s, appears to be plant specific. They have an average size of about 40kDa and they have a characteristic patch of acidic amino acids in their C-terminal domain (Halford et al., 2000). In *Arabidopsis* we can find 10 SnRK2s genes designated as SnRK2.1-10 (Hrabak et al., 2003) from which three of them are strongly activated by ABA , SnRK2.2 (also named SRK2D), SnRK2.3 (SRK2I) and SnRK2.6/OST1 (SRK2E) (Boudsocq et al., 2004). The rice orthologs stress-activated protein kinase (SAPK) 8, SAPK9 an SAPK10 are consistently classified into the same group of SnRK2 protein kinase subfamily, named SAPK1 to SAPK10, and they are also activated by ABA (Kobayashi et al., 2004). The first calcium-independent protein kinase involved in the ABA signalling pathway was the ABA-responsive protein kinase, PKABA1 (Gómez-Cadenas et al., 1999). This serine/threonine protein kinase was identified in wheat and it was reported to be transcriptionally up-regulated by ABA and dehydration (Anderberg and Walker-Simmons, 1992). PKABA1 has the ability to interact and phoshorylate the transcription factor TaABF1 (a member of the AREB/ABF TFs family that recognize ABA-responsive (ABRE) sequences), regulating its activity (Johnson et al., 2002), as well as to mediate ABA suppression of gibberellic acid (GA)-induced gene expression in cereal grains (Gómez-Cadenas et al., 1999, 2001; Johnson et al., 2008). Later on, another SnRK2

family member was identified, the ABA-activated *Vicia faba* protein AAPK (ABA-ACTIVATED PROTEIN KINASE). AAPK was described as a guard cell-specific kinase and implicated in the ABA-induced stomatal closure (Li et al., 2000). Evidence for this conclusion was provided by a dominant negative version of the protein ($AAPK^{K43A}$), which showed to be insensitive to ABA-induced stomatal closure by eliminating the ABA activation of plasma membrane slow anion channels (Li et al., 2000). The putative AAPK ortholog in *Arabidopsis* was identified by a genetic screen based on thermal imaging of drought-stressed plants and named OST1 (SnRK2.6/SRK2E) (Mustilli et al., 2002). This screen method was based on the selection of individuals that displayed a reduced ability to close their stomata in response to drought stress. Hence, they appeared to be colder than the wild type since they would present a higher transpiration rate and therefore a higher heat loss (Merlot et al., 2002). The *ost1* mutants showed to be highly insensitive to ABA regulation of stomatal closure (Mustilli et al., 2002; Yoshida et al., 2002) but they were still able to be sensitive to light and CO_2, suggesting that OST1/SnRK2.6 could be specifically involved in ABA signalling, qualifying as a positive regulator of the pathway (Mustilli et al., 2002). Analysis of the OST1/SnRK2.6 closely related kinases, SnRK2.2 and SnRK2.3, provided evidence that these kinases had an important role in mediating ABA signalling during seed dormancy, germination, growth as well as regulation of gene expression in response to the hormone (Fujii et al., 2007).

Because of functional redundancy between SnRK2.2 and SnRK2.3, single mutants did not have a clear ABA response phenotypes, but the double mutant *snrk2.2 snrk2.3* showed a strong ABA-insensitive phenotype in seed dormancy, germination and root growth inhibition, as well as reduced expression of ABA-inducible genes. For instance, the double mutant *snrk2.2 snrk2.3* seeds were able to germinate after two days of sowing in Murashige and Skoog **(**MS) medium with no stratification treatment; whereas wild type only started to germinate after 6 days of sowing. Moreover, the double mutant still presented 70% of germination compared to complete inhibition of germination of the wild type in 1 μM ABA. Similarly, the *snrk2.2 snrk2.3* mutant was able to carry on its root development in 50 μM ABA, which resulted to be a completely inhibitory concentration for the wild type and single parental mutants (Fujii et al., 2007). However, the double mutant presented a modest effect in leaf water loss compared to the wild type (Fujii et al., 2007; Fujii and Zhu, 2009). Analysis of the *snrk2.2/snrk2.3/snrk2.6* recessive triple mutant revealed extreme insensitive phenotypes to all ABA responses, allowing to qualify the SnRK2.2/SnRK2.3/SnRK2.6 as central positive regulators in ABA signalling. Analysis of the triple mutant suggested that the different SnRK2s might present partial functional redundancy (Fujii and Zhu,

2009; Fujita et al., 2009). This was in contrast with the complete functional segregation suggested in previous studies (Mustilli et al., 2002; Fujii et al., 2007). For instance, under water stress conditions, the triple mutant displayed a greatly reduced tolerance to dehydratation presenting a complete wilted phenotype within 30 minutes (Fujii and Zhu, 2009; Fujita et al., 2009). Although, OST1/SnRK2.6 had been described as crucial in the control of stomata closure and it was strongly expressed in guard cells (Mustilli et al., 2002; Yoshida et al., 2002), the complete disruption of stomatal regulation in the triple mutant suggested that both SnRK2.2 and SnRK2.3 should also have a contribution in stomatal regulation (Fujii and Zhu, 2009; Fujita et al., 2009). This is also supported by public gene expression data, showing expression of the three SnRK2s in guard cells. A similar reasoning could be applied, but now with the contribution of SnRK2.6, to explain the strong insensitive phenotypes displayed by the triple mutant in seed dormancy, seed germination, seedling growth and development, as well as in the expression of ABA-regulated genes. The complete blocking of ABA signalling would be responsible for the strong viviparous phenotype shown by the triple mutant (Fujita et al., 2009; Nakashima et al., 2009), indicating loss of ABA-induced dormancy. Similarly, the blockage of ABA signalling would explain how the triple mutant was able to germinate and grow under concentration of 50 μM ABA, which results to be even an inhibitory concentration even to the *snk2.6* and *snrk2.2snrk2.3* mutants (Fujii and Zhu, 2009). Moreover, in the triple mutant the expression of ABA-inducible genes was completely eliminated (Fujii and Zhu, 2009; Fujita et al., 2009).

Microarrays analysis comparing the triple mutant *versus* the wild type, under ABA treatment or water stress conditions, revealed that the *snrk2.2/snrk2.3/snrk2.6* mutant was drastically impaired in ABA and water stress-dependent gene expression (Fujita et al., 2009). These data together with the strong ABA-insensitive and water stress-sensitive phenotype, suggest that ABA signalling pathways converge at the level of these 3 protein kinases, and that protein phosphorylation mediated by SnRK2.2. SnRK2.3 and SnRK2.6 is absolutely essential for ABA signalling (Fujii and Zhu, 2009; Fujita et al., 2009). Therefore, the elucidation of their direct phosphorylated targets would provide an extreme valuable information for a better understanding of ABA signalling.

Moreover, the *snrk2.2/snrk2.3/snrk2.6* triple mutant also present defects in growth and seed production, even under near 100% humidity conditions (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009), indicating that ABA has important roles in plant growth and reproduction, as it was previously shown by the work of Cheng et al. (2002) and Barrero et al. (2005).

SnRK2s contain a kinase catalytic domain at the N-terminus (with around 290 amino acids) with a well-conserved activation loop, and a variable C-termini (with around 70 amino acids) which encloses one or two boxes: the 'SnRK2 specific' box, shared by all SnRk2s, and the 'ABA-specific' box, specifically conserved in the ABA-activated SnRK2s, which is rich in aspartic residues (Belin et al., 2006; Yoshida et al., 2006). Phosphorylation is required for SnRK2s activity, however it is currently unclear whether they need other upstream kinases *in vivo* for their activation or if autophosphorylation is enough for their activation (Belin et al., 2006; Boudsocq et al., 2007). *In vitro* assays, using SnRK2 immunoprecipatates and treating them with the general kinase inhibitor staurosporine, revealed that SnRK2s were susceptible to the inhibitor. However, the same treatment in protoplasts did not block the ABA activation of the SnRK2s, suggesting the existence of upstream kinases responsible for their activation and insensitive to the inhibitor (Boudsocq et al., 2007). However, OST1 has been also reported to activate by autophosphorylation (Belin et al., 2006). In SnRK2s activation, phosphorylation of conserved serine residues in the activation loop seems to play a crucial role (Belin et al., 2006; Boudsocq et al., 2007). Moreover, these residues can be phosphorylated by different mechanisms depending on the kinase and its responsiveness to ABA (Vlad et al., 2010). For instance, in the case of SnRK2.6, which is activated by ABA and osmotic stress, it presents an independent phosphorylation mechanism of two serine residues, Ser175 and Ser171, which seem to have a synergistic effect. Phosphorylation of Ser175 is necessary for SnRK2.6 activity (Belin et al., 2006), but not sufficient for its full activation. Its full activation requires the phosphorylation of the secondary site, Ser171. Conversely, in the case of SnRK2.10, only activated by osmotic stress, even though phosphorylation of two serine sites is also required, the mechanism appears to be sequential and dependent. Phosphorylation of the secondary site Ser154 is required as a first step for subsequent phosphorylation of Ser158 and activation of the kinase (Vlad et al., 2010). A similar two-step activation process has been found in *Nicotiana tabacum*, where the Ser154 of NtOSAK also triggers the phosphorylation of Ser158 (Burza et al., 2006).

ABA-mediated inhibition of clase A PP2Cs releases the break over the SnRK2s, allowing its activation by phosphorylation (Mustilli et al., 2002; Fujii et al., 2009; Vlad et al., 2009; Umezawa et al., 2009). PPC2s constitutively interact with SnRK2.2, SnRK2.3 and SnRK2.6 (Yoshida et al., 2006; Fujii et al., 2009; Umezawa et al., 2009), inactivating them by dephosphorylation (Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). This interaction occurs through the 'ABA-specific' box at the C-terminus of SnRK2s, and their inactivation takes place by PP2C-mediated dephosphorylation of serine residues located in the activation loop (Vlad et al., 2009; Umezawa et al., 2009), such as S175 in SnRK2.6 (Belin et al., 2006). So, in the absence of ABA, PP2Cs

repress the ABA signalling pathway via the dephosphorylation and inactivation of SnRK2s, meaning that SnRK2s ABA activation would require the release of the PP2Cs break. Late in 2009, different groups reported that ABA negatively regulates PP2Cs activity through the interaction with a new family of ABA receptors named PYR/PYL/RCAR family (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A), whose characterization is the aim of this work.

SnRK2s are known to positively regulate ABA signalling through phosphorylation and activation of ABF/AREB/ABI5 bZIP transcription factors involved in gene activation in response to ABA (Uno et al., 2000; Kobayashi et al., 2005; Furihata et al., 2006; Piskurewicz et al., 2008; Fujita et al., 2009; Yoshida et al., 2010). Moreover, SnRK2s have also been involved in the regulation, also through phosphorylation, of different target proteins implicated in the direct control of ABA-induced stomata closure (Geiger et al., 2009; Lee et al., 2009; Geiger et al., 2010; Sato et al., 2009; Sirichandra et al., 2009A) (see later in 1.5.3 and 1.5.4).

1.5.2.2 Ca^{2+}-REGULATED KINASES

Ca^{2+} acts as a second messenger in ABA signalling (Leung and Giraudat, 1998; Kim et al., 2010), and Ca^{2+} dependent kinases have also been described to play an important role in ABA signalling (Choi et al., 2005; Mori et al., 2006; Zou et al., 2010).

1.5.2.2.1 CALCIUM DEPENDENT KINASES (CDPKs/CPKs)

CDPKs/CPKs have been described as positive regulators of the pathway and have the particularity to have a regulatory calmodulin-like domain located at the C-terminal of the enzyme. Thus, they have the capacity to act as sensors responders since they combine direct Ca^{2+} binding and activation, with kinase activity in the same polypeptide (Cheng et al., 2002). The *Arabidopsis* genome encodes 34 CDPKs (Cheng et al., 2002; Hrabak et al., 2003) and several of them have already been implicated in guard cell regulation and ABA signalling (Sheen et al., 1996; Mori et al., 2006; Zhu et al., 2007; Geiger et al., 2010; Zou et al., 2010). The first evidence of CPDK/CPKs being implicated in ABA signalling was provided by the expression of CPK10/CDPK1 and CPK30/CDPK1a in maize leaf protoplasts, which were described to activate an ABA-inducible promoter (Sheen et al., 1996). CDPK10 has also been involved in plant responses to drought stress via modulation of ABA- and Ca^{2+}-regulated stomatal movements (Zou et al., 2010). *cdpk10* mutants showed an increased sensitivity to drought stress correlated with its impaired capacity to close stomata in the presence of ABA. This impairment in stomatal closure was related with the requirement of functional CDPK10 in order to inhibit inward K^+ channels in an ABA dependent manner

(Zou et al., 2010). During stomatal closure, ion channel-mediated inward K^+ currents in guard cells are inhibited by elevations of ABA and cytosolic Ca^{2+} in order to avoid hyperpolarization and influx of water in the cell (Pandey et al., 2007). Identification of guard cell-expressed CDPK3 and CDPK6 and analysis of their double knockout mutant, revealed them as positive regulators of ABA-regulated stomatal closure (Mori et al., 2006). These two CDPK loss-of-function mutants, led to partial impairment in ABA and Ca^{2+} activation of S-type annion channels, directly involved in stomatal closure (MacRobbie, 1998; Vahisalu et al., 2008; Geiger et al., 2009). In addition, they also seemed to be involved in ABA activation of plasma membrane Ca^{2+} channels (Hamilton et al., 2000). Recently, two other CPKs, CPK21 and CPK23, have been reported to modulate ABA-regulated stomatal closure through the activation of the SLAC1 channel, via its phosphorylation. Both CDPKs seem to be negatively regulated by the clade A PP2C ABI1 (Geiger et al., 2010) (see later in 1.5.4). In addition, CPK4 and CPK11 have also been identified as positive transducers of Ca^{2+} dependent ABA signalling (Zhu et al., 2007). Loss-of-function mutations of *CPK4* and *CPK11* resulted in pleiotropic ABA-insensitive phenotypes in seed germination, seedling growth and stomatal movement. Moreover, CPK4 and CPK11 kinases are able to phosphorylate two members of the AREB/ABF TFs family, namely ABF4 and ABF1, *in vitro* (Zhu et al., 2007). Besides, CDPK4 and CDPK11, several *Arabidopsis* CPKs have been shown to interact with ABF4, such as CPK10, CPK30 or CPK32. Furthermore, CPK32 has been reported to phosphorylate ABF4 *in vitro* and to interact with ABF1, ABF2 and ABF3 (Choi et al., 2005) (see summary of phenotypes in Table 1).

1.5.2.2.2 SnRK3s/CIPKs

Another group of calcium protein kinases are a subgroup 3 from the SnRKs, the SnRK3s, also known as CIPKs for CBL (calcineurin B-like) interacting protein kinases. The SnRK3s/CIPKs, mainly act as negative regulators in ABA signalling. These kinases activity is modulated by calcium binding proteins such as SOS3/CBLs/SCaBPs (Hrabak et al., 2003). For instance, calcium-binding protein SCaBP5/CBL1 and its interacting protein kinase PKS3/CIPK15, function as negative regulators in seed germination, seedling growth and stomatal ABA responses, since their knockouts exhibit hypersentivity to ABA in the former responses (Guo et al., 2002). CIPK3 is shown to interact with CBL9 (Pandey et al., 2008) and it is also involved in negative regulation of ABA signalling in seed germination. However, ABA-induced stomatal regulation is not impaired in the knock-out *cipk3* mutant. Moreover, CIPK3 appears to generally modulate cold and salt-induced gene expression, but not drought-induced gene expression, presenting itself as a cross-talk “node” among different stress signals

(Kim et al., 2003). CIPK14 has also been reported to show hypersensitivity in seed germination and root growth (Qin et al., 2008).

Table 1. CPKs and SnRK3s/CIPKs phenotypes summary.

Protein Kinase	Germination	Stomatal regulation	Growth	Gene expression
CPK10/CDPK1 **CPK30/CDPK1a** Sheen (1996)				●
CPK32 Choi et al., 2005				●
CPK3 **CPK6** Mori et al., 2006		●		
CPK4 **CPK11** Zhu et al., 2007	●	●	●	●
CPK10 Zou et al., 2010		●		
CPK21 **CPK23** Geiger et al., 2010		●		
CIPK15 Guo et al., 2002	●	●	●	
CIPK3 Kim et al., 2003 Pandey et al., 2008	●		●	●
CIPK23 Cheong et al.,2007		●		
CIPK14 Qin et al., 2008	●		●	

As an interacting partner of CBL1 and CBL9, CIPK23 has been identified as a negative regulator of the pathway in guard cells. The *cipk23* mutant is ABA hypersensitive in stomatal responses, showing reduced transpirational water loss in leaves. Based on the analysis of *cbl1cbl9* double mutant phenotype, CBL1 and CBL9 might synergistically activate CIPK23 and target it to the plasma membrane. It has been proposed that CIPK23 negatively regulates ABA signalling in guard cells by activating an inward potassium channel (Cheong et al., 2007) (see summary of phenotypes in Table 1).

1.5.3 TRANSCRIPTION FACTORS INVOLVED IN ABA SIGNALLING

In response to drought, ABA also reprograms expression of downstream target genes (Seki et al., 2002; Leonhardt et al., 2004), that leads to the accumulation of different metabolites and LEA proteins, including dehydrins, to protect cells from dehydration (Verlues et al., 2006; Hundertmark and Hincha, 2008; Urano et al., 2009). Numerous transcription factors from different families have been described to be involved in these ABA-induced changes. The most common regulatory elements conferring ABA-inducibility are the G-box ABA Response Elements (ABREs), which are recognized by members of a bZIP transcription factors family, named AREB/ABFs (Kim et al., 1997, 2002; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Choi et al., 2000; Uno et al., 2000; Carles et al., 2002; Furihata et al., 2006). ABA-regulated genes also contain recognition sites for proteins from the MYB and MYC families (Cominelli et al., 2005; Liang et al., 2005; Jung et al., 2008). Moreover, APETALA 2 (AP2) proteins, such as ABI4, or B3 domain proteins, such as ABA-INSENSITIVE3/VIVIPAROUS1 (ABI3/VP1), have also been involved in ABA signalling (Giraudat et al., 1992; Finkelstein et al., 1998). In addition, other classes of transcription factors also participate in some ABA responses. Among them there are members of the homeodomain-leucine zipper (HD-ZIP) (Ariel et al., 2007), WRKYs, which have been described both as activators and repressors of the pathway (Jiang and Yu, 2009; Chen et al., 2010), basic helix-loop-helix (bHLH) (Li et al., 2007) and Zn-finger transcription factors (Drechsel et al., 2010).

The role of the subfamily of the bZIP transcription factors ABI5/AREB/ABF has been well characterized in ABA signalling (Uno et al., 2000; Fujita et al., 2005; Furihata et al., 2006; Yoshida et al., 2010). The presence of ABA-responsive elements (ABREs) within the promoters of many ABA up-regulated genes suggests that binding of AREB/ABFs controls many targets of ABA signalling (Seki et al., 2002; Leonhardt et

al., 2004). Moreover, these transcription factors have been described to be direct targets of the SnRK2 kinases and crucial for the ABA-induced responses in the plant (Fujita et al., 2009; Yoshida et al., 2010).

1.5.3.1 ABI5/AREB/ABF bZIP-TYPE TRANSCRIPTION FACTORS

The ABI5/AREB/ABFs are sequence-specific DNA binding factors, encoding for basic-domain leucine zipper transcription factors. They belong to a group-A subfamily, which comprises 13 homologs in the *Arabidopsis* genome. They contain three N-terminal (C1, C2 and C3) and one C-terminal (C4) conserved domains (Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Jakoby et al., 2002; Fujita et al., 2005).

Identification and analysis of this conserved *cis*-element (ABRE: PyACGTGG/TC) (Giraudat et al., 1994; Busk and Pagés, 1998) have revealed that ABA-responsive gene expression requires multiple ABREs or a combination of an ABRE element with a coupling element (CE), to be a functional promoter (Shen et al., 1996; Narusaka et al., 2003). Genetic and biochemical studies have identified ABA-INSENSITIVE 5 (ABI5) and closely related trans-acting factors, named AREB/ABF/DPBFs that regulate ABRE-containing genes (Kim et al., 1997, 2002; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Choi et al., 2000; Uno et al., 2000; Carles et al., 2002). ABI5 was identified from independent genetic screens oriented to find ABA-insensitive mutants in germination (Finkelstein, 1994) and early seedling growth in *Arabidopsis* (Lopez-Molina and Chua, 2000). ABI5 was cloned by positional cloning (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). The *Arabidopsis* named ABRE-binding (AREB) proteins or the ABRE-binding factors (ABFs) were first isolated by using ABRE sequences as bait in yeast one-hybrid screenings (Choi et al., 2000; Uno et al., 2000).

Identification and analysis of loss-of-function ABI5 mutants have revealed that ABI5 plays an important role, as a positive regulator, in ABA gene regulation in seeds (Finkelstein, 1994; Lopez-Molina and Chua, 2000; Finkelstein et al., 2005) and early seedling growth (Lopez-Molina and Chua, 2000). These phenotypes appear to correlate with its expression pattern, in which expression levels of ABI5 are much more abundant in developing siliques, reaching the highest peak in desiccating and dry seeds (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). ABI5 is still present in 5-day-old seedlings under ABA treatment, but from this stage onwards its expression starts to fade (Finkelstein et al., 2005).

In order to overcome the potential functional redundancy, overexpression approaches have been first used to analyze other AREB/ABF family members. This group of AREB/ABF members, in contrast with ABI5, does not present phenotypes in germination (Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005). Overexpression of AREB1/ABF2, ABF3 and AREB2/ABF4 has revealed ABA hypersensitivity phenotypes in seedling growth (Kang et al., 2002). Moreover, all the overexpressing lines show enhanced drought resistance and an increase in the expression levels of ABA-regulated genes (Kang et al., 2002; Fujita et al., 2005), suggesting that they might be additional positive regulators in ABA signalling in response to drought stress. To further verify the effects of these regulators and also to try to overcome functional redundancy, loss-of-function mutants of *areb1*, *areb2* and *abf3*, in all possible combinations, have been analyzed (Yoshida et al., 2010). Under drought stress conditions, the triple mutant *areb1 areb2 abf3* has a markedly reduced survival rate in comparison to other single and double mutants and wild type plants. This suggests that AREB1, AREB2 and ABF3 confer drought stress tolerance in a redundant manner. This enhanced tolerance would be mainly attributed to the expression of downstream genes thought to protect the plant from water deficit, such as LEA proteins, thus enhancing their drought tolerance (Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005; Yoshida et al., 2010). Besides the reduced drought tolerance, the triple mutant also shows an enhanced insensitivity in primary root growth and a remarkably impaired expression of ABA-regulated genes when compared to the wild type (Yoshida et al., 2010). Comparative transcriptomical analysis of the *areb1/areb2/areb3* triple mutant versus the wild type, under ABA treatment, indicates that around 90% of the genes that show reduced expression levels in the triple mutant are ABA-responsive genes (Fujita et al., 2009; Yoshida et al., 2010). These results strengthen the previous findings that AREB1, AREB2 and ABF3 contribute to the regulation of ABRE-dependent gene expression in response to water stress and that they act as positive regulators in ABA signalling (Yoshida et al., 2010). Additionally, when comparing the microarray results of the triple *areb1/areb2/areb3* mutant, with the ones obtained for the triple mutant of the SnRK2s, *snrk2.2/snrk2.3/snrk2.6*, it is shown that most of the AREB/ABFs targets genes are also down-regulated in the *snrk2.2/snrk2.3/snrk2.6* triple mutant in response to ABA. This supports the view that these AREB/ABF transcription factors act downstream and are regulated by the ABA-activated SnRK2s in ABA signalling (Fujita et al., 2009). Similarly, when analyzing the expression of genes in seeds of the *snrk2.2/snrk2.3/snrk2.6* triple mutant with those from *abi5* mutants, approximately half of the down-regulated genes in the *abi5* mutants seeds are also suppressed in

snrk2.2/snrk2.3/snrk2.6 ones. These observations also suggest that many ABI5-dependent genes are also controlled by SnRK2s (Nakashima et al., 2009).

The activity of ABI5/AREB/ABF transcription factors, such as AREB1 and ABI5, have been reported to be regulated by ABA-dependent phosphorylation (Uno et al., 2000; López-Molina et al., 2001; Fujita et al., 2005). Activation of these transcription factors by phosphorylation is crucial for their capacity to activate the transcription of ABA-responsive genes (Fujita et al., 2005; Furihata et al., 2006; Fujii et al., 2009). It has been described that AREB1 requires multi-site phosphorylation of Ser/Thr residues of the conserved domains (R-x-xS/T) in order to be activated and thus, promote the transcription of ABA-responsive genes (Furihata et al., 2006). Analysis of the effect of AREB1 phosphorylation on its transactivation activity revealed that mutations on its phosphorylation sites were affecting the ABA induction of *RD29B* promoter-*GUS* expression (Furihata et al., 2006). Amino acid substitutions of these Ser/Thr residues by Ala or Asp result in almost complete suppression or constitutive activation of the transcription factors, respectively (Furihata et al., 2006). Direct interaction *in vivo* of AREB/ABF transcription factors with the ABA-activated SnRK2s has also been described and it seems to happen in the nucleus (Fujita et al., 2009; Yoshida et al., 2010). Further analyses have revealed that SnRK2s, such as SnRK2.2. SnRK2.3 and SnRK2.6, play a key role in the ABA-dependent phosphorylation of AREB/ABF transcription factors (Furihata et al., 2006; Fujii et al., 2007, 2009; Yoshida et al., 2010). Similarly, it has also been reported that the rice AREB1 ortholog, TRAB1, is phosphorylated by the SnRK2 protein kinase ortholog SAPK (Kobayashi et al., 2005), and that the wheat AREB1 ortholog TaABF1 is phosphorylated by the SnRK2 PKABA1 *in vitro* (Johnson et al., 2002). Moreover, as described previously, these AREB/ABF proteins seem to also be activated by Ca^{2+}-dependent proteins involved in ABA signalling (Choi et al., 2005; Zhu et al., 2007).

Moreover, this family of transcription factor proteins seem to have the capacity to form active homo or heterodimers (Kim et al., 2002; Yoshida et al., 2010), creating this way a strong potential for redundant or antagonistic function. Kim et al. (2002) used *in vitro* DNA binding assays to prove this dimerization hypothesis and they reported that ABI5/AtDPBF1, AtDPBF3 and EEL/AtDPBF4, DPBFs *Arabidopsis* homologs expressed in seeds, could dimerize with each other. These heterodimers appeared to have distinct DNA-binding activities. In the work of Yoshida et al. (2010) the same question was raised and they tested this dimerization capacity carrying out *in vivo* BiFC analysis with the transcription factors AREB1/ABF2, AREB2/ABF4 and ABF3. They reported that only samples co-expressing the N-terminal and the C-

terminal half of the yellow fluorescent protein (YFP) fused to AREB1, AREB2 and ABF3, yielded YFP fluorescence and that the signal was localized in the nucleus (Yoshida et al., 2010). This indicated that AREB/ABFs transcription factors had the capacity to form homo- or heterodimers with each other (Yoshida et al., 2010). These results also correlate with evidence based on structural analysis of bZIP proteins, in which it has been shown that they bind to DNA as dimers (Schütze et al., 2008). An example of antagonistic function between members of the family has been shown in the case of the seed expressed ABI5 and EEL (ENHANCED Em LEVEL) (Bensmihen et al., 2002). They both directly interact and regulate the expression of *AtEm* (*ARABIDOPSIS THALIANA LATE EMBRYOGENESIS ABUNDANT*) genes, which encode for LEA proteins, but in an opposite way. While ABI5 positively regulates their expression, EEL regulates AtEms in a negative manner. Experimental evidence suggests that in wild type seeds, the EEL-ABI5 heterodimer and the EEL homodimer would compete with the ABI5 homodimer for binding to the *AtEm1* promoter, preventing ABI5 from optimally activating *AtEm1* (Bensmihen et al., 2002). The equilibrium of this competition, and therefore the expression of *AtEm1*, is tightly related to the expression level of both transcription factors. This competition between ABI5 and EEL could be a mechanism adopted by plants to fine-tune the expression of LEA genes during seed development (Bensmihen et al., 2002).

1.5.4 REGULATION OF TARGET PROTEINS INVOLVED IN STOMATA CLOSURE

In response to drought stress, ABA induces stomatal closure as a way of reducing water loss through transpiration. Stomata closure is induced by a decrease in osmotic pressure in guard cells which provokes water efflux from the cell, reducing its volume and resulting in the pore closing. Decrease in osmotic pressure is mainly due to ion trafficking across the membrane, which is regulated by ion channels (Sirichandra et al., 2009B; Kim et al., 2010).

As a general overview and in a very simplified way, closing of stomata is regulated by a series of transport events. Stomatal opening is driven by hyperpolarization of the guard cell membrane, which is caused by H^+-ATPase-dependent proton efflux (Assmann et al., 1985). In turn, membrane hyperpolarization activates inward-rectifying K^+_{in} channels (Kwak et al., 2001; Lebaudy et al., 2007) and induces solute influx followed by water uptake, which eventually induces stomatal opening. In response to drought or ABA, anion channels (SLAC1 and SLAH3) are activated, expelling anions out of the cell and starting depolarization of the membrane

(Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009, 2011). At the same time, ABA also inhibits the activity of the proton pump ATPase (OST2) and K^+-inward-rectifying channels (KAT1), to prevent from hyperpolarization (Merlot et al.,2007; Sato et al., 2009). Moreover, membrane depolarization also leads to activation of the K^+-outward–rectifying channel (GORK) to expel K^+ (Hosy et al., 2003; Lebaudy et al., 2007), contributing to decrease the osmotic pressure. This decrease in osmotic pressure leads to a reduction of turgor, causing then stomatal closure.

Different genetic and biochemical studies, have suggested S(slow)-type anion channels to play an important role in stimulus-induced stomatal closure (Schroeder and Keller, 1992; Pei et al., 1997; Negi et al., 2008; Geiger et al., 2009). Moreover, connection between ABA signalling components, PP2Cs and SnRK2s, and anion channel regulation in guard cells had already been established (Pei et al., 1997; Li et al., 2000). For instance, *abi1-1D* mutant plants appeared to have impaired anion channel activity in response to ABA (Pei et al., 1997), as well as the OST1 homologue in *Vicia faba*, AAPK, which also seemed to play an important role in modulating the anion channel activity in guard cells (Li et al., 2000). Additionally, it had also been reported that slow anion channels would need to be activated to trigger stomatal closing and that this activation could be mediated by phosphorylation (Schmidt et al., 1995).

Recently, it has been described the direct regulation of SLAC1 by the ABA calcium-independent and calcium-dependent pathway (Geiger et al., 2009; Lee et al., 2009; Geiger et al., 2010). *SLAC1* was genetically isolated from independent mutant screens for ozone-sensitive and CO_2-insensitive stomatal closure mutants, and it was described as a gene encoding an anion transporter essential for stomatal closure in response to ABA, CO_2, Ca^{2+} and ozone treatments located at the plasma membrane of guard cells (Negi et al., 2008; Vahisalu et al., 2008). In addition, Ca^{2+}- and ABA-dependent activation of S-type anion channels were impaired in *slac1* guard cells, providing genetic evidence that *SLAC1* encodes a major anion-transporting component of S-type anion channels in guard cells (Vahisalu et al., 2008). Heterologous expression of *Arabidopsis* SLAC1 in *Xenopus* oocytes illustrates that it functions as an anion channel with selective permeability to Cl^- and $NO3^-$ (Geiger et al., 2009; Lee et al., 2009). The works of Geiger et al. (2009) and Lee et al. (2009) describe the direct *in vivo* interaction and activation by phosphorylation, in a heterologous system, of the ABA-calcium independent kinase OST1/SnRK2.6 and the anion channel SLAC1. Interaction and activation assays were also performed with SnRK2.2 and SnRK2.3,

obtaining a much weaker results than with OST1 (Geiger et al., 2009), probably illustrating some target affinities.

Upstream ABA-signalling regulatory elements, such as PP2Cs and the ABA-receptors PYR/PYL/RCAR, seem to participate in the final regulation of SLAC1 (Geiger et al., 2009; Lee et al., 2009: Geiger et al., 2010). Clade A PP2Cs, negatively regulate SLAC1 through the inactivation of OST1 (Geiger et al. 2009: Lee et al., 2009), although an additional and particular regulatory mechanism has been reported in the case of PP2CA (Lee et al., 2009). Lee et al. (2009) proposed that PP2CA could also regulate SLAC1 activity by directly interacting and dephosphorylating the channel, leading to its inhibition. This suggestion was based on the results observed when coexpressing SLAC1 and PP2CA, in the absence of OST1. They reported a decrease in the anion currents level, being under the one registered for the expression of SLAC1 alone. The question whether PP2Cs were able to directly inactivate SLAC1 was also raised in the work of Geiger et al. (2009). They incubated ABI1 with phosphorylated SLAC1, but no dephosphorylation activity of ABI1 over SLAC1 was detected (Geiger et al., 2009). This result was in contrast with the double inhibitory mechanism proposed for PP2CA by Lee et al. (2009). Additionally, SLAC1 appears to also be activated in an ABA calcium-dependent manner. Guard cell expressed CDPK21 and CDPK23 are able to stimulate SLAC1-related anion currents by direct phosphorylation (Geiger et al., 2010). Previous evidence of CDPKs being involved in anion channel regulation had been reported in the work of Mori et al., (2006). As OST1, CDPK21/23 are susceptible of being negatively regulated by ABI1 and positively regulated by the ABA receptors PYR/PYL/RCAR (Geiger et al., 2010). The two works from Geiger et al. (2009) (2010), together with the work from Lee et al. (2009), provide different steps of evidence to end up reconstituting the basic fast signalling pathway from ABA perception to the activation of anion channels activity in guard cells. Kinase activity assays adding the four elements, RCAR1, ABI1, OST1 or CDPK23 and SLAC1, revealed that SLAC1 was only phosphorylated in the presence of the hormone (Geiger et al., 2010). These results also illustrate that CDPK and OST1 branch of ABA signal transduction in guard cells seem to converge at the level of SLAC1, under the control of PP2Cs/ABA-receptor complex (Geiger et al., 2010). Final activation of SLAC1 by phosphorylation would trigger it to open, releasing anions and starting depolarization of the plasma membrane. This membrane depolarization leads to the activation of the K^+-outward rectifying channel GORK (GATED OUTWARDLY-RECTIFYING K^+ CHANNEL) (Hosy et al., 2003) resulting in K^+ efflux. This guard cell mechanism of releasing osmotically

active compounds (anions and K^+) results in water loss from the cell and subsequently closure of stomata (Sirichandra et al., 2009B).

The elucidation of the crystal structure of SLAC1 homologue from *Haemophilus influenza* and the building up of a homology model for *Arabidopsis* SLAC1, has allowed a better understanding of the channel properties and how it works (Chen et al., 2010). SLAC1 is a symmetrical trimer composed from quasi-symmetrical subunits. Each subunit has ten transmembrane helixes that are arranged in helical hairpin pairs to form a central five-helix transmembrane pore gated by a conserved phenylalanine residue. Phosphorylation of different sites of SLAC1, by protein kinases such OST1 or CDPK23, would induce conformational changes leading to the gate release from the Phe and opening of the pore (Chen et al., 2010).

In addition to SLAC1, the anion channel SLAH3 (SLAC1 HOMOLOG 3) has also been reported to be involved in ABA-induced stomatal closure (Geiger et al., 2011). SLAH3 has been described to be activated by ABA, through the RCAR1/PYL9-ABI1 ABA-receptor-phosphatase complex and the calcium-dependent kinase CPK21. In addition to phoshorylation, SLAH3 also requires an increase in extracellular nitrate concentrations for full channel opening. Due to this fact, the authors propose that SLAH3 might connect the stomatal response to drought with nitrate metabolism and nitrate signalling (Geiger et al., 2011).

To ensure stomatal closure, ion channel-mediated inward K^+ currents in guard cells need to be inhibited in order to avoid K^+ influx followed by water uptake into the cell (Pandey et al., 2007). The inward-rectifying K^+ channel KAT1 has been described to have a key role in stomatal opening (Kwak et al., 2001) and the inhibition of its activity is understood to be one of the requirements to enable stomatal closure (Pandey et al., 2007). A direct connection between the potassium channel KAT1 and the ABA-activated OST1/SnRK2.6, was established in 2009 (Sato et al., 2009). It has been seen that OST1 is able to inhibit KAT1 activity by phosphorylating a specific residue relevant for the activity of the channel, Thr306, located at the C-terminus of the protein. Since OST1 seems to mediate KAT1 inhibition, and OST1 activity is regulated by clade A PP2Cs and the ABA-receptors PYR/PYL/RCAR (Fujii et al., 2009; Vlad et al. 2009; Umezawa et al., 2009), final regulation of the potassium channel under drought stress conditions could be mediated by the core elements of the pathway. The ABA calcium-dependent signalling might also be implicated in the regulation of KAT1 activity during stomatal closure. In the work of Zou et al. (2010) they report that functional CDPK10 was required in order to inhibit inward K^+ channels in an ABA dependent manner.

In addition, to ensure stomatal closing, ABA also inhibits H^+-ATPases activity (Zhang et al., 2004). *OST2* (OPEN STOMATA 2) gene was found to encode for a H^+-ATPase previously named AHA1 (ARABIDOPSIS H^+ ATPASE 1). Two dominant mutants, *ost2-1D* and *ost2-2D*, were independently identified by using a thermal imaging screen (Merlot et al., 2002, 2007). The *ost2-1D* revealed to be prone to wilt even under well-watered conditions, meaning that it was seriously impaired in its ability to regulate stomatal closure (Merlot et al., 2002). Phenotypic characterization of these mutations showed that they were strongly insensitive to ABA, even to high doses such as 100 μM, while they were still reactive to high CO2 and darkness. Morever, these mutants presented constitutive activity of the proton pump. Correlation of both phenotypes revealed that, ABA-induced mechanism of stomatal closure also required the inhibition of H^+-ATPase activity to prevent from stomatal opening (Merlot et al., 2007).

Reactive oxygen species are well known second messengers that positively regulate abscisic acid signalling (Cho et al., 2009). A molecular and genetic study had already shown that two of the 10 NADPH oxidases in the *Arabidopsis* genome, *AtrbohD* and *AtrbohF* (*ARABIDOPSIS THALIANA RESPIRATORY BURST OXIDASE PROTEIN D* and *F*), were highly expressed in guard cells and that mediated ABA-induced ROS production, ABA-activation I_{Ca} channels and ABA-induced stomatal closure (Kwak et al., 2003). Besides the ABA-insensitivity in stomatal closure displayed by the double knockout mutant a*trbohD/F,* other ABA responses were also impaired such as inhibition of seed germination and root growth, suggesting that ROS production by these NADPH oxidases might be of broader significance in ABA signal transduction (Kwak et al., 2003). However, it was still unknown how ABA was activating these two NADPH oxidases.

A previous work of Mustilli and co-workers in 2002 had shown that OST1 acted upstream of ROS in guard cells ABA signalling (Mustilli et al., 2002) and some subsequent studies revealed phosphorylation as a way of regulating NADPH oxidases function (Kobayashi et al., 2007; Nühse et al., 2007). Sirichandra et al. (2009A) reported the physical interaction of OST1 with ArtbohF and the phosphorylation of residues Ser13 and Ser174, located at the N-terminus of the NADPH oxidase. Presumably, and according to other studies (Nühse et al., 2007), phosphorylation by OST1 would activate ArtbohF in order to produce ROS species in response to ABA. ROS would participate in stomata closure in different indirect ways such as inhibiting PP2Cs (Meinhard and Grill, 2001), leaving OST1 free to activate anion channels and inhibit K^+ inward channels, or by increasing the concentration of Ca^{2+} in the cell. This

raise in Ca^{2+} concentration, plays an important role in stomata closure (Kim et al., 2010) through the activation of Ca^{2+} permeable channels (Pei et al., 2000; Murata et al., 2001). Since OST1 interacts with ArtbohF, regulation of its phosphorylation could also be eventually modulated by PP2Cs and the PYR/PYL/RCAR family in response to ABA, in a similar way above described. Additionally, ROS would positively regulate its production, by inhibiting PP2Cs activity such as ABI1 (Meinhard and Grill, 2001).

1.5.5 HORMONE SENSING AND PERCEPTION

In order to better understand a hormone signalling pathway it is critical to know how the hormone is perceived and how the signal is transduced. In the case of ABA, receptors identification has been a challenging task. Genetic approaches, even though very fruitful in the identification of key elements of the ABA signalling pathway, did not succeed in the identification of any putative receptor candidate. This suggested functional redundancy, lethality or even the need to design more sophisticated screen systems to detect more subtle or masked phenotypes. Finally, biochemical and chemical genetic strategies have bypassed such difficulties and have achieved elucidation of different receptors types, either plasma membrane or intracellular ABA receptors. This work has contributed to both the structural and biochemical characterization of the new PYR/PYL/RCAR receptor family. Here, we are going to introduce the ABA receptors identified up to now, together with the PYR/PYL/RCAR family, but a critical discussion on their relevance will be further addressed in the General discussion.

1.5.5.1 ABA RECEPTORS IDENTIFIED UP TO NOW

FCA

The first putative ABA receptor identified was FCA (Flowering time control protein) (Razem et al., 2006), however it was later on retracted by the authors (Razem et al., 2008). FCA was a nuclear RNA-binding protein, previously characterized as a flowering time regulator (Macknight et al., 1997). FCA was identified by sequence similarity to a barley protein, ABAP1, which resulted to be able to bind to an anti-idiotypic ABA antibody (an antibody against an ABA antibody) (Razem et al., 2004). Razem et al. (2006) reported that FCA was stereoespecifically binding (+)-ABA with high affinity (Kd=19 nM) and that this binding was inhibiting the association of FCA with FY (FLOWERING locus Y). Inhibition of the FCA-FY complex formation by the hormone was enhancing accumulation of the floral repressor FLC (FLOWERING LOCUS C) and consequently delaying bolting. FCA was also proposed to be involved

in distinct ABA responses, since *FCA* loss-of-function mutants did not show phenotypes in any of the common ABA physiological responses such as germination, stomatal regulation or primary root development. Only a modest phenotype in lateral root formation was observed. In addition, FCA was described to function through a different signalling pathway, not involving the well known ABA signalling elements, ABI1 or ABI2 (Razem et al., 2006). Unfortunately, different subsequent works set big questions marks on the role of FCA as an ABA receptor (Jang et al., 2008; Risk et al., 2008; Risk et al., 2009), which finally led to retraction of the paper by the authors.

CHLH/ABAR

Similarly to the identification of the FCA receptor, a new approach to isolate ABA-binding proteins led to the identification of a second putative ABA receptor. This time a protein from broad bean leaves was purified by using an ABA affinity matrix. This matrix was constructed by coupling ABA to a Sepharose affinity-chromatography column through its carboxylate group (Zhang et al., 2002). This identification process raised certain reservations about the receptor nature of the protein identified, since the ABA functional group attached to the column has already been described as essential for hormone bioactivity (Milborrow, 1974). The *Arabidopsis* relative of this bean protein was named ABAR (for putative ABSCISIC ACID RECEPTOR) and sequence analysis revealed ABAR to be a subunit of the magnesium-protoporphyrin IX chelatase (Mg-chelatase) and localized in chloroplast (Shen et al., 2006). CHLH, in addition to its central role in producing Mg-protoporphyrin-IX (Walker and Willows, 1997), it was also known to play a key role in mediating plastid-to-nucleus signalling (Mochizuki et al., 2001). To further corroborate that CHLH was binding ABA, Shen et al. (2006) performed a direct binding assay with [^{3}H]-(+)-ABA, in which CHLH seemed to specifically bind (+)-ABA with a Kd=32nM. To explore the CHLH/ABAR functions in ABA signalling, Shen et al. (2006) also generated and characterized transgenic RNAi, antisense and overexpression lines. The plants underexpressing CHLH/ABAR resulted in ABA-insensitive phenotypes in seed germination, post-germination growth and ABA-induced stomatal closure; whereas plants overexpressing CHLH/ABAR displayed opposite phenotypes of hypersensitivity to the hormone. Moreover, down-regulation of CHLH/ABAR expression seemed to also affect the expression levels of positive and negative regulators involved in ABA signalling. All these results led the authors to describe this protein as an ABA receptor (Shen et al., 2006). In 2009, the work from Shang et al. presented a model to describe how the ABA signal was transduced through CHLH/ABAR, to eventually regulate ABA responsive transcription factors. This work reported the interaction between the CHLH/ABAR protein and some WRKY

transcription factors. Apparently, the recruitment of these WRKY proteins by this receptor was enhanced upon binding of the hormone. Moreover, the authors characterized these WRKY transcription factors as repressors of the ABA signalling pathway, and connected them with the regulation of ABA-responsive transcription factors such as ABI4, ABI5, ABF4 and MYB2. Their ABA signalling model through CHLH/ABAR was based on the recruitment of these WRKY repressors by the CHLH/ABAR-ABA bound form, releasing the ABA-responsive transcription factors from blockage. However, this model presented some inconsistencies and still left open questions about the role of CHLH/ABAR in ABA signalling (Shang et al., 2009), which will be further discussed in the General discussion.

G-protein-coupled receptors (GPCRs)

GPCR2 was identified through an *in silico* approach (Liu et al., 2007). Experiments suggesting the extracellular perception of the hormone (Anderson et al., 1994), together with the implication of components of the heterotrimeric G-protein complex in ABA responses (Pandey et al., 2006), led the authors to look for putative GPCR proteins in *Arabidopsis*. GPCRs are a class of proteins typically with a seven-transmembrane domain (7TM) structure. Heterotrimeric G protein complexes are intracellular partners of GPCRs, connecting ligand perception by GPCRs with downstream effectors. In a classical signalling event, in response to activation by ligand binding to a GPCR, the inactive G-heterotrimeric complex Gα-GDP/Gβɣ converts to an active conformation by promoting the release of GDP from the Gα-subunit and the binding of GTP. The GTP binding to this Gα-subunit promotes both the dissociation of the G-protein complex from the GPCR, and the release of Gα from Gβɣ. Either the free Gα-GTP form or the Gβɣ, or even both, can participate in transducing the signal to downstream effectors (Jones and Assmann, 2004; McCudden et al., 2005). Liu et al. (2007) predicted GPCR2 as a seven transmembrane protein and reported binding with the *Arabidopsis* Gα-subunit GPA1. To further analyze the function of GCR2 in *Arabidopsis*, loss-of-function mutants were characterized in their response to the hormone. These mutants were described to present ABA insensitive phenotypes in all the hormone responses. These results suggested GCR2 as a positive regulator in ABA signalling. The fact that it was reported as a membrane protein and to regulate all ABA responses, led the authors to examine its binding to the hormone. As a result, (+)-ABA binding was accounted with a dissociation constant of 20nM. However, subsequent works examining the role of GCR2 in ABA signalling have reported opposite results contradicting the conclusion of Liu et al. (2007) and setting controversy around this receptor (Gao et al., 2007; Johnson et al., 2007; Guo et al., 2008; Risk et al., 2009).

In 2009, another two GPCR-type G proteins were reported as ABA receptors, GTG1-GTG2. Their identification was also performed *in silico*, and their classification as GPCR proteins was based on their sequence similarity to an orphan GPCR, GPR89, and their binding to the Gα-subunit protein, GPA1 (Pandey et al., 2009). According to their prediction as putative receptors, analysis of the double mutant *gtg1gtg2* showed ABA insensitive phenotypes in germination, growth, stomatal closure and expression of ABA-responsive genes. However, ABA binding assays reported that only 1% of the GTG proteins were able to bind the hormone. The authors claimed that result as sufficient and they noted that protein purification required to be optimized to get a more detailed analysis of ABA binding. GTG1 and GTG2 were proposed as a novel GPCR-like G proteins, both involved in ligand binding and initiation of the signalling cascade. In this new model GPA1 would not act as a common Gα-subunit, but as a negative regulator of the GPCR-like G system, regulating GTGs activity (Pandey et al., 2009).

PYR/PYL/RCAR

The identification and characterization of this new class of ABA receptors has shed light on how ABA is perceived in the cell and how the hormone signal is transduced along the pathway.

PYR/PYL/RCAR proteins have been independently identified by four research groups using different methods (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A; Nishimura et al., 2010). Park et al. (2009) pursued a chemical genetics strategy using a new synthetic ABA agonist called pyrabactin, which led them to identify PYR1 and connect it with the ABA signalling pathway. Thus, they named this family as PYR/PYL (*for PYR1-Like*). In an alternative approach, we identified PYL5, PYL6 and PYL8 as constitutive interacting partners of the phosphatase HAB1, in a yeast-two-hybrid screen (Results Chapter 1 of this work). A similar approach was used by Ma et al., (2009) to identify PYL9/RCAR1 and PYL8/RCAR3, but in these case ABI2 was used as a bait. Independently, Nishimura et al. (2010) performed and *in vivo* strategy through which they indentified several PYR/PYL/RCAR proteins by being major *in planta* interactors of ABI1.

PYR/PYL/RCARs belong to a superfamily of soluble ligand-binding proteins defined as START/BetV I superfamily. Star-related lipid transfer (START)-domain proteins are characterized by containing a conserved “helix-grip” fold that forms a central hydrophobic ligand-binding pocket (Iyer et al., 2001; Radauer et al., 2008). The PYR/PYL/RCAR family is composed by 14 members of small proteins, ranging

between 159 and 221 amino acids (Supplemental Figure 1 from Results Chapter 1 of this work). They are directly connected to the ABA signalling pathway. Biochemical analyses have demonstrated that these PYR/PYL/RCAR proteins directly bind clade A PP2Cs, which are well known negative regulators of the pathway (Ma et al., 2009; Park et al., 2009; Nishimura et al., 2010; Results Chapter 1 of this work). Phosphatase activity assays have shown that this interaction results in an ABA-dependent inhibitory effect on these phosphatases (Ma et al., 2009; Park et al., 2009; Results Chapter 1 of this work). Moreover, BiFC (Bimolecular fluorecence complementation) experiments have reported that these interactions occur either in the cytosol or the nucleus, which qualifies these PYR/PYL/RCARs as intracellular proteins (Park et al., 2009; Results Chapter 1 of this work). Besides the biochemical analysis of these PYR/PYL/RCAR proteins, genetic evidence has also supported their role as positive regulators of the pathway. Generation of triple and quadruple loss-of-function mutants (*pyr1pyl1pyl4* and *pyr1pyl1pyl2pyl4*, respectively), show insensitive phenotypes to the hormone in germination and root growth. In addition, the quadruple mutant also displays a reduced ABA-induced expression of ABA responsive genes such as RD29A, NCED3 or P5CS1 (delta1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1), and it is also impaired in ABA-induced stomatal closure (Park et al., 2009). On the other hand, overexpression of PYL5 (Results Chapter 1 of this work) and PYL9/RCAR1 (Ma et al., 2009) displays hypersensitivity to the hormone in similar responses. Moreover, transgenic plants overexpressing both *HAB1* and *PYL5* have shown a phenotype similar to that of PYL5-overexpressing lines, which suggests that PYL5 antagonizes HAB1 function in the plant. Interestingly, overexpression of PYL5 in *Arabidopsis* also confers enhanced drought resistance, indicating that this new family of proteins is connected to the physiological stress-response in plants (Results Chapter 1 of this work).

A critical property of these PYR/PYL/RCAR proteins is that they are able to specifically bind ABA. Isothermal titration calorymetry (ITC) and Nuclear magnetic resonance (NMR) binding asssays have determined that these proteins directly bind to the hormone, which is a must in a hormone receptor (Ma et al., 2009; Miyazono et al., 2009; Results Chapter 1 and 3 of this work; Szostkiewicz et al., 2009). These PYR/PYL/RCAR receptors have been shown to display different affinities for the hormone, according to their oligomeric state (Results Chapter 3 of this work). The family is divided in two sub-classes: monomeric and dimeric receptors. The monomeric receptors present Kds in the range of 1 μM (Ma et al., 2009; Results Chapter 1 and 3 of this work; Szostkiewicz et al., 2009), whereas dimeric receptors show much lower affinities, estimated to be greater than 50 μM (Miyazono et al., 2009; Results Chapter 3

of this work). In addition, experimental data have also shown that some PYR/PYL/RCAR proteins present partial stereospecificity. For instance, similar ITC experiments performed with the (-)-ABA form have revealed that PYL5 is able to bind the non-natural form, although with lower affinity (Kd=19 µM). Moreover, the (-)-ABA is also able to activate the PYL5 receptor, inducing inhibition of the HAB1 phosphatase (Results Chapter 1 of this work). Similarly, Park et al. (2009), in a yeast-two-hybrid assay, have seen that the non-natural form is able to promote the binding between several PYR/PYL/RCARs and PP2Cs.

Interestingly, the hormone affinity is enhanced when PP2Cs are included in ITC binding assays, suggesting that PP2Cs generally stabilize the ABA binding and complex formation (Ma et al., 2009; Results Chapter 1 of this work). For instance, PYL5 binds (+)-ABA with a Kd=1.1 µM, but when HAB1 is added in equimolar conditions the affinity increases to a Kd=38 nM. A physical explanation for this cooperative effect of the PP2Cs has been provided by the recent structural studies of the receptor-ABA-PP2C complex (discussed below).

Collectively, the biochemical and genetic analyses, together with the structure resolution of these proteins in complex with the hormone, have demonstrated that these PYR/PYL/RCARs are ABA intracellular receptors that are able to control different aspects of ABA signalling and physiology.

The existence of two types of PYR/PYL/RCAR proteins with different properties, strongly suggests that they might have specialized functions and a differential contribution to ABA signalling. However, public microarray datasets suggest that, even though they might have different expression levels, PYR/PYL/RCAR receptors often co-expressed in different tissues (Figure 5). Interestingly, public expression data show that, most of the PYR/PYL/RCAR receptors are down-regulated upon ABA treatment (Figure 5). For instance, expression of these receptors in whole-seedling tissue, as well as meshophyll and guard cells, is repressed under ABA treatment. These results are intriguing, since the expression of the clade A PP2C genes and SnRK2s is strongly up-regulated by ABA treatment (public data from Bio-Array Resource for Arabidopsis Functional Genomics (BAR) (http://bar.utoronto.ca); Figure 2 in Results Chapter 1 of this work). Thus, gene expression data reveals an opposite effect of ABA on the expression of the PP2C negative regulators, and the majority of the PYR/PYL/RCAR members.

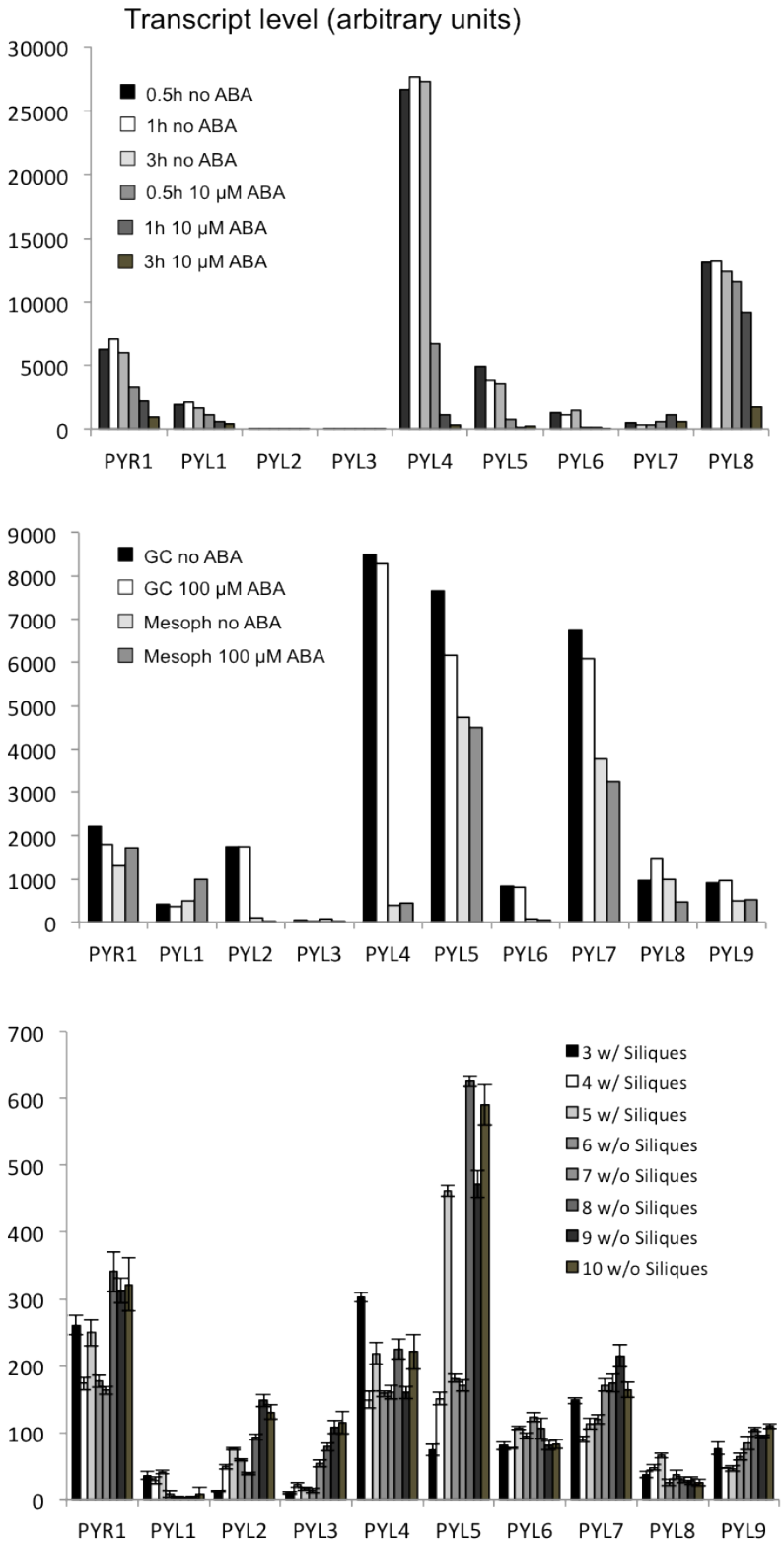

Figure 5. Expression data of the PYR/PYL family members. A, Expression levels of PYR/PYL family members in a time course experiment of whole 7-day-old seedlings mock- or ABA-treated (data produced by the AtGen-Express Consortium (http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm). **B,** Expression levels of PYR/PYL in mesophyl and guard cells that were either mock- or ABA-treated (data from Yang et al., 2008). **C,** Expression levels of PYR/PYL during seed development stages (w/ = with and w/o = without). Data were obtained from de Bio-array Resource for Arabidopsis Functional Genomics (http://bar.toronto.ca).

This expression pattern fits with the negative-feedback regulatory mechanism previously described for PP2Cs (Merlot et al., 2001). Therefore, under stress conditions, ABA would induce the initial ABA-mediated PYR/PYL/RCAR inactivation of PP2Cs. However, this inactivation would be later on attenuated by the combination of both the ABA-induced downregulation of PYR/PYL/RCARs expression and the up-regulation of the PP2Cs and SnRK2s, restoring the initial conditions. This re-setting system of the ABA signalling pathway provides a dynamic mechanism that allows the monitoring of ABA levels and the modulation of the ABA response (Results Chapter 1 of this work). However, it is still unclear whether these PYR/PYL/RCAR proteins might also be regulated by other non-receptor proteins.

1.5.6 ELUCIDATION OF THE CORE ELEMENTS AND RECONSTITUTION OF THE ABA SIGNALLING PATHWAY

Together with the identification of the PYR/PYL/RCAR receptor family, the essential core elements of the pathway have also been elucidated with the PYR/PYL/RCARs at the apex of the cascade (Fujii et al., 2009; Park et al., 2009). ABA signalling in plants has always been considered to be very complicated, due to the numerous other proteins involved (Hirayama and Shinozaki, 2007). However, recently, the pathway has been simplified by documenting that the ABA response can be mediated by the "so called" core pathway: PYR/PYL/RCAR-PP2Cs-SnRK2s (Fujii et al., 2009).

This simplified signalling model by which PYR/PYL/RCAR-PP2Cs would modulate the response to ABA through the regulation of SnRK2 kinase activity, came through several observations. One of these observations was the impaired ABA-induced activation of SnRK2 kinases in the *pyr1pyl1pyl2pyl4* quadruple mutant (Park et al., 2009). Similarly, transfection of *snrk2.2/2.3/2.6* protoplasts with the transcription factor ABF2 did not induce the expression of the ABA-responsive gene, RD29B (RESPONSIVE TO DESSICATION 29B), in the presence of ABA; thus, suggesting a disruption in the signal transduction pathway (Fujii et al., 2009).

In addition, biochemical and structural studies have revealed how ABA binds the PYR/PYL/RCAR receptors, and how this binding leads to the inhibition of the negative regulators, the PP2Cs (Ma et al., Melcher et al., 2009; Miyazono et al., 2009; Park et al., 2009; Santiago et al., 2009A; Santiago et al., 2009B; Yin et al., 2009; Dupeux et al., 2011).

The participation of SnRK2s in the ABA signalling pathway has been well established (Mustilli et al., 2002; Fujii et al., 2007). Out from the 10 SnRK2s encoded by the *Arabidopsis* genome, SnRK2.6/OST1 and its two closest relatives, SnRK2.2 and SnRK2.3, have been shown to be ABA-activated (Boudsocq et al., 2005). The centrality and relevance of these kinases in ABA signalling has been demonstrated by the characterization of triple loss-of-function mutants lacking the three kinases. These mutants have been shown to be strongly impaired in almost all ABA reponses (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009), suggesting that regulation of SnRK2 activity might be critical for the ABA signal to be transmitted to downstream effectors of the pathway. It was previously seen that a PP2C (ABI1) interacted with SnRK2.6/OST1 (Yoshida et al., 2006). However, it has been recently when PP2Cs have revealed as direct regulators of SnRK2s activity (Umezawa et al., 2009; Vlad et al., 2009). In the work of Vlad et al. (2009), a screening to determine HAB1 putative substrates involved in ABA signalling has demonstrated that the activation loop of the ABA-activated OST1 kinase is a direct target of HAB1, suggesting that HAB1 would regulate OST1 activity. This was corroborated by *in vitro* dephosphorylation assays. This prediction was also supported *in vivo*. Firstly, OST1 was shown to interact with PP2Cs, ABI1, ABI2 and HAB1 *in planta*. Secondly, analysis of OST1 activation in PP2C mutant plants revealed that ABA-activation of OST1 was dependent on PP2C activity in the plant. OST1 activity was strongly reduced in hab1^{G246D}, abi^{G180D} and abi2^{G168D} insensitive mutants. By contrast, the ABA-dependent activation of OST1 was significantly increased in PP2C knockout mutants. These evidences indicate that PP2Cs play a crucial role in the regulation of OST1 activity (Vlad et al., 2009). Vlad et al. (2009), also predicted that SnRK2.2 and SnRK2.3 would also be regulated by PP2Cs. In the work of Umezawa et al. (2009), they also performed a larger *in vivo* binding assay in which clade A PP2Cs revealed to be interactors of SnRK2.6/2.2/2.3, among others. Similarly, they have provided evidence supporting the model in which PP2Cs regulate SnRK2s activity by dephosphorylating them, leading to their deactivation (Umezawa et al., 2009; Vlad et al., 2009). This interaction occurs both in nucleus and cytosol (Umezawa et al., 2009; Vlad et al., 2009).

Experimental evidence indicates that ABA activation of the pathway leads to the accumulation of active and phosphorylated SnRK2s through inhibition of PP2Cs. Once activated, SnRK2s are ready to directly phosphorylate protein targets involved in ABA signalling, including the ABF/AREB transcription factors which bind to abscisic-acid responsive promoter elements (ABREs), leading to transcription of ABA-responsive genes . These b-ZIP transcription factors have been revealed to be direct substrates of

SnRK2s and activate by phosphorylation (Johnson et al., 2002; Furihata et al., 2006). Fujii et al. (2009) have also provided *in vivo* evidence of the ABF2 regulation by SnRK2s, and its dependence to elicit the expression of ABA-responsive genes. Transfection of *snrk2.2/2.3/2.6* protoplasts with the transcription factor ABF2 did not induce *RD29B-LUC* (luciferase reporter gene driven by the ABA-responsive RD29B promoter) expression, even in the presence of ABA. However, co-transfection of ABF2 and SnRK2.6 led to the induction of *RD29B-LUC* in an ABA-dependent manner. Similar results were also reported for SnRK2.2 and SnRK2.3 (Fujii et al., 2009).

The PYR/PYL/RCAR-PP2Cs-SnRK2s have been described to be the core components of the ABA response pathway, by being both necessary and sufficient for ABA perception, signalling and, eventually, ABA-responsive gene expression. *In vivo* experiments to reconstitute the ABA signalling pathway have also been perfomed using transient activation in protoplasts. Transfection of ABI1 together with SnRK2.6 and ABF2 led to inhibition of *RD29B-LUC* expression. However, addition of PYR1 receptor to the assay enabled the ABA-dependent induction of the *RD29B-LUC* reporter expression. Similar reconstitution experiments were performed using different combinations of SnRK2 kinases, PP2Cs and PYR/PYL/RCAR receptors. The results from these experiments have led to the conclusion that SnRK2s are inhibited by both ABI1 and HAB1 PP2Cs, and that PYR/PYL/RCAR can antagonize this inhibition by inactivating clade A PP2Cs in an ABA-dependent manner (Fujii et al., 2009). These reconstitution experiments have demonstrated that the core pathway provides the minimal set of proteins that goes from ABA perception to ABA-responsive gene expression.

Futhermore, recent studies have also reported that the core pathway also regulates non-transcriptional responses triggered by ABA. It has been recently demonstrated that OST1 is able to phosphorylate and activate the anion channel SLAC1 in Xenopus oocytes, and that this activation is under the control of the ABA-dependent PYR/PYL/RCAR-PP2C complex (Geiger et al., 2009; Lee et al., 2009). Similarly, OST1 has also been reported to deactivate the potassium channel KAT1 by dephosphorylation (Sato et al., 2009). All these evidences provide an interesting mechanism, involving the core pathway in the regulation of the ABA-mediated response in guard cells. This model mechanism is also consistent with the defect of the *pyr1pyl1pyl2pyl4* quadruple mutant in ABA-induced stomatal closure (Park et al., 2009), as well as with the enhanced drought resistance exhibited by the PYL5-overexpressing lines (Santiago et al., 2009A). Furthermore, the core pathway seems to bifurcate at the PP2Cs level to regulate both SnRK2s and CPKs. It has been reported

that activation by phosphorylation of the anion channels SLAC1 and SLAH3 is also mediated by the Ca^{2+} kinases CPK21 and CPK23, which are in turn regulated by the ABA-activated PYR/PYL/RCAR-PP2C complex (Geiger et al., 2010, 2011). These results suggest that the core pathway regulates both the ABA Ca^{2+}-dependent and Ca^{2+}-independent pathways.

Additionally, it has also been described that OST1 is able to phosphorylate the *Arabidopsis* NADPH-oxidase RbohF, also suggesting the implication of the core pathway in the ABA-mediated production of reactive oxygen species (ROS) (Sirichandra et al., 2009A). The two plasma membrane NADPH oxidases (RbohD and RbohF), had already been implicated in the ABA-induced stomatal closure (Kwak et al., 2003). Thus, these last findings provide a direct molecular connection between the core pathway and the role of ROS in ABA stomatal regulation.

An additional inhibition mechanism has also been suggested for the core element HAB1, on ABA signalling (Saez et al., 2008). Experimental evidence suggests that the phosphatase HAB1 would have a direct regulatory effect on ABA-mediated transcriptional regulation, through the interaction with a putative component of SWI/SNF (SWItch/SUCROSE NON FERMENTING) chromatin remodeling complexes, SWI3B. ChIP experiments have reveal that HAB1 is constitutively present in the vicinity of ABA-responsive *RAB18* and *RD29B* promoters. However, when ABA is applied, HAB1 is removed from these regions. In addition, *swi3b-3* mutants exhibit ABA insensitive phenotypes in germination, root growth and ABA-induced gene expression. Moreover, yeast-two-hybrid and *in planta* BiFC assays have shown that HAB1 and SWI3B interact. This, together with the impaired ABA up-regulation of *RAB18* and *RD29B* in *swi3b-3* mutants, suggests that HAB1 might inhibit the expression of some ABA-reponsive genes through a SWI/SNF chromating-remodeling component targeted to them. Since HAB1 and SWI3B have opposed roles in the ABA signalling pathway, it might be that HAB1 would negatively regulate SWI3B function, modulating its role as a positive regulator, and thus, the transcription activation of some ABA-responsive genes (Saez et al., 2008). The release of HAB1 from the promoter regions of ABA-inducible genes, might be mediated by the ABA-activated PYR/PYL/RCAR family. Thus, the core pathway would also be directly involved in the regulation of the ABA-induced transcriptional response. HAB1, and maybe other clade A PP2Cs, would then play a double inhibitory role in ABA signalling. On one hand, under basal conditions, they would regulate the transcriptional response by blocking the ABA signalling transduction through the inactivation of SnRK2s. Secondly, they would ensure ABA signalling blockage by directly inhibiting the transcription of ABA-responsive genes.

1.5.7 SECOND MESSENGERS IN ABA SIGNALLING

A number of second messengers has also been involved in ABA signalling, including Ca^{2+}, reactive oxygen species (ROS), cADP-ribose (c-ADPR), nitric oxide (NO), phosphatidic acid (PA), phosphatidyl-inositol-3-phosphate (PI3P), inositol-3-phosphate (InsP3), myo-inositol hexakisphosphate (InsP6), and sphingosine-1-phosphate (Leckie et al., 1998; Ng et al., 2001; Coursol et al., 2003; Schoeder et al., 2001; Siegel et al., 2009; Hong et al., 2010; Hubbard et al., 2010; Kim et al., 2010; Lozano-Juste and León, 2010A) (Figure 6). Here, we are not going to extensively develop the subject but briefly summarize their relevance and role in the pathway. Further information on these small molecules can be found in the bibliography cited.

Calcium (Ca^{2+}) and Reactive oxygen species (ROS)

The role of calcium and ROS as second messengers in ABA signalling has been well established (Cho et al., 2009; Kim et al., 2010). For instance, Ca^{2+} has revealed to be critical on ABA-induced stomatal regulation. Quantification in *Arabidopsis* of the relative importance of $[Ca^{2+}]_{cyt}$-elevation-dependent and -independent signalling in ABA-induced stomatal regulation, has revealed that $[Ca^{2+}]_{cyt}$-dependent mechanisms are responsible for ~70% of the response (Siegel et al., 2009). In addition, a number of Ca^{2+}-associated proteins have already been described to be implicated in ABA responses (Choi et al., 2005; Geiger et al., 2010, 2011). However, the overall understanding of the molecular components underlying this network does require further investigation.

ABA increases ROS levels (Pei et al., 2000; Mustilli et al., 2002) and their generation has also been reported to be critical in ABA-induced stomatal response (Kwak et al., 2003). Moreover, ROS seem to act downstream the core elements SnRK2s, since ABA-induced increases in ROS are abolished in the *ost1-2* mutant (Mustilli et al., 2002).

Recent findings have provided a possible mechanistic of how ABA might promote this Ca^{2+} sensitivity, also connecting both the Ca^{2+}-dependent and –independent pathways in the control of stomata closure (Geiger et al., 2009, 2011; Sirichandra et al., 2009A). It has been known for some time that ABA induces $[Ca^{2+}]_{cyt}$ increases in guard cells, and that it is mediated by Ca^{2+} influx through Ca^{2+}-permeable channels (I_{Ca}) and Ca^{2+} releases from internal stores (MacRobbie et al., 2000; Pei et al., 2000). Moreover, these I_{Ca} channels have been reported to be stimulated by reactive oxygen species (ROS) (Pei et al., 2000). Recently, the ABA-activated

SnRK2.6/OST1 kinase has been described to directly activate by phosphorylation the NADPH oxidase RbohF, inducing this way the production of ROS in the cell (Sirichandra et al., 2009A). ROS also presents a positive feedback since they have been reported to block the negative regulator of the pathway, the ABI1 clade A PP2C (Meinhard and Grill, 2001), allowing the activation of the SnRK2. These evidences connect the ABA Ca^{2+}-independent pathway with the stimulation of Ca^{2+} influxes in the cell. In addition, ABA not only induces an increment in the $[Ca^{2+}]_{cyt}$ concentration, but also enhances the sensitivity of Ca^{2+}-dependent processes (Young et al., 2006). For instance, experimental data suggest that $[Ca^{2+}]_{cyt}$-dependent activation of S-type anion channels and down-regulation of K^{+}_{in} channels are induced by pre-exposure to ABA (Siegel et al., 2009).

Additionally, a good number of Ca^{2+}-dependent kinases, SnRK3s and CPKs, have been implicated in ABA signalling (1.5.2 of the Introduction). Some of these kinases have been involved in the regulation of ABA-stomatal closure (Mori et al., 2006; Geiger et al., 2010; Zou et al., 2010), as well as in the regulation ABA-responsive transcription factors (Zhu et al., 2007), which suggests that Ca^{2+} also plays a role in ABA-mediated gene expression.

Cyclic ADP-ribose (cADPR)

Cyclic ADP-ribose has also been identified as a signalling molecule involved in ABA signal transduction (Wu et al., 1997; Leckie et al., 1998; Meimoun et al., 2009). cADP-ribose has been involved in the mobilization of intracellular Ca^{2+} stores, contributing to increase the $[Ca^{2+}]_{cyt}$ and thus promoting stomatal closure (Leckie et al., 1998; Meimoun et al., 2009). Experimental data have shown that addition of cADPR to guard cell vacuoles, leads to the activation of Ca^{2+} permeable currents, resulting in the release of Ca^{2+} to the cytosol (Leckie et al., 1998).

Phosphatidic acid (PA)

Abcisic acid induces the production of PA through the activation of Phospholipase Dα1 (PLDα1) (Zhang et al., 2004). Phosphatidic acid has been described to play a positive role in ABA signalling. PA prevents water loss, both by promoting stomatal closure and inhibiting stomatal opening (Mishra et al., 2006). Phosphatidic acid promotes stomatal closure by regulating ABI1 function, inhibiting its phosphatase activity and sequestering it to the plasma membrane (Mishra et al., 2006). This way, PA contributes to the inactivation of a negative regulator of the pathway, promoting ABA signalling. Moreover, PA also regulates NADPH oxidase activity and

the production of ROS in ABA-mediated stomatal closure. PA also induces the production of reactive oxygen species, which also act as positive regulators promoting stomatal closure (Zhang et al., 2009).

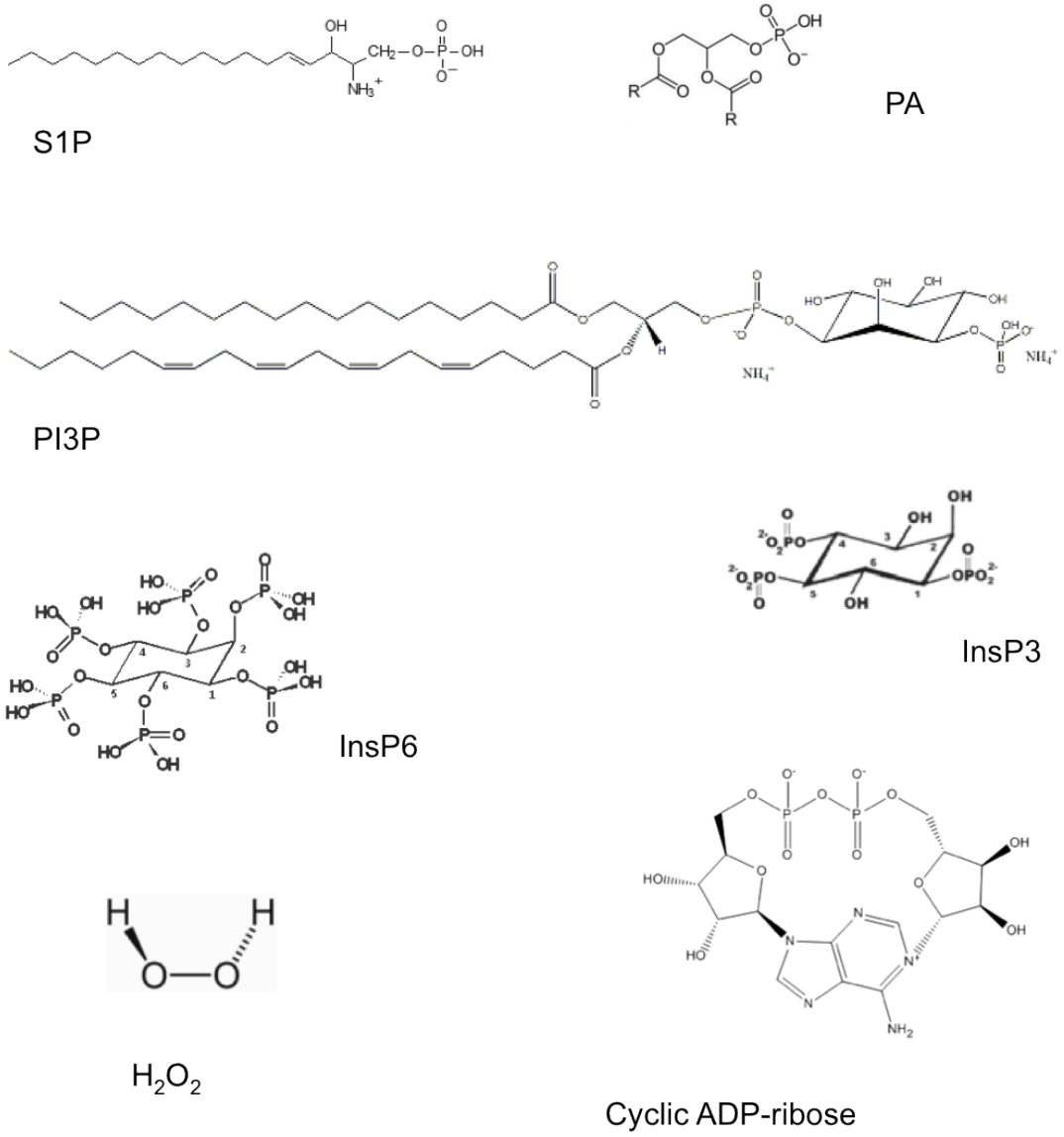

Figure 6. Second messengers involved in ABA signalling. S1P -sphingosine 1-phosphate, PA-phosphatidic acid, PI3P -phosphatidylinositol 3-phosphate, InsP3 -Inositol 1,4,5-trisphosphate, Ins6-intositol hexakisphosphate , H_2O_2 and cyclic ADP-ribose.

Phosphatidyl-inositol-3-phosphate (PI3P), Inositol-3-phosphate (InsP3), myo-inositol hexakisphosphate (InsP6) and sphingosine-1phosphate (S1P)

All these secondary messengers have been reported to have a positive role in ABA signal transduction in guard cells. They have been implicated in the promotion of

stomatal closure, either through the stimulation of ROS production or influx of Ca^{2+} into the cytosol. For instance, PI3P, a product of phosphatidylinositol 3-kinase (PI3K), is involved in the activation of NADPH oxidases and stimulation of ROS production (Park et al., 2003). Similarly, ABA promotes the release of caged InsP3, which in turn initiates influx of Ca^{2+} into the cytosol, leading to inhibition of K^{+}_{in} channels and stomatal closure. ABA has also been reported to stimulate the production of InsP6, which seems to also inhibit K^{+}_{in} channels in a Ca^{2+}-dependent manner (Gilroy et al., 1990; Lemtiri-Chlieh et al., 2000; reviewed in Shroeder et al., 2001). Moreover, sphingosine-1-phosphate has been reported as a calcium-mobilizing molecule in plants also involved in ABA stomatal closure (Ng et al., 2001; Coursol et al., 2003).

Nitric oxide (NO)

The small molecule NO has also been implicated in ABA signalling. ABA seems to induce accumulation of NO (Desikan et al., 2002; Lozano-Juste and León, 2010A). Recent genetic evidence of a NO-deficient triple mutant *nia1nia2noa1-2*, has shown that NO plays a negative role in ABA signalling. The triple mutant has revealed to be hypersensitive to ABA in germination, seed establishment, ABA-responsive gene expression and ABA-induced stomatal closure (Lozano-Juste and León, 2010A). These genetic results support previous studies, in which NO was proposed to decrease the sensitivity of seeds to ABA (Bethke et al., 2006). In contrast, previous works had also proposed NO production as essential for ABA-mediated regulation of stomata closure (Desikan et al., 2002). In the work of Desikan et al. (2002) the double mutant *nia1nia2* was insensitive to ABA, and therefore unable to close their stomata under ABA treatment. This insensitivity was correlated with the inability of the double mutant to generate NO, qualifying NO as an essential positive regulator of the ABA-mediated response in stomatal closure. Thus, these results are in contrast with the phenotypes exhibited by the triple NO-deficient mutant, in which lack of NO does not prevent from ABA-dependent stomatal closure (Lozano-Juste and León, 2010A). The molecular mechanism through which NO affects ABA signalling has not yet been elucidated, but it seems that it might be Ca^{2+} independent. Since NO might play a negative role in ABA signalling, one hypothesis could be that it might be regulating the sensitivity to ABA by acting on positive regulators of the pathway such as the PYR/PYL/RCAR receptors or the SnRK2s (Lozano-Juste and León, 2010B).

1.5.8 ARCHITECTURE AND FUNCTION OF THE PYR/PYL/RCAR RECEPTORS

The structure resolution of the ABA-bound PYR/PYL/RCAR proteins has been definitive to corroborate their role as ABA receptors, as well as crucial for understanding the molecular mechanisms underlying hormone perception and signal transduction (reviewed in Appendix 1: Santiago et al., 2011). Up to date, five ABA receptors have been crystallized, PYR1, PYL1, PYL2, PYL3 and PYL10 (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009; Hao et al., 2011; to be published: Zhang et al., 2011) (see Table 2). The structure consists of a central seven-stranded β-sheet flanked by two α-helixes. This bent β-sheet presents a curved disposition, and together with the long carboxy-terminal α-helix (α5), produces a central cavity where the hormone accommodates. This cavity is closed in its bottom side by two small α-helixes (α3-α4), while the upper part is surrounded by two flexible loops (β3-β4 and β5-β6). In Results Chapter 2 of this work, the structure resolution of PYR1 further describes the structural arrangement. Three of these receptors have been described as homodimers, PYR1, PYL1 and PYL2 (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). The dimer interface involves amino acids in loops α4-β2, β3-β4, β5-β6 and the N-terminal part of the α5 helix (further described in the General discussion).

Crystal structures of PYR1, PYL1 and PYL2 have been obtained both in the ABA-bound form and in the apo-form (ABA free) (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). In the particular case of PYR1, there was one ABA-bound and one ABA-free subunit in the crystallographic asymmetric unit (Nishimura et al., 2009; Results Chapter 2 of this work). Analysis of the ABA-bound forms has revealed the nature of the interactions that coordinate ABA into the receptor binding pocket. ABA happens to be almost completely buried inside the cavity, isolated from solvent. The interactions established by wall residues of this cavity, perfectly match the polar and hydrophobic character of the different functional groups of the hormone. The cyclohexene ring and the isoprene moiety establish hydrophobic interactions with apolar side chains, while the carboxylic, hydroxyl and ketone groups are coordinated by polar interactions. Most of the polar interactions between the protein and ABA are mediated by water molecules rather than by direct contacts (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B). Notably, the residues that directly recognize and coordinate ABA inside the pocket are highly conserved among the PYR/PYL/RCAR members (Santiago et al., 2009B; Yin et al., 2009). Recognition and coordination of the ABA

molecule by key residues of PYR1 pocket, is further described in the General discussion.

Table 2. Summary of the PDB files of structural data on ABA-receptors of the PYR/PYL/RCAR family

Authors	Apo-structures	ABA-PYR/PYL/RCAR bound structures	PYR/PYL/RCAR-PP2C complex structures
Nature. Melcher et al.,2009	apoPYL1 **3KAY** apoPYL2 **3KAZ**	ABA-bound PYL2 **3KB0**	PYL2-ABA-HAB1 **3KB3**
Nature. Mizayono et al.,2009		ABA-bound PYL1 **3JRS**	PYL1-ABA-ABI1 **3JRQ**
Nat. Struct. & Mol. Bio. Yin et al.,2009	apoPYL2 **3KDH**	ABA-bound PYL2 **3KDI**	PYL1-ABA-ABI1 **3KDJ**
Science. Nishimura et al.,2009	PYR1 (subunit B) **3K3K**	PYR1 (subunit A) **3K3K**	
Nature. Santiago et al.,2009	PYR1(subunit B) **3K90**	PYR1(subunit A) **3K90**	
Plant Physiology Dupeux et al., 2011			PYR1-ABA-HAB1 **3QN1**
Mol. Cell Hao et al., 2011	apoPYL10 **3RT2**		PYL10-HAB1 **no ABA** **3RT0**
To be published Zhang et al., 2011	apoPYL2 **3KL1** apoPYL3 **3KLX**		

Comparison of both ABA-bound and unbound structures, has made it possible to reveal the conformational changes induced upon binding of the hormone (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et

al., 2009). This comparison has revealed notable differences in the two loops surrounding the upper part of the cavity (β3-β4 and β5-β6 loops) and the N-terminal part of the α5 helix (β7-α5 loop). Particularly, the β3-β4 (S_{85}GLPA$_{89}$ in PYR1) and β5-β6 (H_{115}RL$_{117}$ in PYR1) loops adopt a complete different conformation upon ABA binding. In the free-ABA form these loops present an open conformation, creating a passage into the cavity. However, upon binding of the hormone, they close up over the entry, similar to a lid, stabilizing the ligand inside the cavity and isolating it from solvent. Hence, these loops have been designated as the "gating loops".

Moreover, the region comprising the β7 and the N-terminal part of the α5 (M_{147}PEGNSEDDTRM$_{158}$ in PYR1) also participates in the stabilization of this closed conformation, by bending over upon binding of the hormone. In addition to trapping the ligand inside the cavity, these conformational changes are crucial to generate a favourable interaction surface for the binding of the PP2C. For instance, mutations in any of the PYR1 loops described above, severely reduce or abolish the capacity of the receptor to interact with the PP2C (Results Chapter 2 and Appendix Results Chapter 2 of this work). Similar conclusions have been obtained for PYL1 and PYL2 (Melcher et al., 2009; Miyazono et al., 2009). Another important change in the loops conformation is the flipping movement of a conserved Ser (Ser85 in PYR1, equivalent to Ser89 in PYL2 and Ser112 in PYL1), which gets exposed to solvent in these PYR/PYL/RCAR proteins upon ABA binding, and that plays a critical role in the interaction with the active site of the PP2C (described below) (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Results Chapter 2 of this work).

Besides the structural data, multi-angle laser light scattering (MALLs) and small-angle X-ray scattering (SAXS) experimental analyses also indicate that these receptors exist as dimers in solution. However, the receptor-PP2C complexes present 1:1 stoichiometry (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011). This implies that receptor dimers require dissociation before interacting with the phosphatase (Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). When both the dimerization surface and the PP2C-binding interface are compared, it can be appreciated that both surfaces largely overlap, suggesting that homodimerization is blocking the interaction with the PP2C (Results Chapter 3 of this work). In the work of Yin et al. (2009), the PYL2 dimerization surface is analyzed upon binding of the hormone. They observe that ABA binding induces a change in the relative orientation of one of the PYL2 subunits with respect to the other. This change

in orientation of the two PYL2 protomers, leads to a decrease of van de Waals contacts and hydrogen bonds, resulting in the weakening of the dimer interface. These results suggest that ABA may be responsible of dimer dissociation. In line with this analysis, experiments in solution presented in Results Chapter 3 of this work, demonstrate that ABA is both necessary and sufficient for PYR1 dimer dissociation. MALLS analysis, in the absence of ABA, shows that PYR1 apparent molecular mass corresponds to that of the dimer (39 kDa). However, when the same experiment is performed in the presence of the hormone the apparent molecular mass shifts to 21 kDa, which coincides with that of the PYR1 monomer. To corroborate these results, the same analysis is performed with a mutant defective in ABA binding, which does not dissociate in the presence of ABA (Results Chapter 3 of this work).

Biochemical characterization by MALLS and ultra centrifugation (AUC) of the PYR/PYL/RCAR family, has revealed that they can be separated into two distinct sub-classes: one corresponding to dimeric receptors, to which PYR1, PYL1 and PYL2 belong, and another of monomeric receptors, where it has been included PYL4, PYL5, PYL6, PYL8, PYL9 and PYL10 (Results Chapter 3 of this work; Hao et al., 2011). The different oligomeric state confers them a different ABA sensitivity. Calorimetry and Nuclear magnetic resonance (NMR) assays have shown that dimer receptors have lower ABA affinities and positive ABA enthalpies, whereas the monomers show Kds in the range of 1 uM and negative enthalpies (Ma et al., 2009; Miyazono et al., 2009; Santiago et al., 2009A; Szostkiewicz et al., 2009). The existence of two types of PYR/PYL/RCAR receptors with different ABA affinities, suggests a mechanism that might be able to modulate the dose-response curve to ABA in the plant (Results Chapter 3 of this work).

Despite the degree of similarity presented by these PYR/PYL/RCAR proteins (Appendix 1), recent structural studies (see Table 3), using the ABA seed-analog pyrabactin, have revealed subtle differences among the receptor binding pockets that result in important functional consequences. Pyrabactin is able to activate PYR/PYL/RCAR receptors in a selective manner, acting as an ABA agonist with PYR1 and PYL1, and as an antagonist in PYL2 (Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010). This selective activation mechanism is further described in the General discussion.

Table 3. Summary of the PDB files of structural data on ABA-receptors of the PYR/PYL/RCAR family bound to agonist/antagonist

Authors	Agonist/antagonist bound structures	Agonist/antagonist -PYR/PYL/RCAR-PP2C complex structures
JBC Hao et al., 2010	Pyrabactin-bound PYL1 **3NEG** HR pyrabactin-bound PYL1 **3NEF**	
JBC Yuan et al., 2010	Pyrabactin-bound PYL2 **3NR4** HR pyrabactin-bound PYL2 **3NS2**	
Nat. Struct. & Mol. Biol. Peterson et al., 2010	Pyrabactin-bound PYR1(P88S) **3NJO** Pyrabactin-bound PYL2(V114I) **3NJ1** Pyrabactin-bound PYL2 **3NJ0**	
Nat. Struct. & Mol. Biol. Melcher et al., 2010	Pyrabactin-bound PYL2(A93F) **3NMP** Pyrabactin-bound PYL2 **3NMH**	PYL2 (A93F)-Pyrabactin-ABI2 **3NMV** PYL2(A93F)-Pyrabactin-HAB1 **3NMT** PYL1-Pyrabactin-ABI1 **3NMN**

1.5.9 ARCHITECTURE OF TERNARY COMPLEXES: INSIGHTS OF THE ABA-INDUCED INHIBITION MECHANISM OF PP2Cs

The structure resolution of the ternary complex PYR/PYL/RCAR-ABA-PP2C, has made it possible to elucidate the molecular mechanism underlying the ABA-induced inhibition of PP2Cs by these receptors. Up to now, three ternary complexes and one ABA-free receptor-phosphatase complex have been reported, i.e. PYL1-ABA-ABI1, PYL2-ABA-HAB1, PYR1-ABA-HAB1 and PYL10-HAB1 (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011; Hao et al., 2011). In these crystallizations studies, it has only been used the catalytic core of the PP2Cs (ABI: residues 125-429; HAB1: residues 172-511). So the N-terminal part still remains to be elucidated, which might also be involved in the interaction with other partners. The

interaction of the ABA-bound receptor with the PP2C is established through the gating loops in their closed conformation and the N-terminal part of the α5 helix. On the other hand, PP2C contacts the ABA receptor through two key docking points, its active site and a small protruding region, named the flap sub-domain (Schlicker et al., 2008), which contains a critical Trp residue. This Trp residue inserts between the receptor's gating loops, with the N in the indole group establishing a water-mediated hydrogen bond with the ketone group of ABA, and two conserved residues located in the gating loops, a Pro and an Arg (Figure 7A).

In addition to this interaction, the β7-α5 loop of the receptor experiences a conformational rearrangement moving towards the gating loops and the PP2C flap domain. This results in new interactions that help both to stabilize the receptor closed conformation and the receptor-phosphatase complex (Melcher et al., 2009; Miyazono et al., 2009; Dupeux et al., 2011). This could provide a molecular explanation for the increase in ABA affinity of the receptor-phosphatase complex as compared to the receptor alone (Ma et al., 2009; Santiago et al., 2009A). The interaction through this Trp residue has been reported to be crucial in the formation of the ternary complex and in the control of the PP2C phosphatase activity. Mutants in that Trp have revealed to be insensitive to inhibition by ABA-bound PYR/PYL/RCAR receptors (Miyazono et al., 2009; Dupeux et al., 2011). In support to these results, engineering of hab1^{W385A} dominant allele has led to strong ABA insensitive phenotypes in germination, seedling establishment, water-loss and expression of ABA-responsive genes (Dupeux et al., 2011). Modelling of the Trp385Ala mutation in HAB1, illustrates the disruption of the interaction between the Trp residue and the ABA molecule, leading to the loss of the locking point (Figure 7B). Interestingly, this Trp lock seems to be part of a plant specific recognition mechanism involving clade A PP2Cs. Eight out of the nine clade A PP2Cs contain the Trp residue, which is absent in other plant PP2Cs and also in the human PP2C (Figure 7C).

Besides the contact with the conserved Trp residue, the loop β3-β4 of the PYR/PYL/RCAR proteins inserts into the phosphatase catalytic site blocking the access of potential substrates to the active site (Melcher et al., 2009; Miyazono et al., 2009; Dupeux et al., 2011). The conserved Ser in this loop (Ser85 in PYR1, Ser89 in PYL2 and Ser112 in PYL1), flipped in position upon ABA binding, establishes hydrogen bonds with two residues of the PP2C catalytic site, a conserved Gly (Gly246 in HAB1 and Gly180 in ABI1) and a metal-coordinated Glu residue (Glu203 in HAB1 and Glu142 in ABI1). This explains the inhibitory action of the receptor-phosphatase complex on the PP2C activity. The structural information suggests a competitive inhibition

mechanism. Unfortunately, the structure of a PP2C in complex with a natural substrate i.e. the positive regulators SnRK2s, has not been elucidated to resolve this issue. However, some experimental evidence also supports the competitive nature of the inhibitory mechanism. In the work of Melcher et al. (2009), they show how the inhibition of HAB1 by ABA-bound PYL2 can be overcome by increasing concentrations of an OST1 phosphopeptide containing residues of the kinase activation loop. In line with these results, Fujii et al. (2009) also report the disruption of the interaction between PP2Cs and SnRK2s by the PYR/PYL/RCAR proteins in the presence of ABA, performing yeast triple-hybrid assays (Fujii et al., 2009). Moreover, structural analysis has suggested that the conserved Ser in the β3-β4 loop might mimic the phosphorylated serine residues critical for SnRK2s activity (Ser175 in OST1) (Dupeux et al., 2011). Comparison of the complex structure PYR1-ABA-HAB1 with the catalytic domain of the human PP2C has revealed, that the Ser85 of the receptor is very close to the position expected to be occupied by the phosphoryl group of the substrate of the phosphatase reaction (Das et al., 1996; Dupeux et al., 2011). This observation lends weight to the interpretation that the formation of the receptor-phosphatase complex blocks the access of natural PP2C substrates to the active site, supporting the competitive nature of the inhibition mechanism. However, these results contrast with those reported by Ma et al. (2009), in which they conclude that inhibition of ABI2 by RCAR1/PYL9, using a non-peptidic PP2C substrate (methyl-umbelliferyl-phosphate), is independent of substrate concentration. Thus, suggesting a non-competitive inactivation mechanism.

Monomeric PYR/PYL/RCAR receptors have been reported to interact in a constitutive manner with PP2Cs, in yeast two hybrid assays and PP2C co-purification assays *in planta* (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A; Nishimura et al., 2010). Even though, under such experimental conditions it is difficult to rule out the presence of small amounts of ABA, biochemical data provided in Results Chapter 3 of this work show that some monomeric receptors are able to establish less stable complexes with the PP2C. In the case of the monomeric receptors, the PP2C interaction surface is not occluded as it happens in the dimeric receptors. Moreover, the gating loops may likely be flexible in solution and thus able to adopt a compatible conformation to be recognized by PP2Cs, even in the absence of the hormone. This might explain the formation of weak interactions between some monomeric receptors and PP2Cs in the absence of ABA. Recently, the structure resolution of the apo complex PYL10-HAB1 has revealed the determinant factor for a monomeric receptor to establish a stable and inhibitory complex with PP2Cs in the absence of ABA.

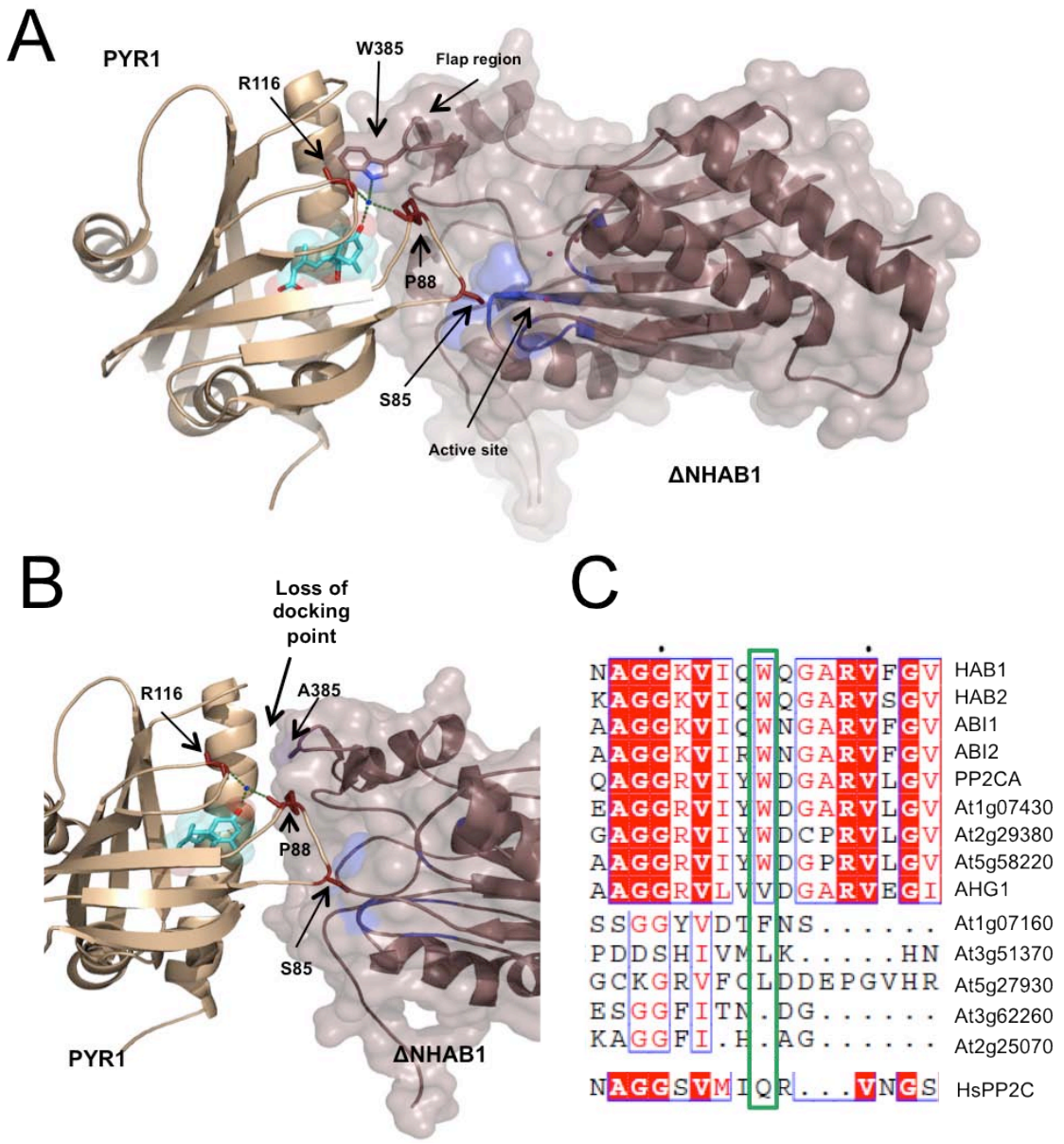

Figure 7. Structural details of the PYR/PYL/RCAR-ABA-PP2C complex. A, Overview of contact points between PYR1 and the catalytic core of HAB1. The PP2C contacts the receptor through its active site and the flap region containing the Trp385 residue. Detail of the interactions involving this residue, the PYR1 gating loops, containing Pro88 and Arg116 residues, and the ketone group of ABA. The contacts are coordinated through a water molecule (in blue) located at the narrow channel between the loops. Mn^{2+} ions are marked as pink dots. **B,** Modelling of a Trp385Ala mutation in HAB1. This mutation leads to loss of the docking point provided by the interaction of the Trp residue with the ABA molecule. **C,** The tryptophan lock is part of a plant specific recognition mechanism in clade A PP2Cs. Eight of the nine clade A PP2Cs contain the Trp (W) residue which is absent in other plant PP2Cs and also in human PP2C.

This critical factor is the presence of bulky and hydrophobic residues in the gating loops, so that they can establish enough hydrophobic contacts to stabilize the closed conformation, required for PP2C inhibition (Hao et al., 2011). Apparently, these

hydrophobic residues are able to mimic the role of ABA when enclosed in the pocket. The ligand-binding pocket of PYR/PYL/RCAR receptors is mainly hydrophilic and filled with water molecules. However, the gating loops residues that end up facing the cavity upon binding of the hormone are mostly hydrophobic. This polarity problem can be solved by the recognition of an amphipathic molecule, such as ABA, which may be able to neutralize the polarity of the binding pocket and attract the hydrophobic side of the gating loops, stabilizing the closed conformation. In the particular case of PYL10, the closed conformation of the β3-β4 loop can be induced in the absence of ABA by hydrophobic interactions between the ligand-free pocket and the β3-β4 loop. Hao et al. (2011) present Leu79 as a candidate to coordinate this interaction network. Interestingly, in all the other PYR/PYR/RCAR proteins, this position is occupied by a Val residue. This Leu79 in PYL10 is located at the end of the β3, and its side chain establishes van der Waals contacts with residues in the loop β3-β4 such as Leu83 (Leu87 in PYR1), Ala85 (Ala89) and Ile59 (Ile62 in PYR1) in α4-β2 loop. All these residues are involved in the binding of the hormone to stabilize the closed conformation (Results Chapter 2 of this work). In addition to this network, Leu159 (Val 163 in PYR1) located in the α5 helix, also contacts Leu83 (Leu87 in PYR1). The network of interactions formed by these hydrophobic residues effectively docks the PYL10 β3-β4 loop in a closed conformation (Hao et al., 2011). Notably, PYR/PYL/RCAR receptors described as monomeric contain either Leu or Ile at the position corresponding to Leu159 in PYL10. However, the main difference between PYL10 and the rest of the monomeric receptors lies in the residue Leu79, which happens to be Val in the rest of the proteins. This Val is able to establish van de Waals contacts with the conserved Leu and Ala residues from the β3-β4 loop, involved in the binding with ABA. However, these connections are not enough to induce the closed conformation, still requiring an amphipathic ligand. This could be the explanation why other monomeric PYR/PYL/RCAR proteins are not able to establish stable receptor-PP2C complexes. In addition, activity assays performed by Hao et al. (2011) are in line with the structural analysis. For instance, other monomeric receptors such as PYL5, PYL6 or PYL8, require stoichiometries of 1:10 to achieve 60%, 55% and 50% of HAB1 inhibition in the absence of ABA, respectively; while PYL10 is able to achieve the same level of inhibition with 1:1 stoichiometries. Similarly, in the case of ABI1, PYL10 is able to achieve 90% of inhibition with 1:1 stoichiometries, while the other monomeric receptors require 1:100 stoichiometries. The existence of two sub-classes of PYR/PYL/RCAR receptors with distinct properties suggests that they might have specialized functions

and contribute differentially to ABA signalling. Since monomeric receptors have higher ABA affinities, they would efficiently compete with dimeric receptors for low endogenous ABA levels. That might be necessary for the regulation of certain developmental processes related to plant survival and growth (Results Chaper 3 of this work). Indeed, both ABA-deficient and ABA-insensitive mutants exhibit strong impaired phenotypes in growth and reproduction (Barrero et al., 2005; Fujii and Zhu, 2009). Further analysis on PYL10 would be required to have more information about its ABA-independent functions. So far, current microarrays databases do not show PYL10 expression, raising some questions about its physiological relevance. On the other hand, if PYL10 inhibits clade A PP2Cs such as ABI1, HAB1 and PP2CA, in the absence of ABA, it could generate a constitutive plant response, which might result deleterious for the plant (Rubio et al., 2009). For the rest of the monomeric receptors, it would be important to investigate whether the inhibition stoichiometries presented by Hao et al. (2011) are in the physiological range.

1.5.9.1 A MOLECULAR EXPLANATION FOR *abi1*^{G180D}, *abi2*^{G168D}, *hab1*^{G246D} MUTANTS

The recent identification of the PYR/PYL/RCAR-ABA-PP2C signalling complex, together with the elucidation of its crystal structure (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2010), has provided a molecular explanation for the dominantly insensitive phenotypes of *abi1-1D*, *abi2-1D* and *hab1*^{G246D} mutants (Koornneef et al., 1984; Robert et al., 2006). These dominant mutations happen to be a substitution of a Gly (corresponding to Gly180 in ABI1, Gly168 in ABI2 and Gly246 in HAB1) to Asp at the catalytic site of the PP2C (Leung et al., 1994; Leung et al., 1997; Rodriguez et al., 1998B). They present strong ABA insensitive phenotypes, both in seed and vegetative tissues (Leung et al., 1994; Meyer et al., 1994; Bertauche et al., 1996; Leung et al., 1997; Leube et al., 1998; Rodriguez et al., 1998B). The dominant character of these mutations has not made possible to obtain clear conclusions about their nature and effect *in vivo*. However, loss-of-function alleles of clade A PP2Cs have clearly revealed that they are negative regulators of the ABA signalling pathway (Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007).

Robert and co-workers generated 35S:hab1^{G246D} transgenic lines and they analyzed its effect on the function of HAB1 *in planta*. The 35S:hab1^{G246D} seeds presented strong insensitivity to ABA-mediated inhibition of germination compared to

wild type and 35S:HAB1 seeds. The 35S:hab1^{G246D} seeds were able to germinate at 10 µM and 100 µM ABA, which were completely inhibitory concentrations for wild type and 35S:HAB1 seeds. However, the 35S:HAB1 lines generated by Robert et al. (2006) did not exhibit any difference in sensitivity when compared to the wild type. These results were in contrast with the already overexpressing lines described in Saez et al. (2004), which presented a marked insensitivity to the hormone as compared to the wild type plants. Moreover, Robert et al. (2006) also examined the stomatal closing response, which is a key ABA-controlled process that preserves water under drought conditions. They analyzed the stomatal response under stress conditions using infrared thermography. 35S:hab1^{G246D} plants resulted to have lower leaf temperature than wild type plants, indicating that plants expressing *hab1^{G246D}* had a higher transpiration rate. Furthermore, when *35S:hab1^{G246D}* plants were transferred from water saturated air to a dryer environment, they rapidly exhibit a wilty phenotype. Altogether indicated, that plants expressing *hab1^{G246D}* were unable to close their stomata to prevent from water loss in response to ABA during stress conditions. Finally, expression of ABA-inducible genes was also reduced in *hab1^{G246D}* plants as compared to the wild type. The phenotypes exhibited by *35:hab1^{G246D}* resulted to be completely opposite to the ABA hypersensitive phenotypes caused by the disruption of the HAB1 gene (Leonhardt et al., 2004; Saez et al., 2004). Thus, Robert et al. (2006) proposed that G246D mutation was rather acting as dominant positive or hypermorphic mutation (Wilkie, 1994), which would mimic a more active version of HAB1 PP2C *in planta* (Robert et al., 2006).

However, these mutations present a reduced PP2C activity, which in principle would be in apparent contradiction with their proposed hypermorphic nature (Bertauche et al., 1996; Leube et al., 1998; Leung et al., 1997; Rodríguez et al., 1998; Robert et al., 2006; Yoshida et al., 2006; Vlad et al., 2009; Dupeux et al., 2011). For instance, G246D presents five times less phosphatase activity than HAB1 wild type using as a substrate a peptide mimicking the activation loop of the natural SnRK2 (Vlad et al., 2009). An explanation for the negative effect that these mutations exert over their phosphatase activity comes from the vicinity of this Gly (180 in ABI, 168 in ABI2 and 246 in HAB1) to the Mg^{2+} or Mn^{2+} coordination site in the catalytic core of the phosphatase. Thus, its mutation to a bulky residue like Asp would likely affect their Mg^{2+} or Mn^{2+} activation dependence (Leube et al., 1998). Structure resolution of the PP2Cs HAB1 and ABI1 from the ternary complex, have provided a spatial location of the active site residues, which indeed illustrates the proximity of this Gly to a Glu residue (Glu203 in HAB1) that plays an important role in coordinating the

dephosphorylation reaction through metal ions (Figure 3) (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011).

However, the hypermorphic nature of these phosphatase mutations comes from their inability to interact with the PYR/PYL/RCAR receptors in the presence of ABA (Park et al., 2009; Ma et al., 2009; Santiago et al., 2009A). This Gly to Asp mutation renders the phosphatase refractory to inhibition by PYR/PYL/RCAR proteins, being constitutively active and leading to blockage of ABA signalling. *In vitro* and *in vivo* reconstitution assays have demonstrated that hab1^{G246D} and abi1^{G180D} mutants are able to dephosphorylate SnRK2s even in the presence of ABA and PYR1 receptor. This indicates that these PP2C mutants behave as hypermorphic mutations compared to wild type PP2Cs, since they are able to constitutively inactivate SnRK2s (Fujii et al., 2009; Umewaza et al., 2009; Dupeux et al., 2011). The model by which these PP2C mutants escape from the ABA-mediated PYR/PYL/RCAR mechanism of inhibition is in agreement and explains their ABA-insensitive phenotype (Vlad et al., 2009). In addition, structure resolution of the ternary complex has provided a molecular explanation for this hypermorphic behavior. Structural studies have revealed that the PYR/PYL/RCAR receptors exert their inhibitory capacity by inserting their β3-β4 loop into the phosphatase active site. A conserved Ser from the loop establishes interactions with a Gly and a Glu residues from the catalytic site, thus blocking the access to natural substrates (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011). Mutation of the conserved Gly to an Asp residue, would likely result in disruption of these interactions, which would eventually lead to these phosphatase mutants to escape from the ABA-mediated PYR/PYL/RCAR inhibition (further addressed in the General discussion).

In Results Appendix Chapter 2 of this work, we provide additional support to the model above exposed by giving biochemical evidence of the capacity of hab1^{G246D} mutation to dephosphorylate OST1 in the presence of ABA and PYR1, and that this PP2C mutant is refractory to inhibition by different PYR/PYL proteins.

2. OBJECTIVES

2. OBJECTIVES

1. Characterization of interacting partners of clade A PP2Cs as a new family of ABA intracellular receptors.

2. Crystallization studies and structure resolution of the PYR1 ABA receptor in complex with the hormone. Structural analysis of the hormone perception and signal transduction molecular mechanism.

3. Mutational analysis on PYR1 receptor to study the biological relevance of critical residues in ABA binding and interaction with the PP2C. Biochemical characterization of the hab1^{G246D} mutant in its interaction with the PYR/PYL/RCAR family.

4. Identification and characterization of two subclasses of apo PYR/PYL/RCAR proteins with different oligomeric states. Correlation of their oligomeric state with their ABA affinities. Description of their distinct ABA activation mechanisms, and prediction of the role of this family in the modulation of the ABA response in the plant.

5. Study of the ABA-PYR/PYL/RCAR-PP2C signalling pathway in cultivated plants (*Oryza sativa*).

3. RESULTS: CHAPTER 1

Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs

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Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs

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SUMMARY

Abscisic acid (ABA) is a key phytohormone involved in adaption to environmental stress and regulation of plant development. Clade A protein phosphatases type 2C (PP2Cs), such as HAB1, are key negative regulators of ABA signaling in Arabidopsis. To obtain further insight into regulation of HAB1 function by ABA, we have screened for HAB1-interacting partners using a yeast two-hybrid approach. Three proteins were identified, PYL5, PYL6 and PYL8, which belong to a 14-member subfamily of the Bet v1-like superfamily. HAB1–PYL5 interaction was confirmed using BiFC and co-immunoprecipitation assays. *PYL5* over-expression led to a globally enhanced response to ABA, in contrast to the opposite phenotype reported for *HAB1*-over-expressing plants. F_2 plants that over-expressed both *HAB1* and *PYL5* showed an enhanced response to ABA, indicating that *PYL5* antagonizes *HAB1* function. PYL5 and other members of its protein family inhibited HAB1, ABI1 and ABI2 phosphatase activity in an ABA-dependent manner. Isothermal titration calorimetry revealed saturable binding of (+)ABA to PYL5, with K_d values of 1.1 μM or 38 nM in the absence or presence of the PP2C catalytic core of HAB1, respectively. Our work indicates that PYL5 is a cytosolic and nuclear ABA receptor that activates ABA signaling through direct inhibition of clade A PP2Cs. Moreover, we show that enhanced resistance to drought can be obtained through PYL5-mediated inhibition of clade A PP2Cs.

Keywords: phytohormone, abscisic acid, signaling, phosphatase 2C, receptor.

INTRODUCTION

The phytohormone abscisic acid (ABA) plays a central role in coordinating the various aspects of the plant response to water stress, as well as in regulation of plant growth and development (Verslues *et al.*, 2006). Both negative and positive regulators of ABA signaling have been identified (Finkelstein *et al.*, 2002; Hirayama and Shinozaki, 2007; Verslues and Zhu, 2007), but there is confusion concerning ABA receptors. Thus, FCA, ABAR/CHLH/GUN5 and GCR2 were initially reported as ABA receptors localized in the nucleus, chloroplast and plasma membrane, respectively (Razem *et al.*, 2006; Shen *et al.*, 2006; Liu *et al.*, 2007). Regrettably, the paper by Razem *et al.* (2006) has been recently retracted (Razem *et al.*, 2008), and several results from the paper by Liu *et al.* (2007) have been questioned (Gao *et al.*, 2007; Johnston *et al.*, 2007; Illingworth *et al.*, 2008), including the ability of GCR2 to bind ABA (Risk *et al.*, 2008, 2009). Arabidopsis mutants of ABAR/CHLH/GUN5 have global impairment of ABA responses (Shen *et al.*, 2006), but no connection with the previously identified regulators of ABA signaling has yet been reported. Intriguingly, the barley Mg-chelatase 150 kDa subunit, which shows 82% identical residues to the Arabidopsis chelatase H subunit, is not an ABA receptor (Müller and Hansson, 2009). A recent report has shown that two G protein-coupled receptors (GPCRs), GTG1 and GTG2, are membrane-localized ABA receptors (Pandey *et al.*, 2009). Finally, regulators of protein phosphatases type 2C (PP2Cs) that function as ABA sensors have been described very recently (Ma *et al.*, 2009; Park *et al.*, 2009).

ABA signaling involves protein phosphorylation/dephosphorylation events, and clade A PP2Cs are one of the best studied components of the pathway, in which they act as negative regulators (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia *et al.*, 2003; Leonhardt *et al.*, 2004; Saez *et al.*, 2004, 2006; Kuhn *et al.*, 2006; Yoshida *et al.*, 2006a; Nishimura *et al.*, 2007). The existence of at least six clade A PP2Cs that act as negative regulators of ABA signaling provides an indication of the complexity of the PP2C-based regulatory system. Accordingly, a high number of protein kinases that act as positive regulators of the ABA pathway have been identified, some of which interact with certain clade A PP2Cs (Guo *et al.*, 2002; Yoshida *et al.*, 2006b). The identification of the dominant gain-of-function mutations *abi1-1D* and *abi2-1D* as mutant versions of PP2Cs that show a strong ABA-insensitive phenotype indicated the importance of these components in ABA signaling (Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994; Rodriguez *et al.*, 1998a). Further studies have shown that strong hypomorphic or null mutants of clade A PP2Cs have ABA-hypersensitive phenotypes, e.g. *abi1-1R1*, *hab1-1*, *pp2ca-1* and *abi1-2*, and these phenotypes are reinforced by combined inactivation of these genes (Merlot *et al.*, 2001; Saez *et al.*, 2006). For instance, the ABA-hypersensitive phenotype of *hab1-1* is enhanced when combined with loss-of-function alleles of *ABI1* (Saez *et al.*, 2006). Conversely, reduced sensitivity to ABA is found in *HAB1*-overexpressing lines, which is consistent with its role as negative regulator of ABA signaling (Saez *et al.*, 2004, 2006).

To obtain further insight into the function of HAB1 and its regulatory mechanisms, we have used yeast two-hybrid (Y2H) screening for identification of interacting partners. As a result, we have identified two classes of HAB1-interacting partners. The first is represented by SWI3B, a putative component of SWI/SNF chromatin-remodeling complexes (Saez *et al.*, 2008). These results revealed a connection between ABA signaling and the chromatin remodeling machinery, and suggest direct involvement of HAB1 in the regulation of ABA-induced transcription, as well as regulation of HAB1 function by ABA (Saez *et al.*, 2008).

In this paper, we describe a second class of HAB1-interacting partners, which belong to a branch of the Bet v 1-like superfamily (Radauer *et al.*, 2008). The Bet v 1 family consists of particularly potent allergens from white birch (*Betula verrucosa*) pollen. X-ray crystallography analysis of Bet v 1 has revealed a protein structure comprising seven anti-parallel β-strands and three α-helices, which has been named the helix-grip fold and contains a hydrophobic cavity that can accommodate plant steroid hormones (Gajhede *et al.*, 1996; Markovic-Housley *et al.*, 2003). Moreover, protein structure comparisons have revealed similarity between the birch allergen and the star-related lipid-transfer (START) domain of the MLN64 protein (Iyer *et al.*, 2001). The START domain was initially identified as a lipid-binding domain present in eukaryotic proteins involved in signal transduction processes (Ponting and Aravind, 1999). Thus, Bet v 1 proteins and START proteins have similar helix-grip folds that are involved in the binding of lipid molecules (Iyer *et al.*, 2001). Recent analyses revealed a large superfamily of mostly lipid-binding proteins with a common fold, which has been classified as Bet v 1-like clan CL0209 in the Pfam protein family database and as a Bet v 1-like superfamily in the SCOP database (Radauer *et al.*, 2008).

The HAB1-interacting proteins of the Arabidopsis family hereby identified belong to a 14-member subfamily of the Bet v 1-like superfamily described by Radauer *et al.* (2008), which has also been identified by Park *et al.* (2009) and Ma *et al.* (2009). In this paper, we have analysed some members of this family, and show that they act as inhibitors of the phosphatase activity of HAB1, ABI1 and ABI2 PP2Cs in an ABA-dependent manner. Moreover, we demonstrate that one of these proteins, PYL5, binds ABA with micromolar or nanomolar affinity in the absence or presence of HAB1, respectively, and its over-expression leads to a globally enhanced response to ABA and enhanced drought resistance. As PYL5 is localized at both cytoplasm and nucleus, our work suggests that this protein acts as an intracellular hormone receptor involved in activation of the ABA signaling pathway through inhibition of certain clade A PP2Cs.

RESULTS

HAB1-interacting partners belong to a branch of the Bet v 1-like superfamily

We have previously reported (Saez *et al.*, 2008) that fusion of full-length HAB1 to the GAL4 DNA-binding domain resulted in the activation of the Y2H gene reporters. Deletion of the HAB1 N-terminal domain was required to eliminate such activation, and therefore only the PP2C catalytic core of HAB1 (ΔNHAB1 amino acids 179–511) could be used as bait in the Y2H screen (Saez *et al.*, 2008). Despite this limitation, we were able to identify three closely related members of an Arabidopsis gene family that showed interaction with ΔNHAB1, namely At5g05440 (PYL5), At5g53160 (PYL8) and At2g40330 (PYL6) (Figure 1a). Deletions at the N-terminus (lacking amino acids 1–99) or C-terminus (lacking amino acids 163–203) that are predicted to affect protein structure of PYL5 (Figure S1) abolished its interaction with ΔNHAB1 (Figure 1b). On the other hand, introduction of a Gly246Asp (G246D) mutation, which severely reduces HAB1 PP2C activity, also led to loss of the interaction with PYL5, PYL8 and PYL6 (Figure 1c).

BLAST searches in Arabidopsis using as a query the amino acid sequence encoded by these genes revealed a gene family with 14 members (Figure S1), named the PYR/PYL family (Park *et al.*, 2009). All the members encode small proteins, ranging between 159 and 221 amino acid residues, and protein domain analysis using the InterPro database

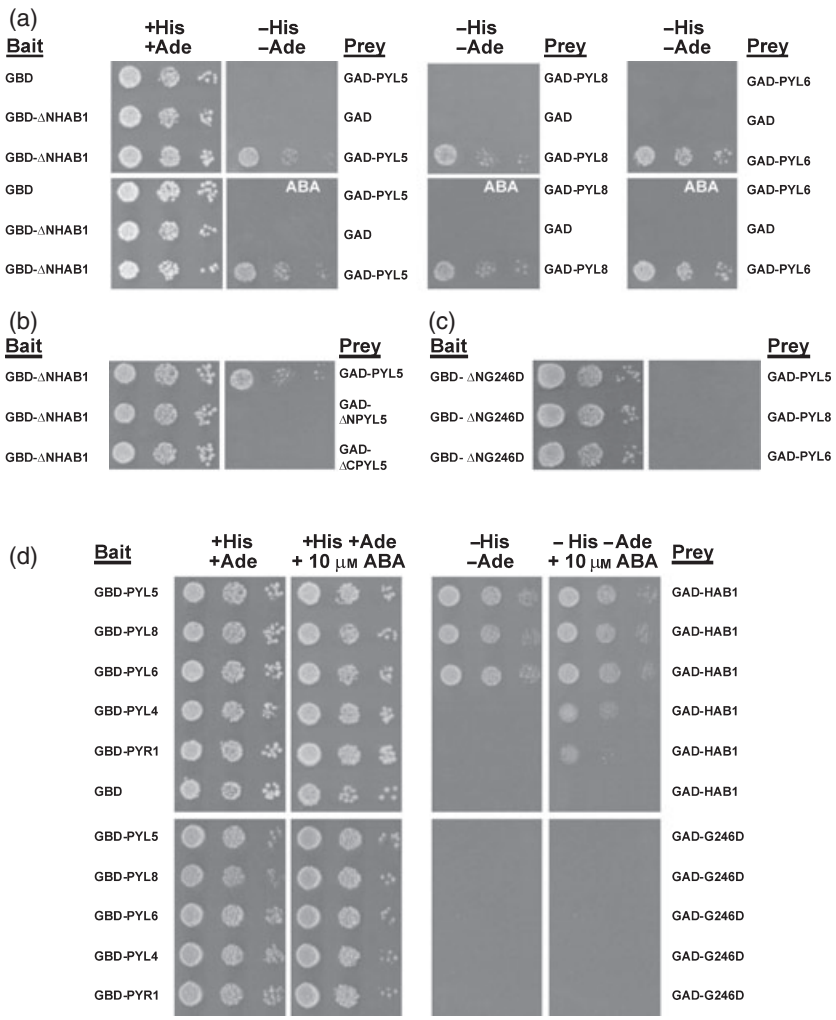

Figure 1. Interaction of HAB1 with PYL5, PYL8 and PYL6 in a yeast two-hybrid assay.
Interaction was determined by growth assay on medium lacking His and Ade. When indicated, the medium was supplemented with 10 μM racemic ABA. Dilutions (10^{-1}, 10^{-2} and 10^{-3}) of saturated cultures were spotted onto the plates, and photographs were taken after 3 days.
(a) Interaction assay using ΔNHAB1 as bait (fused to the Gal4 DNA-binding domain, GBD) and PYL5, PYL8 and PYL6 as prey (fused to the Gal4 activation domain, GAD).
(b) Interaction assay using ΔNHAB1 as bait and either N- or C-terminal deletions of PYL5 as prey.
(c) Interaction assay using G246D ΔNhab1 as bait and PYL5, PYL8 and PYL6 as prey.
(d) Interaction assay using PYL5, PYL8, PYL6, PYL4 and PYR1 as bait (fused to GBD) and either full-length HAB1 or G246 hab1 as prey (fused to GAD).

(http://www.ebi.ac.uk/interpro) revealed the presence of the Bet v 1 family signature (Figure S1). Secondary structure analysis (http://bioinf.cs.ucl.ac.uk/psipred/; Jones, 1999) revealed a β–α–β_6–α arrangement, which is similar to the prototypic Bet v 1 structure (Figure S1) (Markovic-Housley *et al.*, 2003). The PYR/PYL family represents a subfamily of the Bet v 1-like superfamily (Radauer *et al.*, 2008), and its members are distantly related in sequence to Bet v 1 (Figure S1). As other members, e.g. PYR1 and PYL4, have also been identified in Y2H assays as HAB1-interacting partners, and this interaction was ABA-dependent (Park *et al.*, 2009), we assayed the effect of exogenous ABA in our Y2H screen. No significant effect of ABA was found in our assay (Figure 1a). To bypass the limitation of using only the PP2C catalytic core of HAB1 in the Y2H assay, we generated a construct where full-length HAB1 was fused to the GAL4 activation domain (GAD). The corresponding fusion protein did not lead to self-activation of Y2H gene reporters (Figure 1d), and therefore we could use it to test the interaction with PYR/PYL genes fused to the GAL4 DNA-binding domain (GBD). The interaction of HAB1 and PYL5, PYL8 or PYL6 was not dependent on the addition of exogenous ABA, whereas the interaction with PYR1 and PYL4 did not occur in the absence of exogenous ABA (Figure 1d). Finally, introduction of a G246D mutation into the full-length HAB1 construct abolished the interaction with PYR/PYL constructs (Figure 1d).

Gene expression of some members of the PYR/PYL family is down-regulated by ABA

Interestingly, data from the Bio-Array Resource for Arabidopsis Functional Genomics (BAR) (http://bar.utoronto.ca) show that expression of *PYL5*, *PYL8* and *PYL6*, as well as additional members of the family, *PYL4*, *PYR1* and *PYL1*, was strongly down-regulated in whole-seedling tissue by ABA treatment (Figure 2a). These results are particularly intriguing, taking into account that *HAB1* expression, as well as that of other clade A PP2C genes, is strongly up-regulated by ABA treatment (Figure 2a) (Merlot *et al.*, 2001; Saez *et al.*, 2004). Therefore, gene expression data reveal an opposite

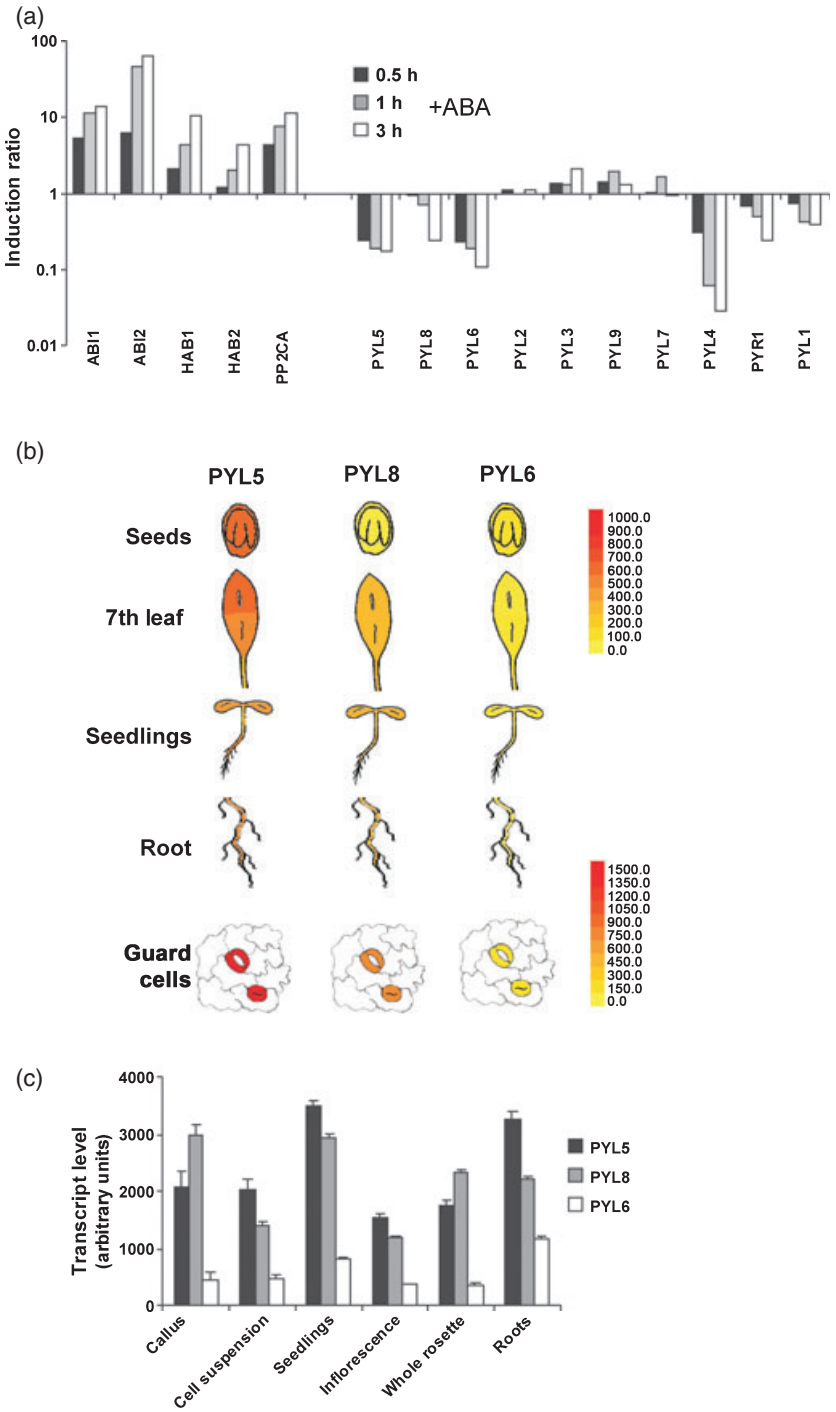

Figure 2. ABA-mediated down-regulation of *PYR/PYL* gene expression.
Expression pattern of *PYL5*, *PYL8* and *PYL6* genes.
(a) ABA-mediated up-regulation of clade A *PP2Cs* and down-regulation of some *PYR/PYL* genes by 10 μM ABA treatment for 0.5, 1 and 3 h in whole 7-day-old seedling tissue. Data were obtained by Kilian *et al.* (2007) and are available from http://www.Arabidopsis.org/info/expression/ATGenExpress.jsp.
(b) Pictographic representation of *PYL5*, *PYL8* and *PYL6* expression values obtained from public databases. The color scale at the top is for the first four panels, and the color scale at the bottom is for the guard cells. Seed data refer to dry seeds. Drawings were created using the eFP browser (http://bar.utoronto.ca).
(c) Transcription level of *PYL5*, *PYL8* and *PYL6* in various tissues, based on data obtained by Kilian *et al.* (2007) and available from the Genevestigator database (http://www.genevestigator.ethz.ch).

effect of ABA on the expression of clade A PP2Cs and at least six members of the PYR/PYL family. Microarray data analysis using the Arabidopsis eFP browser (Winter *et al.*, 2007; http://bar.utoronto.ca/) revealed differential expression among *PYL5*, *PYL8* and *PYL6* (Figure 2b). *PYL5* showed higher expression than *PYL8* and *PYL6* in seeds, seedlings, leaves and guard cells (Figure 2b). Additionally, microarray data analysis at the Genevestigator database (http://www.genevestigator.ethz.ch) (Zimmermann *et al.*, 2004) revealed a general expression pattern for *PYL5* in callus, cell suspension, seeds, seedlings, inflorescences, rosette leaves and roots (Figure 2c). This pattern was also observed for *PYL8*; however, its expression in seed and guard cells was lower than that of *PYL5* (Figure 2b). Given these results, further biological analysis of the HAB1-interacting partners was performed on PYL5.

Subcellular localization and *in planta* interaction between HAB1 and PYL5

In vivo targeting experiments were performed in tobacco to determine the subcellular localization of PYL5. To this end, a GFP–PYL5 fusion was generated and delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration. Figure 3(a) shows that the GFP–PYL5 fusion localizes to both nucleus and cytosol, similar to the subcellular localization described for HAB1 (Saez *et al.*, 2008). In contrast, SWI3B, another HAB1-interacting partner, was only found in the nucleus (Saez *et al.*, 2008). Additionally, the subcellular localization of PYL5 was examined by standard biochemical techniques in protein extracts from transiently transformed

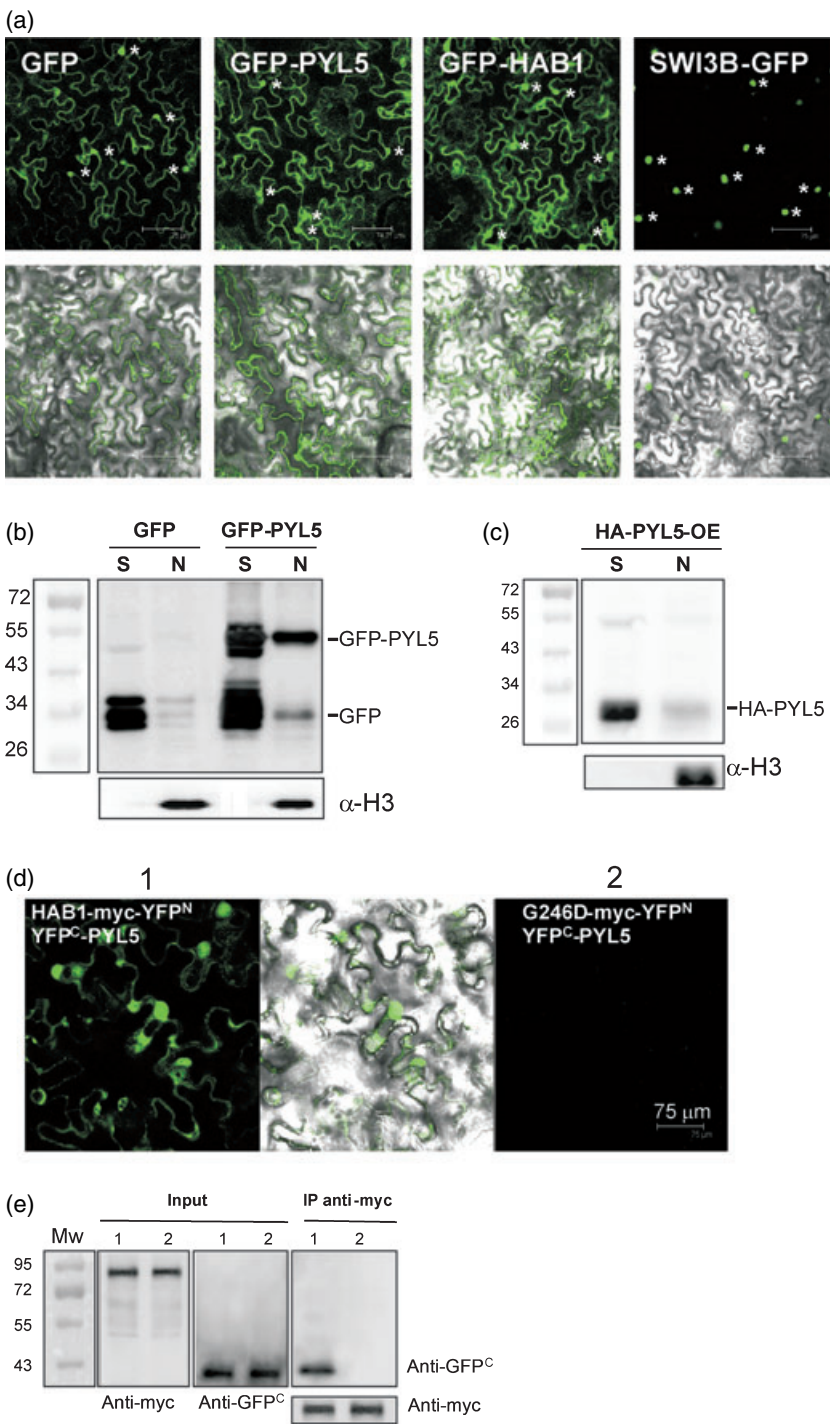

Figure 3. Subcellular localization of PYL5 and interaction with HAB1.
(a) Subcellular localization of GFP and GFP fusions in *Agrobacterium*-infiltrated tobacco leaves. Epifluorescence and bright-field images (merged with epifluoresecence) of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring the indicated constructs and the silencing suppressor p19. The nuclei of tobacco epidermal cells are marked with asterisks.
(b, c) Biochemical fractionation of protein extracts from transiently transformed tobacco cells (b) or Arabidopsis cells that express HA–PYL5 (c). The soluble cytosolic (S) and nuclear fractions (N) were analysed using anti-GFP, anti-histone 3 (H3) or anti-HA antibodies.
(d, e) BiFC visualization and co-immunoprecipitation experiments show interaction between HAB1 and PYL5 in tobacco leaves, but introduction of the G246D substitution into HAB1 abolishes the interaction. The co-immunoprecipitation assay (e) demonstrates the interaction between PYL5 and HAB1 *in planta*. Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harboring constructs HAB1–myc–YFP^N/YFP^C–PYL5 (lanes 1) or G246D–myc–YFP^N/YFP^C–PYL5 (lanes 2) were analysed using anti-myc or anti-GFP^C antibodies. Input levels of epitope tagged-proteins in crude protein extracts (20 μg total protein) were analysed by immunoblotting. Immunoprecipitated myc epitope-tagged proteins were probed with anti-GFP^C antibodies to detect co-immunoprecipitation of HAB1–myc–YFP^N with YFP^C–PYL5.

tobacco cells (Figure 3b) or in Arabidopsis cells that stably express a hemagglutinin (HA) epitope-tagged version of PYL5 (Figure 3c). In agreement with microscopy studies, GFP–PYL5 was detected in both the cytosolic and nuclear fractions by immunoblot analysis (Figure 3b). HA–PYL5 protein was also detected in both the cytosolic and nuclear fractions of Arabidopsis transgenic cells (Figure 3c).

Bimolecular fluorescence complementation (BiFC) assays were used to test the interaction between HAB1 and PYL5 in plant cells. To this end, HAB1 was translationally fused to the N-terminal 155 amino acid portion of YFP (YFP^N) in the pSPYNE vector, which generated an HAB1–myc epitope–YFP^N fusion protein (Figure 3e). Similarly, PYL5 was translationally fused to the C-terminal 84 amino acid portion of YFP (YFP^C) in the pYFP^C43 vector (a derivative of pMDC43, Curtis and Grossniklaus, 2003), generating a YFP^C–PYL5 fusion protein (Figure 3e). The corresponding constructs were co-delivered into leaf cells of tobacco by *Agrobacterium* infiltration, and, as a result, fluorescence was observed in the nucleus and cytosol of tobacco cells (Figure 3d). Suitable controls did not show fluorescent signal. Moreover, introduction of the G246D mutation into the sequence of HAB1 abolished the interaction with PYL5 in the BiFC assay (Figure 3d). We confirmed the interaction of HAB1 and PYL5 by co-immunoprecipitation experiments in tobacco protein extracts prepared from the BiFC assay described above. We found that HAB1 and PYL5 co-immunoprecipitated, as YFP^C–PYL5 was detected in the immunocomplex precipitated with an antibody to the myc epitope, which pulls down the HAB1–myc–YFP^N fusion protein (Figure 3e). In contrast, the co-immunoprecipitation assay did not show interaction between G246D hab1 and PYL5 (Figure 3e).

Over-expression of *PYL5* leads to enhanced response to ABA

Taking into account that homologous genes of the same family usually display functional redundancy to varying degrees, we used a gain-of-function approach to provide genetic evidence of the role of PYL5 in ABA signaling. We generated *PYL5*-over-expressing (OE) lines, and selected three homozygous T_3 lines for further studies (Figure 4). Real-time quantitative PCR analysis showed that *PYL5* expression in these lines was between 15- and 20-fold higher than in wild-type (Figure S2), and HA-tagged PYL5 protein was detected at similar levels in these lines (Figure 4a).

PYL5-OE lines showed higher sensitivity to ABA-mediated inhibition of seed germination than wild-type, as also found in the loss-of-function *hab1-1* mutant (Figure 4b). Moreover, a significant reduction of germination (approximately 20%) was consistently found in these lines (Figure 4b). In contrast to the phenotype of *hab1-1*, *35S:HAB1-dHA* lines (*HAB1*-OE, containing a double HA epitope) showed reduced sensitivity to ABA-mediated inhibition of seed germination (Figure 4b) (Saez *et al.*, 2004). Transgenic lines that showed simultaneous over-expression of *PYL5* and *HAB1* (*HAB1*-OE *PYL5*-OE) were generated by transferring pollen from *35S:HAB1-dHA* lines (Hyg^R; Saez *et al.*, 2004) to the stigmas of emasculated flowers from *35S:HA-PYL5* T_3 lines ($phosphinothricin^R$) (Figure 4a). Interestingly, transgenic plants over-expressing both *HAB1* and *PYL5* showed a phenotype similar to that of *PYL5*-OE lines, which suggests that PYL5 antagonizes HAB1 function in the plant (Figure 4b). To determine growth sensitivity to ABA, we used the double *hab1-1 abi1-2* mutant as a reference for the enhanced response to ABA, as it shows a stronger phenotype than *hab1-1* (Saez *et al.*, 2006). Root-growth assays revealed that *PYL5*-OE lines were as hypersensitive to ABA-mediated inhibition of growth as the double *hab1-1 abi1-2* mutant (Figure 4c,d). Although *HAB1*-OE lines were insensitive to ABA-mediated inhibition of root growth, transgenic plants over-expressing both *HAB1* and *PYL5* showed enhanced root sensitivity to ABA, similar to the phenotype found in the *PYL5*-OE lines (Figure 4c,d). Gene expression in response to ABA was enhanced in *PYL5*-OE lines compared to wild-type, as real-time quantitative PCR analysis showed enhanced up-regulation of the ABA-inducible genes *RAB18* and *RD29B* (Figure 5a). Finally, water-loss assays under drought-stress conditions indicated reduced water loss (Figure 5b) and enhanced drought resistance (Figure 5c) in *PYL5*-OE lines compared to wild-type.

Inhibition of PP2C activity by PYR/PYL proteins is ABA-dosage dependent

The phenotypes observed for *PYL5*-OE lines are similar to those reported in loss-of-function *pp2c* alleles. Moreover, simultaneous over-expression of *PYL5* and *HAB1* abolished the ABA-insensitive phenotype of *HAB1*-OE lines, which indicates that PYL5 antagonizes HAB1 function in the plant (Figure 4b–d). As PYR/PYL proteins interact with HAB1, we hypothesized that they might have a regulatory effect on PP2C activity. We found an ABA-dependent inhibitory effect of PYR/PYL proteins on HAB1 activity (Figure 6a), whereas PYR/PYL proteins do not inhibit HAB1 activity in the absence of ABA (Figure S3). Moreover, the ABA inhibitory concentration required to achieve 50% inhibition (ABA-IC_{50}) of HAB1 activity was dependent on the PYR/PYL protein assayed (Figure 6a). Thus, the ABA-IC_{50} values were 35, 135, 188 and 390 nM in the presence of PYL5, PYL8, PYL4 or PYR1, respectively. Interestingly, other hydrophobic hormones, such as brassinosteroids or giberellins, did not promote inhibition of HAB1 activity by PYL5 or PYR1 proteins (Figure S3). Therefore, PYR/PYL proteins act as potent negative regulators of HAB1 activity in an ABA-dependent manner.

To ascertain whether the effect of PYR/PYL proteins was or was not specific for HAB1, we have assayed their effect on ABI1 and ABI2 activity. The ABA-IC_{50} value for ABI2 was 115,

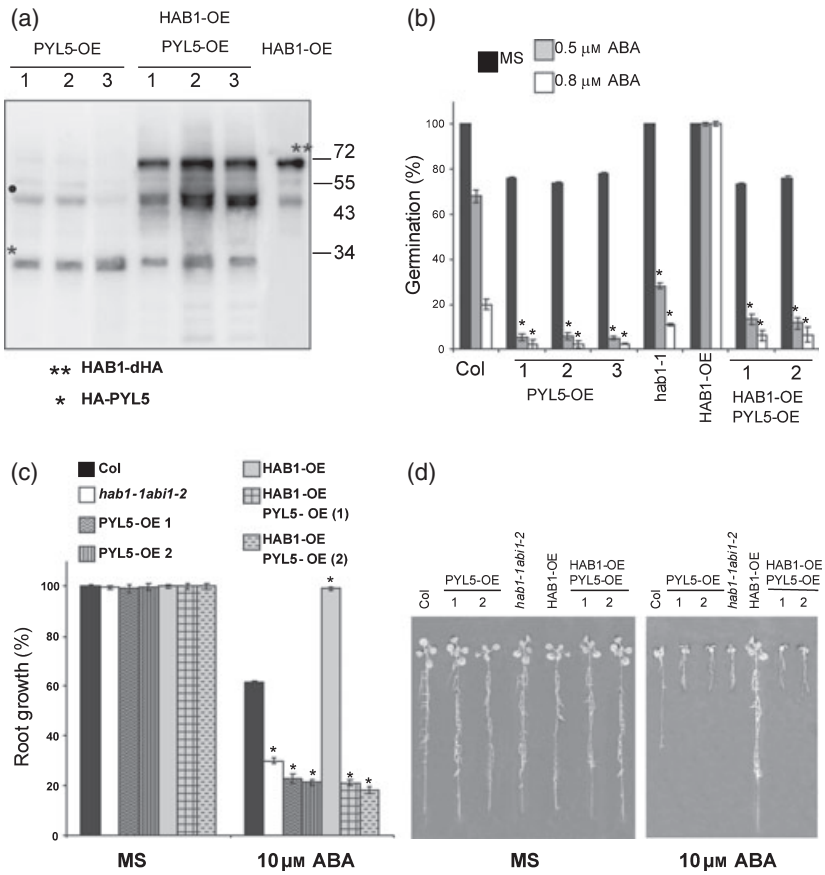

Figure 4. ABA-hypersensitive inhibition of germination and root growth in *PYL5*-OE lines compared to wild-type plants.
Over-expression of *PYL5* abolishes the ABA insensitivity of *HAB1*-OE lines.
(a) Immunoblot analysis shows expression of HA-tagged PYL5 (*) and HAB1-dHA (**). An non-specific band that migrates at the same position as the Rubisco large subunit is indicated by a dot.
(b) Percentage seed germination in the presence of the indicated ABA concentrations. Approximately 200 seeds of ecotype Columbia, three independent *PYL5*-OE lines, the *hab1-1* mutant, *HAB1*-OE line and two independent lines (F_2 seeds) that showed simultaneous over-expression of *PYL5* and *HAB1* (*HAB1*-OE *PYL5*-OE) were sown and scored 4 days later. Data are means ± SE from three independent experiments. $^*P < 0.01$ (Student's *t* test) when comparing data for each genotype versus the wild-type under the same assay conditions.
(c, d) ABA-hypersensitive root growth inhibition of two independent *PYL5*-OE lines compared to wild-type. (c) Seedlings were grown on vertically oriented MS plates for 4 days. Afterwards, plants were transferred to new MS plates lacking or supplemented with 10 μM ABA. Root growth was scored after 10 days. Data are means ± SE from three independent experiments ($n = 20$ seedlings per experiment). $^*P < 0.01$ (Student's *t* test) when comparing data for each genotype versus the wild-type under the same assay conditions.
(d) Representative seedlings 10 days after the transfer of 4-day-old seedlings from MS to plates lacking or supplemented with 10 μM ABA.

118, 110 and 360 nM in the presence of PYL5, PYL8, PYL4 or PYR1, respectively (Figure 6b). In the case of ABI1, these values were 123, 75, 272 and 330 nM, respectively (Figure 6c). In summary, PYR/PYL proteins have an ABA-dependent inhibitory effect on key representatives of clade A PP2Cs. The ABA-IC_{50} for HAB1 in the presence of PYL5 was 3-, 5- and 11-fold lower than values obtained with PYL8, PYL4 and PYR1, respectively (Figure 6a). In the case of ABI1, the ABA-IC_{50} in the presence of PYL8 was 1.6-, 3- and 4-fold lower than in the presence of PYL5, PYL4 and PYR1, respectively (Figure 6c). Finally, no significant effect was found for ABA alone, i.e. without PYR/PYL protein, on either HAB1, ABI1 or ABI2 activity (Figure 6a–c).

We also tested whether PYL5 inhibited other PP2Cs in an ABA-dependent manner (Figure S4). Interestingly, a closely related PP2C from clade A, PP2CA (At3g11410), as well as a distantly related PP2C (At4g38520) from clade D, were not inhibited by PYL5, which indicates a selective effect of PYL5 on the HAB1/ABI1/ABI2 PP2Cs. Finally, we tested whether (+)ABA and (–)ABA enantiomers were equally effective at promoting PYL5-mediated inhibition of HAB1 activity (Figure S5). The ABA-IC_{50} for HAB1 in the presence of PYL5 was 23 nM for (+)ABA and 186 nM for (–)ABA, which indicates that (+)ABA is eight times more effective than (–)ABA at promoting inhibition of HAB1 mediated by PYL5.

ABA binding to recombinant His6–PYL5

Isothermal titration calorimetry (ITC) was used to determine whether ABA binds directly to 6× His-tagged PYL5 (His6–PYL5). In these experiments, known volumes of a solution containing (+)ABA, the natural enantiomer, were injected into a cell containing His6–PYL5 and the heat exchanged was measured. Figure 7(a) shows the ITC thermogram for one representative experiment. Addition of the ligand releases a large amount of heat, and a typical saturation curve was obtained upon sequential addition of ligand, demonstrating a specific and saturable binding reaction. Analysis of this thermogram (Figure 7b) indicated a dissociation constant (K_d) of 1.1 μM, with an enthalpy value (ΔH) of –7.9 kcal mol^{-1} and an entropy value (ΔS) of 1.3 cal mol^{-1} K^{-1}. The negative value and magnitude of ΔH indicate the net creation of non-covalent bonds during the binding process. The stoichiometry (N) was approximately 0.7, which suggests binding of one ABA molecule per protein molecule. Minor experimental errors inherent to the methods for determining ligand and active protein concentration (minor proteolysis of the

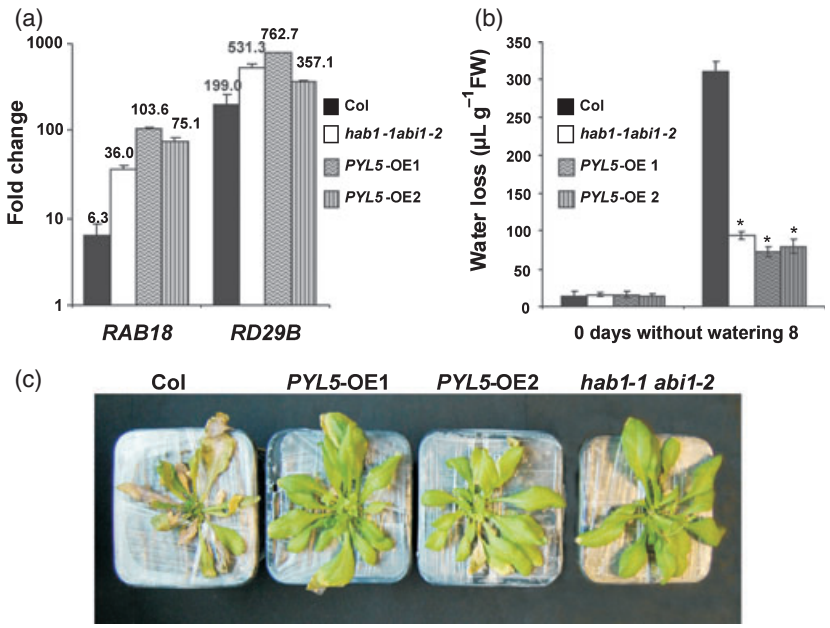

Figure 5. Enhanced expression of *RAB18* and *RD29B* and drought resistance in two independent *PYL5*-OE lines compared to wild-type.
(a) Induction ratio of *RAB18* and *RD29B* genes in *PYL5*-OE lines compared to Columbia wild-type and the double mutant *hab1-1 abi1-2*. mRNAs were prepared from 15-day-old seedlings (whole tissue) that were either mock-treated or 10 µM ABA-treated for 3 h. Data are means ± SE from three independent experiments. Expression of β-actin-8 was used to normalize data in real-time quantitative PCR experiments.
(b) Water loss quantification after 8 days without watering. Data are means ± SE from three independent experiments ($n = 5$ plants per experiment). $^*P < 0.01$ (Student's *t* test) when comparing data for each genotype versus the wild-type under the same assay conditions.
(c) Enhanced drought resistance of *PYL5-OE* plants compared with wild-type. Representative plants 15 days after the start of withholding of water. The shoot was cut to better show the effect of drought on rosette leaves.

PYL5 protein was observed at the end of ITC experiments performed at 25°C over 4 h) might explain the deviation from an ideal 1:1 stoichiometry. Finally, the binding was partially sterospecific, as similar ITC experiments (Figure 7c,d) performed with the non-natural (–)ABA enantiomer revealed a K_d of 19 µM, which is one order of magnitude higher than for the natural enantiomer. These data indicate that PYL5 has at least 10-fold higher affinity for the natural isomer, but it is able to bind (–)ABA, which is expected given the lack of *in vivo* selection on the non-natural enantiomer action. These data are also consistent with the inhibitory effect of (–)ABA on HAB1 activity mediated by PYL5 (Figure S5).

Ma *et al.* (2009) have shown that addition of ABI2 to a similar ITC experiment in which they measured binding of (+)ABA to RCAR1/PYL9 led to an apparent K_d of 64 nM ABA, which is approximately 10-fold lower than in the absence of ABI2. We added the PP2C catalytic core of HAB1 (ΔNHAB1) to our ITC assay of (+)ABA binding to PYL5, and analogous results were obtained, with an apparent K_d of 38 nM ABA, which is approximately 25-fold lower than the value obtained in the absence of the PP2C (Figure 7e,f). These results suggest that the ternary complex ABA–receptor–PP2C behaves as a high-affinity system for ABA in the nanomolar range. Finally, we did not observe binding of (+)ABA to ΔNHAB1 using the ITC assay (Figure 7g,h). ΔNHAB1 was used for ITC experiments because we could not obtain the high amount of soluble protein required using the full-length construct. ΔNHAB1 is able to interact with PYL5 (Figure 1), and its phosphatase activity is inhibited by PYL5 in an ABA-dependent manner (Figure S4).

DISCUSSION

The Arabidopsis PYR/PYL family represents a branch of the Bet v 1 superfamily, which currently comprises 11 subfamilies (Radauer *et al.*, 2008). Although the members of this superfamily share a common structural fold, evolution has diversified their function (Radauer *et al.*, 2008). For instance, whereas Bet V is a major pollen allergen that belongs to the PR-10 family of plant pathogenesis-related proteins, other members of the superfamily are involved in lipid transport, antibiotic biosynthesis, degradation of polycyclic aromatic compounds, or activation of Hsp90 ATPase (Radauer *et al.*, 2008). However, a common feature among many components of the superfamily is the binding of hydrophobic compounds, including membrane lipids, plant hormones, secondary metabolites and polycyclic aromatic compounds (Radauer *et al.*, 2008). The data we have obtained for some members of the PYR/PYL family indicate that they are part of an ABA-dependent mechanism to regulate PP2C activity, and hence ABA signaling.

Interestingly, the HAB1-interacting partners PYL5, PYL6 and PYL8 did not require exogenous ABA supplementation for the Y2H interaction. As biosynthesis of ABA has been described in several fungi (Hirai *et al.*, 2000; Nambara and Marion-Poll, 2005), we cannot exclude the possibility that endogenous ABA from yeast might be sufficient to promote the interaction of these clones with HAB1. However, the results obtained in yeast show a notable difference between these proteins and PYR1–PYL4, whose interaction with HAB1 required ABA supplementation (Figure 1d). Therefore, even though ABA does not appear to be required for the interaction of PYL5, PYL6 and PYL8

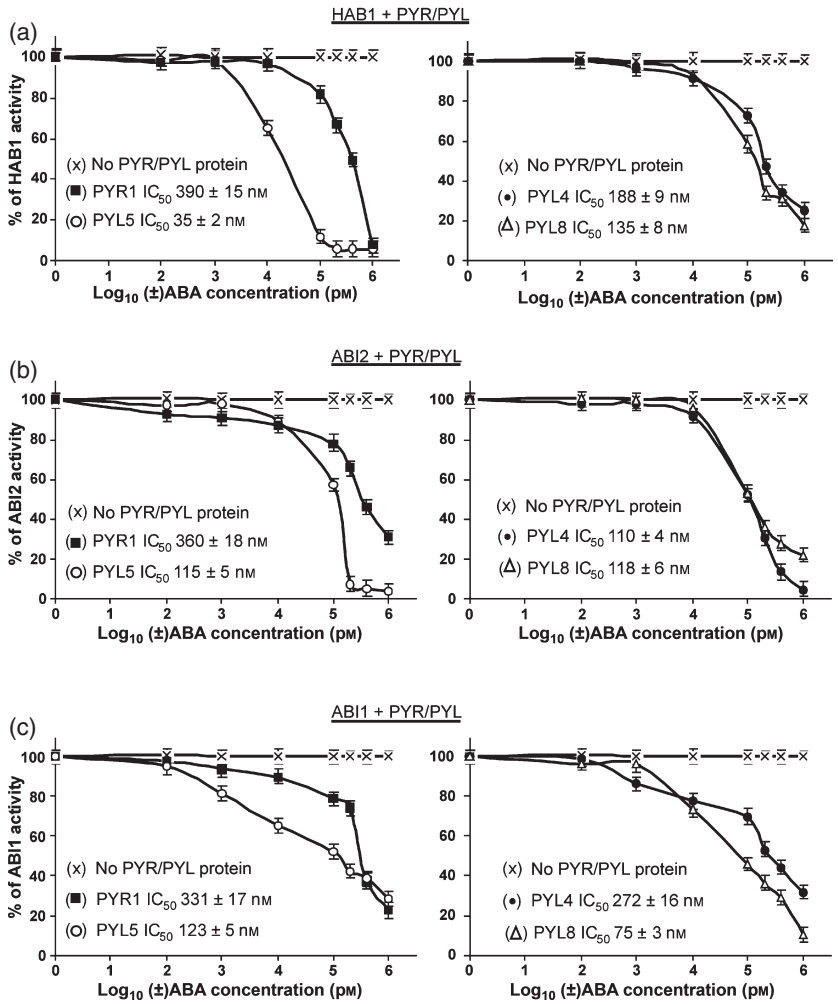

Figure 6. ABA-dependent inhibitory effect of PYR/PYL proteins on HAB1, ABI2 and ABI1 PP2C activity.

Phosphatase activity was measured using the Ser/Thr phosphatase assay system (Promega) with the RRA(phosphoT)VA peptide as the substrate. Data are means ± SE from three independent experiments. His6–HAB1, MBP–ABI2, MBP–ABI1, His6–PYL5, His6–PYL4 and His6–PYR1 proteins were obtained as described in Experimental procedures. Phosphatase assays were performed in a 100 μl reaction volume containing either 3 μg His6–HAB1 or 10 μg MBP–ABI2/MBP–ABI1 and 5 μg His6–PYL5, His6–PYL8, His6–PYL4 or His6–PYR1. In the case of HAB1 and PYL5, 2.5 μg of inhibitor were used. The indicated (±)ABA concentrations were included in the PP2C activity assay. The activities of His6–HAB1, MBP–ABI2 and MBP–ABI1 recombinant proteins in the absence of ABA (100% activity) were 4.4 ± 0.3, 3.5 ± 0.2 and 3.6 ± 0.2 nmol Pi min^{-1} mg^{-1}, respectively.

with HAB1, ABA probably affects the interaction between them and the PP2C, because they do not promote inhibition of HAB1 activity in the absence of ABA. Upon ABA binding, PYL5 probably suffers a conformational change that enhances the affinity for and binding of HAB1 and leads to an inhibitory ABA–PYL5–HAB1 complex. Additional ITC experiments are required to determine the K_d of the interaction between PYL5 and HAB1 in the absence of ABA, and to establish whether they exist mostly as a stable co-receptor complex or as individual components in the absence of ABA.

The interaction of PYR/PYL proteins in the Y2H screen was abolished when G246D hab1 was used as the interacting partner (Figure 1d). Plants harboring a *35S:G246D hab1* construct show strong dominant ABA-insensitive phenotypes (Robert *et al.*, 2006), which might partly reflect the ability of G246D hab1 to escape negative regulation by PYR/PYL proteins. Additional factors might also contribute to the ABA-insensitive phenotype of plants expressing G246D hab1 (e.g. enhanced substrate affinity or enhanced protein stability compared to wild-type); however, escape from the PYR/PYL-based inhibitory mechanism offers a reasonable explanation for this phenotype.

The ABA response in *PYL5*-OE plants was globally enhanced, which indicates that PYL5 is a positive regulator of ABA signaling. Interestingly, we have demonstrated that PYL5 enhances drought resistance through inhibition of clade A PP2Cs, which is in agreement with the phenotype of the double *hab1-1 abi1-2* mutant (Saez *et al.*, 2006). Indeed, a clear parallel was found between the phenotypes of *PYL5*-OE plants and those of loss-of-function *pp2c* mutants. Moreover, transgenic plants over-expressing both *HAB1* and *PYL5* showed a phenotype similar to that of *PYL5*-OE lines. Therefore, both genetic and biochemical data indicate that the positive role of PYL5 in ABA signaling involves ABA-dependent inhibition of HAB1 function. PYL5 is an ABA-binding protein that shows saturable and partially stereospecific binding to (+)ABA with an apparent K_d of 1.1 μM. However, equimolar presence of HAB1 in the binding assay resulted in a K_d of 38 nM, which indicates that the affinity of the complex for ABA is 25-fold higher under these conditions. It has been

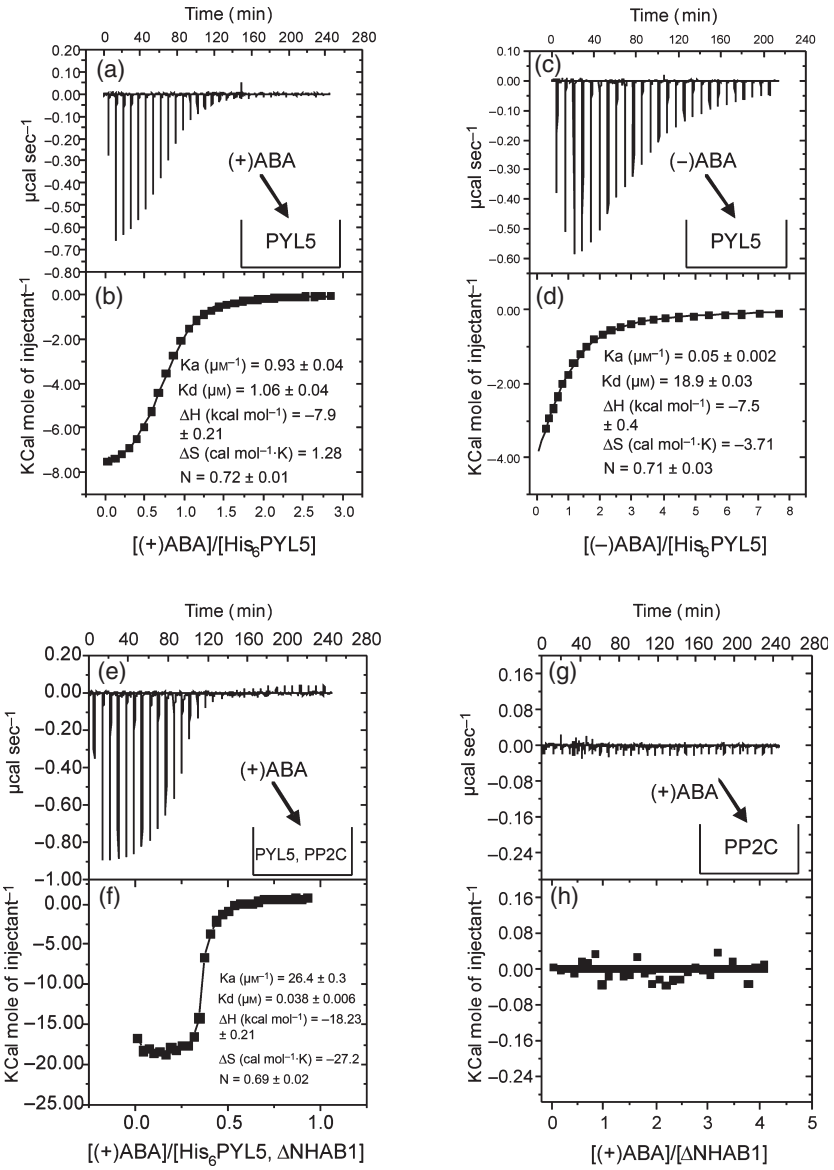

Figure 7. Isothermal titration calorimetry profiles and thermodynamic data for various binding experiments at pH 7.5 and 25°C.
(a) Raw data for 35 sequential injections of 6 µl of 0.58 mM (+)ABA stock solution into a cell containing 33 µM His6–PYL5 in 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MnCl_2$ and 1 mM β-mercaptoethanol (ITC buffer). The injections were performed over a period of 6 sec, with an 8 min interval between injections, and the final concentration reached was 74.5 µM.
(b) Plot of the heat evolved (kcal) per mole of (+)ABA added, corrected for the heat of (+)ABA dilution, against the molar ratio of (+)ABA to His6–PYL5. The data (filled squares) were fitted using the software 'one set of sites', and the solid line represents the best fit.
(c) Raw data for 26 injections (increasing from 3 to 20 µl) of 1 mM (–)ABA stock solution into a cell containing 30 µM His6–PYL5 in ITC buffer. The final ligand concentration in the cell was 180 µM.
(d) Plot of the heat evolved (kcal) per mole of (–)ABA added, corrected for the heat of (–)ABA dilution, against the molar ratio of (–)ABA to His6–PYL5.
(e) Raw data for 35 sequential injections of 6 µl of 0.2 mM (+)ABA stock solution into a cell containing 30 µM of both His6–PYL5 and ΔNHAB1 in ITC buffer. The final ligand concentration in the cell was 25 µM.
(f) Plot of the heat evolved (kcal) per mole of (+)ABA added, corrected for the heat of (+) ABA dilution, against the molar ratio of (+)ABA to His6–PYL5 and ΔNHAB1.
(g) Raw data for sequential 35 injections of 6 µl of 0.5 mM (+)ABA stock solution into a cell containing 30 µM ΔNHAB1. The final ligand concentration in the cell was 70 µM.
(h) Plot of the heat evolved (kcal) per mole of (+) ABA added, corrected for the heat of (+) ABA dilution, against the molar ratio of (+)ABA to ΔNHAB1. Data indicate that no binding was observed under the conditions used.
Data are means ± SE from three independent experiments.

previously discussed by McCourt and Creelman (2008) that ABA binding proteins with a nanomolar K_d are probably saturated under physiological conditions, as the ABA concentration in mesophyll and guard cells oscillates in the micromolar range (Zeevaart and Creelman, 1988). However, careful studies using LC-MS have shown that the ABA concentration in 4-week-old turgid rosettes oscillates between 10 and 40 nM (Priest *et al.*, 2006). Subtle effects of ABA on plant growth and vegetative development are expected at these concentrations as ABA plays an important although often under-appreciated role in these processes, as inferred from the phenotypes of severe ABA-deficient (Cheng *et al.*, 2002; Barrero *et al.*, 2005) or ABA-insensitive mutants (Fujii and Zhu, 2009). In addition, it should be kept in mind that the proportion of ligand-binding sites occupied by a ligand (θ), ABA in this case, follows a hyperbolic function with respect to ligand concentration, i.e. θ = [ABA]/[ABA] + K_d. This means that 38 nM free ABA leads to 50% occupancy of PYL5, which presumably leads to 50% inhibition of HAB1. Only a subtle effect on HAB1 function is expected as a result of this inhibition, as the *hab1-1* mutation is recessive and the ABA response was not markedly affected in heterozygous +/– individuals. In order to achieve 90 or 95% occupancy of PYL5, the free ABA concentration must increase to 340 or 720 nM, respectively. Therefore, in order to achieve marked inhibition of HAB1, ABA levels must move closer the (sub)micromolar range, and 4-week-old wilted or salt-treated rosettes do show such ABA concentrations (Priest *et al.*, 2006). Finally, it is important to emphasize that these

calculations assume that the protein concentrations of PYL5 and HAB1 are not limiting and their ratio is close to 1:1. As it is the ternary complex ABA–receptor–PP2C that behaves as a nanomolar sensor for ABA, variations in the ratio of receptor to PP2C would influence the range of ABA perception. Finally, the (–)ABA enantiomer was also bound by PYL5, but with a lower affinity (K_d = 19 μM). Although this compound is not the natural enantiomer, it is known to elicit many ABA effects (Nambara *et al.*, 2002; Huang *et al.*, 2007). The binding of (–)ABA to PYL5 as well as its inhibitory effect on HAB1 activity provide an explanation for the ABA-inducible effects described for this enantiomer.

The biochemical analysis was extended to other PYR/PYL proteins and clade A PP2Cs, such as ABI1 and ABI2, that play a key role in ABA signaling. As a general result, we found that PYR/PYL proteins have an ABA-dependent inhibitory effect on clade A PP2Cs such as HAB1, ABI2 and ABI1. Given the multiplicity of both clade A PP2Cs (nine members) and PYR/PYL proteins (14 members), it is anticipated that certain pairs of PP2C–PYR/PYL proteins could show a preferential interaction, based on biochemical parameters, subcellular localization, and organ, tissue and developmental expression, as well as developmental and environmental regulation of ABA biosynthesis. For instance, we found that the ABA-IC_{50} value for PYL5–HAB1 or PYL8–ABI1 interactions was notably lower than values obtained for other combinations (Figure 6). The multiplicity of PYR/PYL proteins provides a plausible regulatory mechanism to handle differentially with the complex PP2C-based regulation of ABA signaling (see for instance data at Figure S4).

Taking together the data presented here and the phenotypic analysis of combined *pp2c* mutants (Rubio *et al.*, 2009), we postulate a model whereby PYR/PYL function serves to eliminate the brake to ABA signaling imposed by clade A PP2Cs (Figure 8). Thus, under basal conditions, the ABA-signaling pathway is repressed by the concerted action of clade A PP2Cs (Figure 8a). In support of this concept, we have recently identified a partial constitutive response to basal ABA levels in triple loss-of-function mutants impaired in clade A PP2Cs (Rubio *et al.*, 2009). An increase in ABA levels would be transmitted by PYR/PYL proteins to cause ABA-dependent inhibition of clade A PP2Cs, releasing their inhibitory effect on ABA signaling and leading to the ABA response (Figure 8b). Finally, although PP2Cs are negative regulators of ABA signaling, they are strongly induced by ABA by a negative-feedback regulatory mechanism (Merlot *et al.*, 2001). Conversely, six members of the Arabidopsis family that includes HAB1-interacting partners were notably down-regulated by ABA. Therefore, an opposite effect of ABA on gene expression is observed for clade A PP2Cs and some *PYR/PYL* genes. Given that PYR/PYL proteins act as inhibitors of PP2C

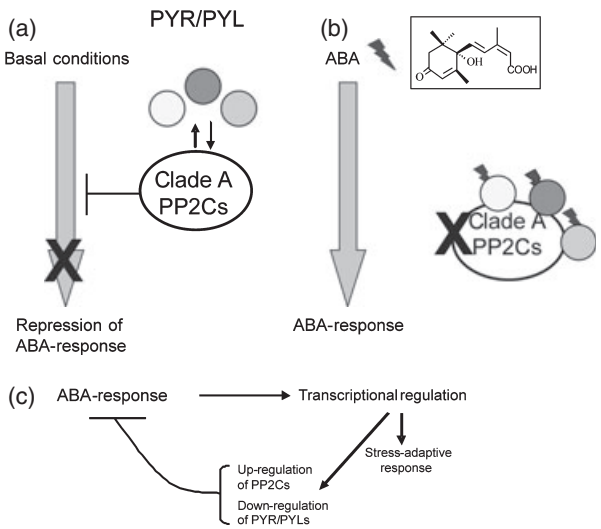

Figure 8. Model for ABA-dependent PYR/PYL-mediated inhibition of PP2C activity and ABA signaling. Details of the model are discussed in the text.

activity, the observed ABA-mediated down-regulation of the *PYR/PYL* genes fits into the negative-feedback regulatory mechanism described for PP2Cs. Thus, the initial response to ABA achieved by ABA-dependent PYR/PYL-mediated inactivation of PP2Cs (Figure 8b) is attenuated later on by the concerted action of both ABA-mediated up-regulation of *PP2C* genes and down-regulation of the PP2C inhibitors encoded by *PYR/PYL* genes (Figure 8c). Such re-setting of the ABA signaling cascade provides a dynamic mechanism to monitor ABA levels and modulate the ABA response.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana (ecotype Columbia) and *Nicotiana benthamiana* plants were grown as decribed by Saez *et al.* (2008).

Generation of *PYL5*-OE lines and crossing with *HAB1*-OE lines

The coding sequence of *PYL5* cDNA was PCR-amplified using the primers FCDS5g05440 and RCDS5g05440 (the sequences of all primers used in this work are at Appendix S1). The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen, http://www.invitrogen.com/) and recombined by LR reaction into the pEarleyGate 201 destination vector (Earley *et al.*, 2006). The *pEarley201-35S:HA-PYL5* construct was transferred to *Agrobacterium tumefaciens* C58C1 (Deblaere *et al.*, 1985) by electroporation, and used to transform the wild-type. Seeds from transformed plants were harvested and plated on phosphinothricin (10 μg ml^{-1}) selection medium to identify T_1 plants. T_3 progeny that were homozygous for the selection marker were used for further studies. Transgenic lines that show simultaneous over-expression of *PYL5* and *HAB1* were generated by transferring pollen from *35S:HAB1-dHA* lines (Hyg^R; Saez *et al.*, 2004) to the stigmas of emasculated flowers from *35S:HA-PYL5* T_3 lines. F_2 seedlings that showed

resistance both to hygromicin (10 μg ml^{-1}) and phosphinothricin (10 μg ml^{-1}) were selected, and co-expression of *35S:HAB1-dHA* and *35S:HA-PYL5* was verified by immunoblot analysis using peroxidase-conjugated anti-HA antibody (Roche, http://www.roche.com).

Yeast two-hybrid assays

The yeast two-hybrid screening method used here has recently been described by Saez *et al.* (2008) (see also Appendix S1).

Sub-cellular localization and BiFC assays

Experiments were performed essentially as described by Voinnet *et al.* (2003) (see also Appendix S1).

Biochemical fractionation

The protocol used was based on fractionation techniques described by Bowler *et al.* (2004) and Cho *et al.* (2006), and has been described recently by Saez *et al.* (2008) (see also Appendix S1).

Protein extraction, protein blot analysis and immunoprecipitation

Protein extracts for immunodetection experiments were prepared from *N. benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* C58C1 (pGV2260) transformed with the constructs indicated above or with Arabidopsis *35S:HAB1-dHA* and *35S:HA-PYL5* transgenic lines. Protocols were essentially similar to those reported by Saez *et al.* (2008) (see also Appendix S1).

Germination and root growth assays

To measure ABA sensitivity at germination, approximately 200 seeds per experiment were plated on solid medium comprising MS basal salts, 1% sucrose and increasing concentrations of ABA. In order to score seed germination, the percentage of seeds that showed radicle emergence was determined after 4 days. To score ABA-mediated root growth inhibition, approximately 20 seedlings per experiment were grown on vertically oriented MS plates for 4 days. Afterwards, plants were transferred to new plates containing MS medium supplemented or not with 10 μM ABA. Seedlings were grown vertically for 10 days, and plates were scanned on a flatbed scanner to produce image files suitable for quantitative analysis using NIH ImageJ software version 1.37 (http://rsb.info.nih.gov/nih-image/).

Drought-stress assay

Drought stress was imposed by withholding water for plants maintained under greenhouse conditions essentially as described by Saez *et al.* (2006). To this end, plants (five individuals per experiment, three independent experiments) were grown under normal watering conditions for 5 weeks and then subjected to drought stress by completely terminating irrigation and minimizing soil evaporation by covering the pots with plastic film. Two samples of 10 leaves of each genotype were then removed, weighed, incubated in de-mineralized water for 3 h, and weighed again. The difference in weight was considered to be water loss.

RNA analyses

Plants were grown on MS plates supplemented with 1% sucrose. After 10 days, plantlets were transferred to 100 ml Erlenmeyer flasks containing 2 ml of liquid MS medium supplemented with 1% sucrose. Ten days later, plantlets were either mock- or 10 μM ABA-treated for 3 h. RNA extraction, cDNA synthesis and real-time quantitative PCR amplifications were performed as described by Saez *et al.* (2008) (see also Appendix S1).

Purification of recombinant proteins as MBP and 6× His-tagged proteins

The generation of HAB1, ABI1 and ABI2 cDNAs has been described previously (Meyer *et al.*, 1994; Rodriguez *et al.*, 1998a,b). The coding sequence of HAB1 or ΔNHAB1 (lacking the N-terminal amino acid residues 1–178) was excised from pCR/GW/TOPO vector by *Nco*I/*Eco*RI digestion and cloned into pETM11 to encode N-terminally 6× His-tagged recombinant proteins. ABI1 and ABI2 ORFs were cloned into the pMalc2 vector to generate constructs encoding MBP–ABI1 and maltose binding protein (MBP)–ABI2. Purification details are described in Appendix S1.

PP2C enzyme assay

Phosphatase activity was measured using the Ser/Thr phosphatase assay system (Promega, http://www.promega.com/) using the RRA(phosphoT)VA peptide as the substrate. Briefly, phosphatase assays were performed in a 100 μl reaction volume containing 25 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 25 μM peptide substrate and the indicated amount of the PP2C. When indicated, PYR/PYL recombinant proteins and ABA were included in the PP2C activity assay. After incubation for 60 min at 30°C, the reaction was stopped by addition of 100 μl molybdate dye (Baykov *et al.*, 1988), and the absorbance at 630 nm was recorded.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC calorimeter equipped with the control, data acquisition and analysis software ORIGIN 7 (Microcal Inc., http://www.microcal.com/). Details of the technique are given in Appendix S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid sequence alignment of At5g05440 (PYL5), At5g53160 (PYL8) and At2g40330 (PYL6) gene products with Arabidopsis family members identified in the TAIR database and Bet v 1 protein.

Figure S2. Diagram of the construction used to over-express PYL5, and results of real-time quantitative PCR analysis of PYL5 expression in mRNAs prepared from 10-day-old seedlings of wild-type and three independent *PYL5*-OE lines.

Figure S3. PYR/PYL proteins do not inhibit HAB1 activity in the absence of ABA, and epibrassinolide and gibberellic acid do not promote inhibition of HAB1 activity by PYR/PYL proteins.

Figure S4. Differential inhibitory effect of PYR/PYL proteins on ΔNHAB1, ΔNPP2CA and clade D PP2C (At4g38520) activity.
Figure S5. Differential effect of (+)ABA and (−)ABA enantiomers on PYL5-mediated inhibition of HAB1 activity.
Appendix S1. Supplementary experimental procedures.
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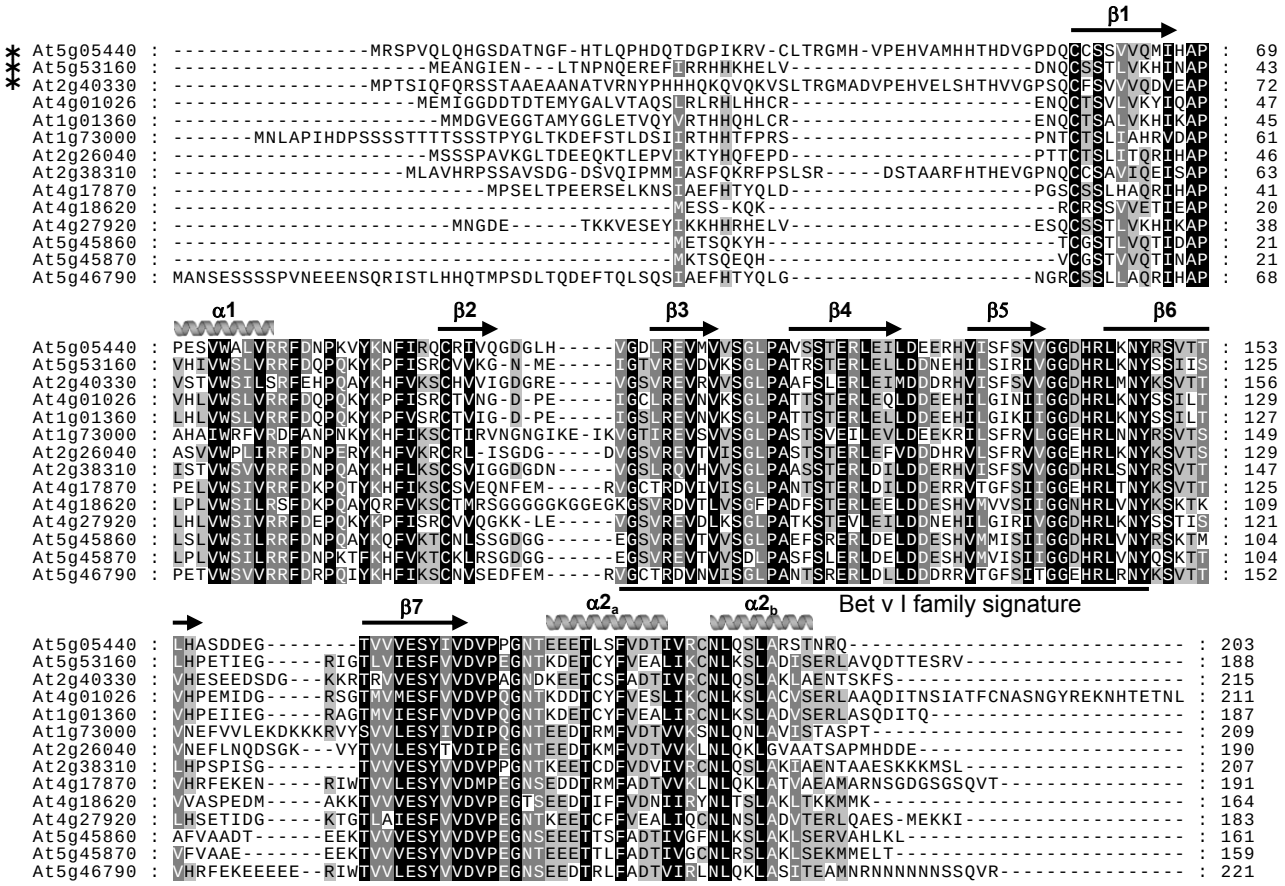
Figure S1a, b

(a)



(b)

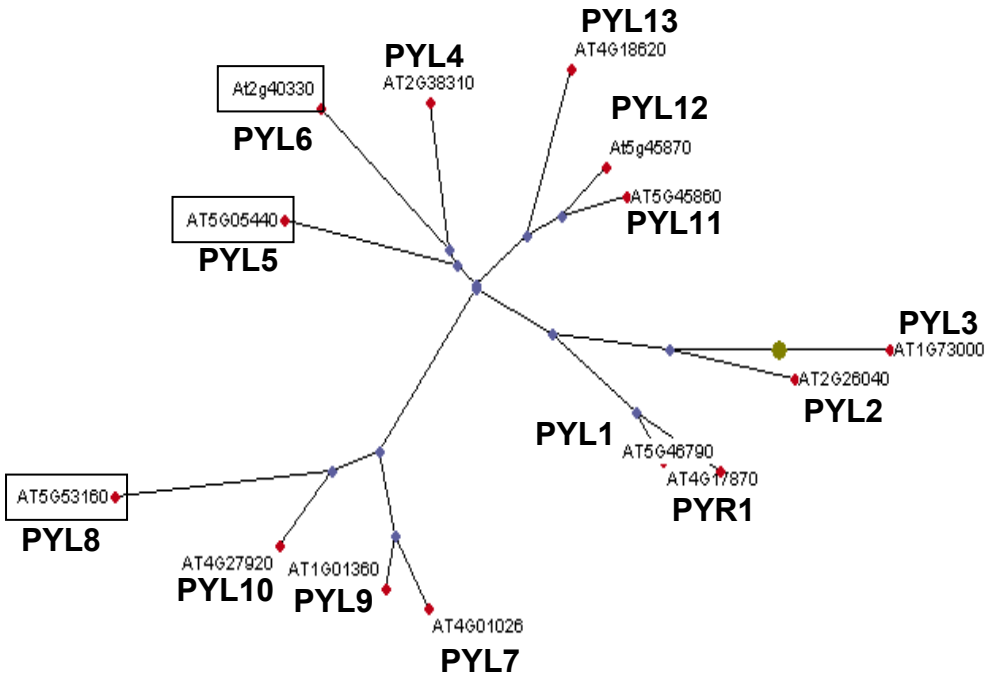


Figure S1c

(c)

```
BetV_I    :  ------------------------------------------------------------MG :   2
At5g05440 :  MRSPVQLQHGS---DATNGFHTLQPHDQTDGPIKRVCLTRGMH-VPEHVAMHHTHDVGPD :  56
At5g53160 :  -MEANGIEN------LTNPNQER-----------------------EFIRRHHKHELVDN :  30
At2g40330 :  MPTSIQFQRSSTAAEAANATVRNYPHHHQK-QVQKVSLTRGMADVPEHVELSHTHVVGPS :  59
At4g17870 :  MPSELTPEERS---ELKNS---------------------------IAEFHTYQLDPG :  28
At2g38310 :  ---MLAVHRPSS--AVSDGDSVQIPMMIASFQKRFPSLSRDST-----AARFHTHEVGPN :  50

                    β1          α1a       α1b          β2          β3
BetV_I    :  VFNYETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGN-GGPGTIKKISFPE :  61
At5g05440 :  QCCSSVVQMIHAPPESVWALV---RRFDNPKVYKNFIRQCRIVQGDGLHVGDLREVMVVS : 113
At5g53160 :  QCSSTLVKHINAPVHIVWSLV---RRFDQPQKYKPFISRCVVKGN--MEIGTVREVDVKS :  85
At2g40330 :  QCFSVVVQDVEAPVSTVWSIL---SRFEHPQAYKHFVKSCHVVIGDGREVGSVREVRVVS : 116
At4g17870 :  SCSSLHAQRIHAPPELVWSIV---RRFDKPQTYKHFIKSCSVEQNFEMRVGCTRDVIVIS :  85
At2g38310 :  QCCSAVIQEISAPISTVWSVV---RRFDNPQAYKHFLKSCSVIGGDGDNVGSLRQVHVVS : 107

                    β4          β5          β6
BetV_I    :  GFPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGG------SIL : 115
At5g05440 :  GLPAVSSTERLEILDEERHVISFSVVGG---DHRLKNYRSVTTLHASDDEG-----TVVV : 165
At5g53160 :  GLPATRSTERLELLDDNEHILSIRIVGG---DHRLKNYSSIISLHPETIEG--RIGTLVI : 140
At2g40330 :  GLPAAFSLERLEIMDDDRHVISFSVVGG---DHRLMNYKSVTTVHESEEDSDGKKRTRVV : 173
At4g17870 :  GLPANTSTERLDILDDERRVTGFSIIGG---EHRLTNYKSVTTVHRFEKEN--RIWTVVL : 140
At2g38310 :  GLPAASSTERLDILDDERHVISFSVVGG---DHRLSNYRSVTTLHPSPISG-----TVVV : 159

                    β7          α2
BetV_I    :  KISNKYHTKGDHEVKAEQVKASKEMG--ETLLRAVESYLLAHSDAYN----  : 160
At5g05440 :  ESYIVDVPPGNTEEETLSFVDTIVRCNLQSLARSTNRQ-------------  : 203
At5g53160 :  ESFVVDVPEGNTKDETCYFVEALIKCNLKSLADISERLAVQDTTESRV---  : 188
At2g40330 :  ESYVVDVPAGNDKEETCSFADTIVRCNLQSLAKLAENTSKFS---------  : 215
At4g17870 :  ESYVVDMPEGNSEDDTRMFADTVVKLNLQKLATVAEAMARNSGDGSGSQVT  : 191
At2g38310 :  ESYVVDVPPGNTKEETCDFVDVIVRCNLQSLAKIAENTAAESKKKMSL---  : 207
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Figure S2

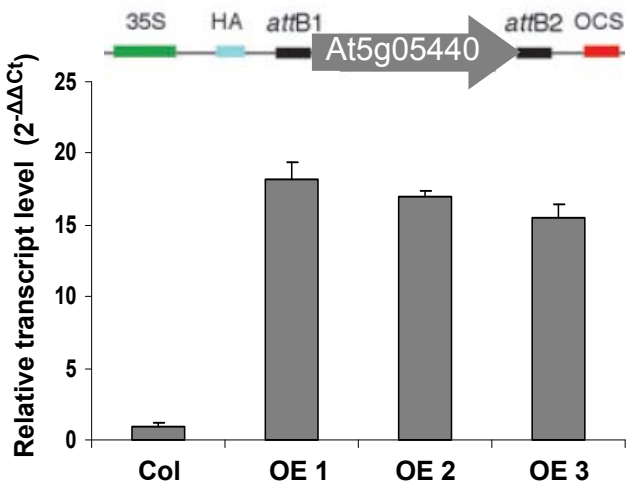


Figure S3

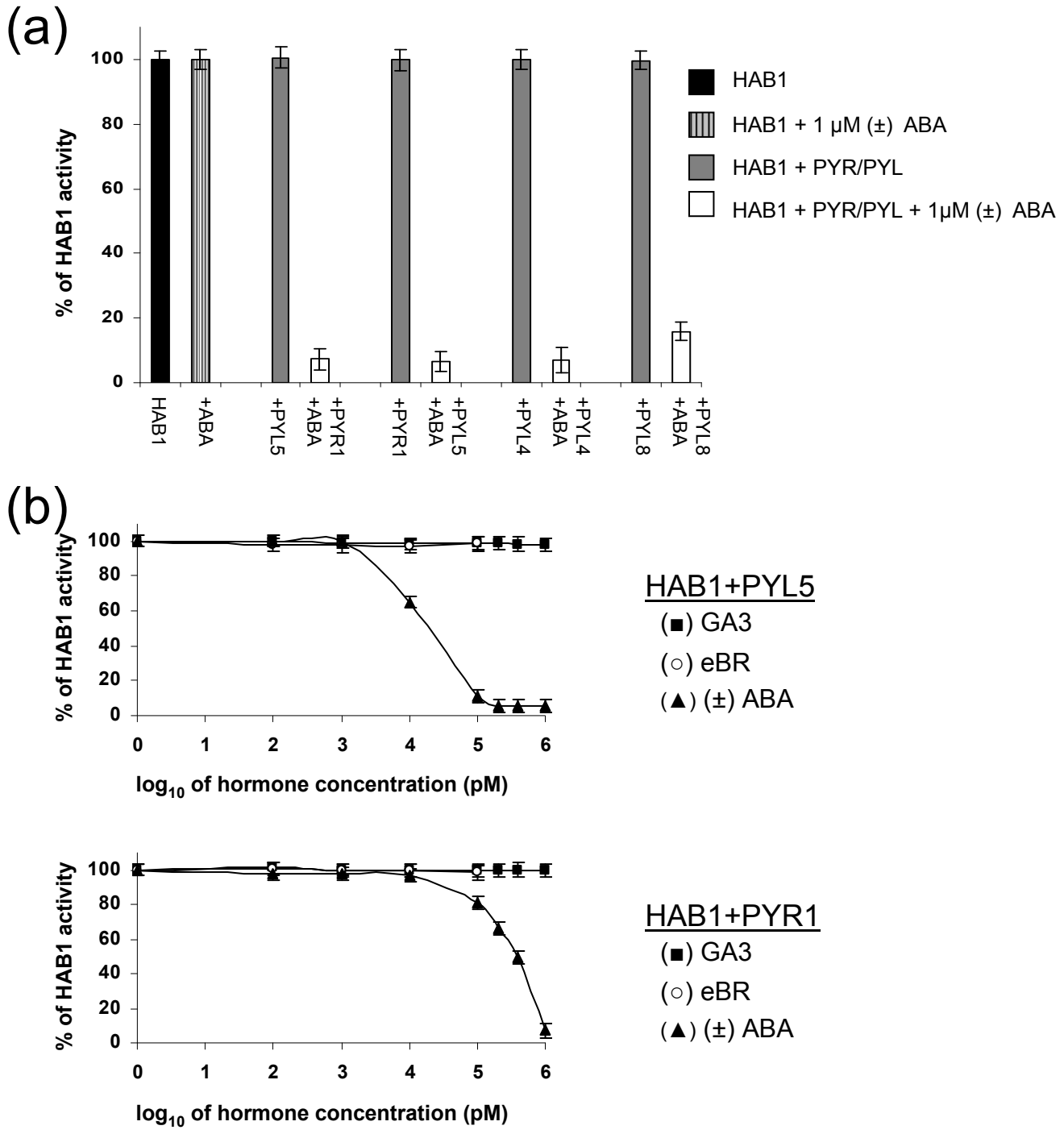

(a)
% of HAB1 activity
100
80
60
40
20
0
HAB1
+ABA
+PYL5
+ABA
+PYR1
+PYR1
+ABA
+PYL5
+PYL4
+ABA
+PYL4
+PYL8
+ABA
+PYL8
HAB1
HAB1 + 1 μM (±) ABA
HAB1 + PYR/PYL
HAB1 + PYR/PYL + 1μM (±) ABA
(b)
% of HAB1 activity
0
1
2
3
4
5
6
log10 of hormone concentration (pM)
HAB1+PYL5
(■) GA3
(○) eBR
(▲) (±) ABA
% of HAB1 activity
log10 of hormone concentration (pM)
HAB1+PYR1
(■) GA3
(○) eBR
(▲) (±) ABA

Figure S4

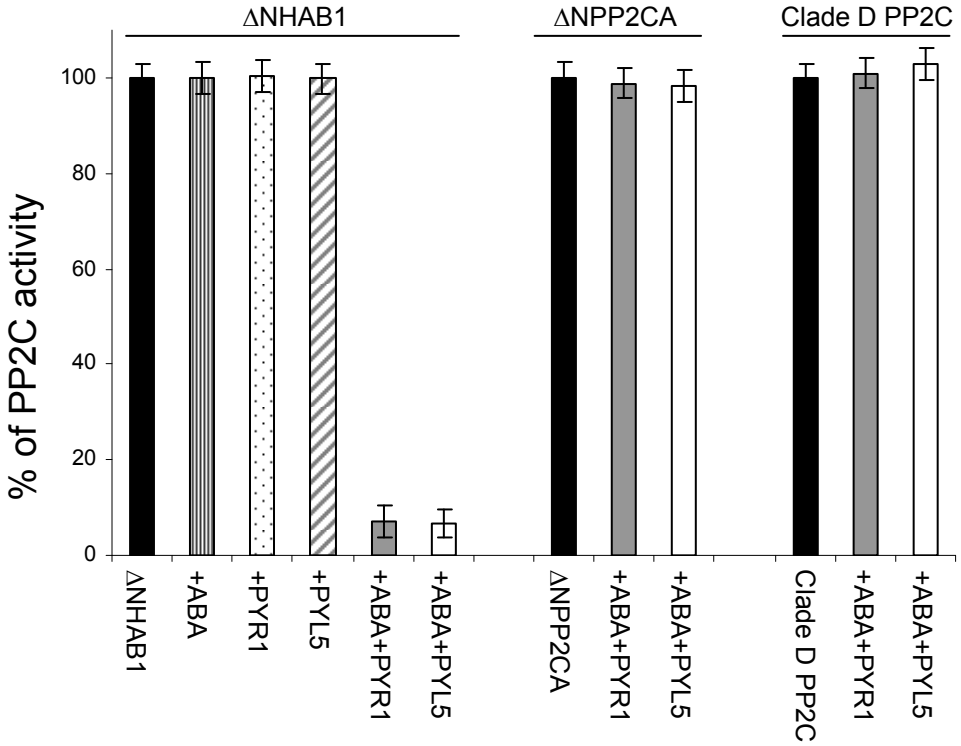
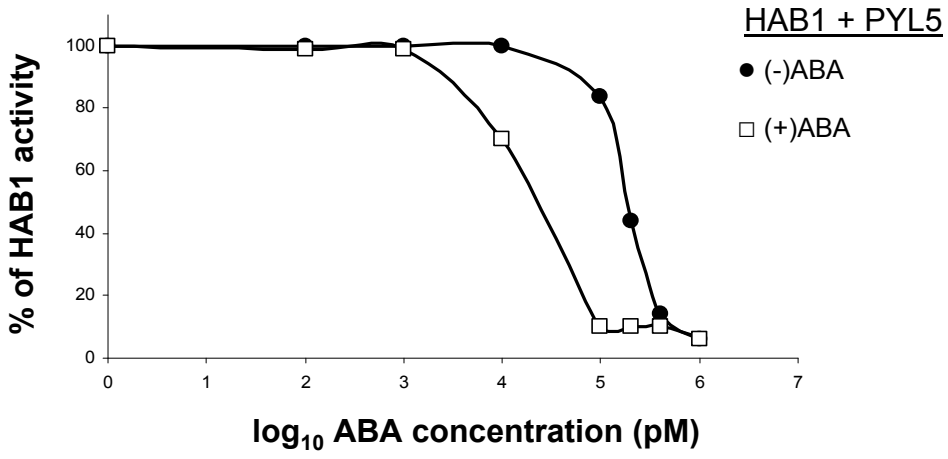


Figure S5



4. RESULTS: CHAPTER 2

The abscisic acid receptor PYR1 in complex with abscisic acid

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LETTERS

The abscisic acid receptor PYR1 in complex with abscisic acid

Julia Santiago[1]*, Florine Dupeux[2]*, Adam Round[2], Regina Antoni[1], Sang-Youl Park[3], Marc Jamin[4], Sean R. Cutler[3], Pedro Luis Rodriguez[1] & José Antonio Márquez[2]

The plant hormone abscisic acid (ABA) has a central role in coordinating the adaptive response in situations of decreased water availability as well as the regulation of plant growth and development. Recently, a 14-member family of intracellular ABA receptors, named PYR/PYL/RCAR[1–3], has been identified. These proteins inhibit in an ABA-dependent manner the activity of a family of key negative regulators of the ABA signalling pathway: the group-A protein phosphatases type 2C (PP2Cs)[4–6]. Here we present the crystal structure of *Arabidopsis thaliana* PYR1, which consists of a dimer in which one of the subunits is bound to ABA. In the ligand-bound subunit, the loops surrounding the entry to the binding cavity fold over the ABA molecule, enclosing it inside, whereas in the empty subunit they form a channel leaving an open access to the cavity, indicating that conformational changes in these loops have a critical role in the stabilization of the hormone–receptor complex. By providing structural details on the ABA-binding pocket, this work paves the way for the development of new small molecules able to activate the plant stress response.

ABA is currently recognized as the pivotal plant hormone that coordinates the complex regulatory network required to cope with water stress[7]. During drought or salt stress, ABA levels in the plant can rise up to 40-fold, triggering the closure of stomata as well as the accumulation of osmocompatible solutes, dehydrins and LEA proteins[7], all of which contribute to the avoidance and tolerance of water stress. ABA can also stimulate root growth to improve water uptake[7]. Group-A protein phosphatases 2C (PP2Cs), such as ABI1, ABI2, HAB1 and PP2CA, are key negative regulators of ABA signalling, and have a critical role in this response[4–6]. Recently, two G-protein-coupled receptors (GPCRs), GTG1 and GTG2, have been identified as plasma-membrane-localized ABA receptors[8]. However, deletion of both proteins impairs, but does not completely abolish, the ABA response, suggesting the existence of alternative ABA receptors[9]. The existence of intracellular ABA receptors was recently confirmed by the discovery of the PYR/PYL/RCAR proteins[1–3]. This protein family contains 14 members in *Arabidopsis thaliana*, some of which show dual cytosolic and nuclear localization, and are able to inhibit the activity of ABI1, ABI2 and HAB1 in an ABA-dependent manner[1–3]. In the absence of PP2Cs, RCAR1 (also known as PYL9) and PYL5 bind ABA with a dissociation constant (K_d) of 0.66 μM and 1.1 μM, respectively; however, in the presence of a PP2C, the affinity increases by more than tenfold, showing a K_d in the nanomolar range (64 nM for the RCAR1–ABI2 pair[2], and 38 nM for PYL5–ΔNHAB1[3]). This has prompted some authors to propose that PYR/PYL/RCAR and PP2Cs could act as co-receptors. The PYR/PYL/RCAR proteins are predicted to contain a Bet v I domain, a versatile scaffold for the binding of hydrophobic ligands[10,11]. Here we describe the crystal structure of the PYR1 protein in complex with ABA at 2.0 Å resolution and elucidate the interactions that stabilize the hormone–receptor complex.

Gel filtration coupled to multi-angle laser light scattering (MALLS)[12] and small angle X-ray scattering analysis show that *Arabidopsis thaliana* PYR1 is a dimer both in the presence and absence of ABA (see Supplementary Fig. 1). MALLS analysis also indicates that the interaction between PYR1 and the catalytic core of the protein phosphatase HAB1 (ΔNHAB1) occurs only in the presence of the hormone with a stoichiometry of 1:1 (see Supplementary Fig. 1). Because PYR1 does not interact with HAB1 in the absence of ABA, our MALLS data indicate that the PYR1 dimer dissociates upon ABA-promoted interaction with the phosphatase. *Arabidopsis thaliana* PYR1 crystals were obtained in the presence of 1 mM (+)-ABA and X-ray diffraction data were collected and refined to 2.0 Å resolution (see Methods for a description of the experimental procedures and Supplementary Table 1 for data collection and refinement statistics). The refined model (R_{work}/R_{free} = 20.0/23.4) contains two similar copies of the PYR1 dimer in the crystal asymmetric unit (see Supplementary Information).

The structure of the PYR1 monomer consists of a Bet v I fold[10,13] plus a 27-amino-acid amino-terminal extension that contains two short helical segments (Fig. 1). The PYR1 Bet v I domain consists of

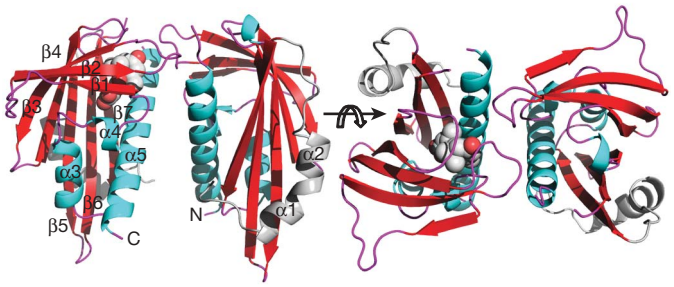

Figure 1 | The structure of the PYR1 dimer. Two different views of the PYR1 dimer are shown. The region corresponding to the Bet v I fold is coloured according to secondary structure (strands in red, helices in cyan and loops in magenta). The 27-amino-acid N-terminal extension containing two short helical segments is depicted in grey. Secondary structure elements as well as the N and C termini are indicated (as in Supplementary Fig. 3). One of the subunits in each of the dimers contains an (+)-ABA molecule (atoms represented as spheres) in the binding pocket, whereas the second subunit is empty.

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seven strands forming a bent β-sheet and producing a central cavity that resembles that of a folded hand. The walls of the cavity are completed by a long carboxy-terminal α-helix (α5) juxtaposed to the β-sheet. Two small helices (α3 and α4) between strands β1 and β2 close the bottom side of the cavity. The dimerization region involves residues in loops α4–β2 and β3–β4 and the N-terminal part of helix α5 (see Fig. 1 and Supplementary Fig. 3).

A continuous electron density cloud that could not be attributed to either protein or solvent molecules was observed inside the cavity of one of the subunits of each of the dimers (see Fig. 2a). This density could be unambiguously interpreted as a molecule *S*-2-*cis*-4-*trans* abscisic acid. The structure of the PYR1-bound (+)-ABA is similar to that of crystalline ABA[14]. The carboxylic and diene groups, in the isoprenoid moiety, are found in a plane which is orthogonal to the plane formed by the cyclohexenone ring. The cyclohexenone ring is puckered, with the C6′ carbon below the plane of the ring.

ABA is almost completely buried inside the PYR1 cavity and stabilized by a series of interactions that perfectly match the polar and hydrophobic character of its different functional groups (see Fig. 2). Most of the polar interactions between the protein and ABA are mediated by water molecules. Only two direct polar contacts are found and they involve the amine group of Lys 59 and the backbone amide of Ala 89, which interact with ABA's carboxylic and ketone groups, respectively. A network of water molecules is very favourably coordinated between the hydroxyl and carboxylic groups of the ABA molecule and the side chains of Glu 94, Tyr 120, Ser 122 and Glu 141 (see Fig. 2). Adding to this, the backbone amide of Arg 116 establishes a water-mediated hydrogen bond with the oxygen in the ketone group of ABA. This set of ABA-contacting residues is invariant in the PYR/PYL/RCAR protein family with the exception of Lys 59, which seems to be replaced by glutamine in PYL13 (see Supplementary Fig. 3). The functional relevance of these contacts is supported by genetic studies which show that the E94K and E141K substitutions confer resistance to pyrabactin[1], a selective ABA agonist.

Stereospecificity in the hormone–receptor interaction seems to be contributed mostly by steric constraints imposed by hydrophobic residues that conform the upper part of the PYR1 cavity to match accurately the van der Waals surface of the (+)-ABA molecule rather than through polar interactions (Fig. 2b). The mono-methyl group at position C2' is confined in a tight space between Phe 61, Val 163 and Val 83, while the cavity is significantly wider at the opposite side, where the much larger dimethyl group at C6' finds an optimal fitting. Phe 61 is strictly conserved whereas Val 163 and Val 83 are highly conserved or replaced by bulkier isoleucine and leucine residues in other PYL proteins (see Supplementary Fig. 3). The cavity in PYR1 is significantly larger than ABA, which occupies its upper part (see Fig. 3). The side chains of Lys 59 and Arg 79 seem to limit the access of the ligand to the lower part of the cavity, which contains a number of water molecules. Ligand binding studies reported here and elsewhere indicate that PYR/PYL/RCAR proteins have a stoichiometry of ABA-binding close to 1:1 both for isolated receptors as well as in the presence of the phosphatases, suggesting that in solution both subunits are able to bind the hormone. Therefore, it is likely that the alternative configuration observed is favoured by crystal packing.

The overall structure of the two subunits in the PYR1 dimer is very similar; however, notable differences appear in residues 84–89, 113–118

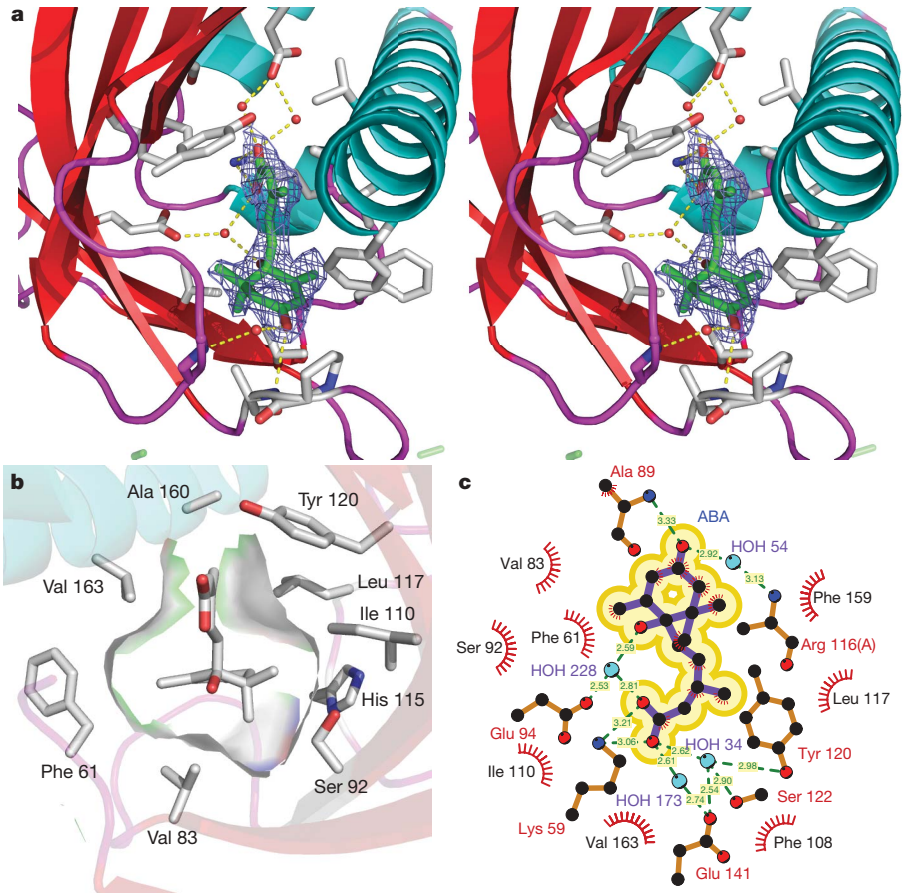

Figure 2 | Abscisic acid binding. **a**, Stereo representation of the ABA binding site showing omit electron density (blue mesh) around the ligand region. Abscisic acid is represented as sticks. Major polar interactions between ABA and the protein are indicated (yellow dotted lines). **b**, The protein surface in the PYR1 cavity conforms tightly to the shape of the ABA molecule (centre) favouring binding of the (+) enantiomer. **c**, Detailed description of the interactions generated with the program Ligplot. Hemispheres represent hydrophobic interactions whereas lines represent polar interactions. HOH indicates water molecules. The yellow ribbon around the ABA molecule indicates low solvent accessibility.

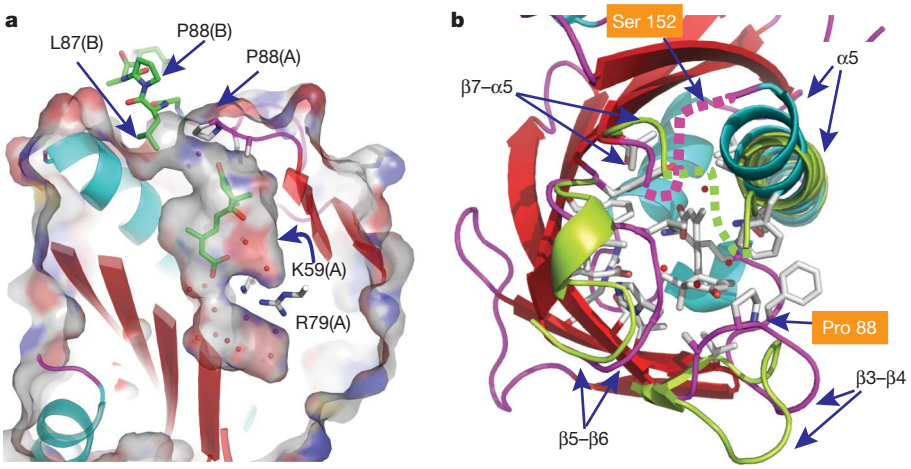

Figure 3 | Conformational changes in the loops surrounding the ABA binding cavity. a, The PYR1 ligand-binding cavity is shown. Key amino acids are labelled; the letters in parenthesis refer to subunits A or B of the dimer. Water molecules are indicated as red spheres. **b**, The ligand-bound and free forms of PYR1 have been superimposed. The hormone-bound form is coloured according to secondary structure (as in Fig. 1). The corresponding loops of the hormone-free molecule and the C-terminal part of helix α5 showing a different conformation are displayed in light green. The positions of loops β3–β4, β5–β6 and the N-terminal region of α5 are highlighted. The approximate positions of loop β7–α5 (disordered in the structure) are indicated by dotted lines. In the ABA-bound form of PYR1, the loops surrounding the entry to the cavity fold over the ABA molecule, trapping it inside. The same loops in the unliganded form adopt an open conformation that leaves the cavity accessible to the solvent. The positions of residues Pro 88 and Ser 152 involved in interactions with the PP2Cs are indicated.

and 153–159 located in loops β3–β4 and β5–β6 and the N-terminal part of helix α5, respectively. These loops surround the upper part of the PYR1 cavity and in the ligand-bound form adopt a closed conformation folding over the ABA molecule and isolating it almost completely from the solvent as shown in Fig. 3. Notably, in the ABA-free subunit, these loops adopt an open conformation that creates a passage into the cavity. The closed conformation of the loops in the ABA-bound subunit is stabilized by hydrophobic and polar interactions between residues in the loops and the cyclohexenone moiety of the ABA molecule (see Fig. 3). The equivalent residues are exposed to the solvent in the ABA-free subunit. The β3–β4 loop (amino acids 84–89) of the empty subunit points towards the ABA-bound subunit rather than towards the inside of the cavity, with Leu 87 inserted in a hydrophobic pocket formed by loops β3–β4, β5–β6 and the N-terminal part of helix α5 of the ABA-bound molecule (see Fig. 3a). This probably stabilizes both the open conformation of this loop and the closed conformation of the ABA-bound molecule. The conformational changes of the loops flanking the cavity's entry probably have a role similar to a lid stabilizing the ligand inside the cavity, as the subunit showing the open conformation remains empty, despite the fact that the residues in the inner part of the cavity adopt a configuration favourable for ABA binding.

Point mutations P88S and S152L located in the β3–β4 and β7–α5 loops, respectively, cause a reduction in the capacity of PYR1 to interact with and inhibit the protein phosphatase HAB1[1]. Isothermal titration calorimetry measurements show that the P88S mutation does not impair ABA binding, but significantly decreases the stability of the ternary complex ABA–PYR1(P88S)–PP2C as compared to the wild type (see Supplementary Information). This suggests that the loop β3–β4 is not only important for the stabilization of the ligand, as the PYR1 structure suggests, but that it is also involved in interactions with the PP2Cs. Similarly, Ser 152 is located in the loop β7–α5, which is disordered in this structure, and does not contact the ABA molecule, indicating again that the loops surrounding the entry to the ABA binding cavity are required for direct interactions with the phosphatases. On the basis of the genetic, biochemical and structural data, we propose that the interactions between the phosphatase and lid in its closed conformation stabilize the ternary ABA–receptor–PP2C complex, which could also explain the increased affinity of PYR1 for ABA in the presence of the phosphatase. Alternatively, the dissociation of the PYR1 dimer and the formation of the PYR1–phosphatase complex could lead to a reorganization of the PYR1 ABA-binding site.

The structure described here shows precise details on the specific interactions between ABA and PYR1, confirming the role of the PYR1 protein as an ABA receptor. Our observations also confirm that the major determinants of stereoselectivity are spatial constraints around positions 2 and 6 in the ring of the ABA molecule, rather than interactions involving polar groups, which as already suggested[15], may explain the striking biological activity of the unnatural (−)-ABA stereoisomer. The structural details of the ABA–receptor interactions described here pave the way for the development of agonist molecules that could be useful to cope with water stress in crops.

METHODS SUMMARY

PYR1, PYR1(P88S) and ΔNHAB1 were expressed and purified as described previously[3,16]. Crystallization conditions for PYR1 were identified at the High Throughput Crystallization Laboratory of the EMBL Grenoble Outstation (https://htxlab.embl.fr). The crystals used for data collection were obtained by the vapour diffusion method with a protein concentration of 25 mg ml^{-1} in the presence of 0.2 M sodium magnesium acetate, 0.1 M sodium cacodylate pH 6.5 and 18% PEG 8000 and 1 mM (+)-ABA and were frozen with 20% glycerol as cryo-protectant. X-ray data were collected at the ID14eh4 beamline of the ESRF. XDS[17], Coot[18] and REFMAC[19] programs were used for crystallographic analysis. Initial phases were obtained by the molecular replacement method with the model of the XoxI protein (3CNW) and the program Phaser[20]. Small angle X-ray scattering data were collected at the BioSAXS station (ID14eh3) of the ESRF. Samples were exposed using 30-μl volumes loaded into a quartz capillary mounted in vacuum and moved through the beam during exposure to minimize the effect of radiation damage. The scattering from the buffer was measured before and after each sample measurement and the average scattering used for background subtraction with PRIMUS[21]. The theoretical scattering curves from the three possible dimeric PYR1 ensembles were calculated and fitted to the merged experimental scattering curve for PYR1 using the program CRYSOL[22]. MALLS and isothermal titration calorimetry experiments were carried out as described previously[3,12]. Figures were generated with Pymol (DeLano Scientific LLC) and Ligplot[23].

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

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

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Author Contributions J.S. contributed with the cloning, protein purification, ITC, MALLS and helped with crystallization and SAXS experiments. F.D. performed protein purification, crystallization and crystal refinement experiments and helped with X-ray data collection. A.R. supervised SAXS data collection and performed data analysis. M.J. carried out MALLS experiments and analysis. R.A. carried out cloning and protein purification. S.-Y.P. and S.R.C. carried out cloning of mutant PYR1 proteins and contributed to discussions. P.L.R. contributed to discussions and writing of the manuscript. J.A.M. supervised the work and performed data collection, structure solution and refinement as well as writing of the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structure have been deposited in the Protein Data Bank under accession code 3K90. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.A.M. (marquez@embl.fr).

METHODS

Cloning, expression and purification of PYR1, PYR1(P88S) and ΔNHAB1 recombinant proteins. The ORF *Arabidopsis PYR1* was amplified by polymerase chain reaction (PCR) from a pUni clone obtained from ABRC, using FwAt4g17870 ACCATGGCTTCGGAGTTAACACC and RvAt4g17870 TCACG TCACCTGAGAACCACT primers and cloned to pCR/GW/TOPO vector. The coding sequence was excised using NcoI–EcoRI digestion and cloned into pETM11. PYR1(P88S) was produced as described previously[1]. The ΔNHAB1 construct was obtained according to ref. 3. BL21 (DE3) cells transformed with the corresponding pETM11 or pTE28 constructs were grown in 1 l of LB medium containing 50 μg ml^{-1} kanamycin to an optical density at 600 nm of 0.6–0.8. Then, 1 mM IPTG was added and induction was carried out over night at 20 °C. Cells were harvested 12 h after induction and stored at −80 °C before purification. Purification was carried out at 4 °C. A 2-l pellet was re-suspended in 100 ml of Lysis buffer (20 mM Tris pH 7.5, 300 mM NaCl, 15 mM Imidazol, 2 mM mercaptoethanol and Merk's protease cocktail inhibitor) and the cells were sonicated in a Misonix Sonifier. A cleared lysate was obtained after centrifugation at 20,000*g* for 45 min and protein was loaded to a 1-ml Ni HiTrap HP column using the AKTA Prime system. The protein extract loaded to the Ni HiTrap HP column was washed with 15 ml of wash buffer (20 mM Tris pH 7.5, 300 mM NaCl and 1 mM mercaptoethanol). The bound protein was then eluted with a gradient from 0–100% of elution buffer (20 mM Tris pH 7.5, 300 mM NaCl, 1 mM mercaptoethanol and 250 mM Imidazol). The fractions containing the protein were then dialysed for 2 h in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM $MnCl_2$ and 1 mM mercaptoethanol (dialysis buffer) at 4 °C, right after purification. The protein was then passed through a gel filtration step using a S200 column. Protein concentration was carried out with Amicon Ultra Centrifugal Filters (Millipore) and flash-frozen with liquid nitrogen. In the case of cleaved protein, the protein collected from the Ni purification was incubated with TEV protease, in a ratio of 1:100, for 2 h at 4 °C. Then, the protein was dialysed for 2 h in 20 mM Tris pH 7.5, 150 mM NaCl, 15 mM Imidazol and 1 mM mercaptoethanol at 4 °C. Second, Ni purification was carried out using a 1 ml Ni HiTrap HP column and an AKTA Purifier System. The cleaved protein was collected from the flow through. A gel filtration step, using a S200 column, was performed next with 20 mM Tris pH 7.5, 150 mM NaCl and 1 mM mercaptoethanol buffer. Concentration and flash freezing was carried out as described above. In the case of ΔNHAB1, 2 mM $MnCl_2$ was added to all the buffers.

Crystallography. Initial crystallization conditions for PYR1 were identified at the High Throughput Crystallization Laboratory of the EMBL Grenoble Outstation (https://htxlab.embl.fr). The crystals used for data collection were obtained by the vapour diffusion method with a protein concentration of 25 mg ml^{-1} in the presence of 0.2 M sodium magnesium acetate, 0.1 M sodium cacodylate pH 6.5, 18% PEG 8000 and 1 mM (+)-ABA, and were frozen with 20% glycerol as cryo-protectant. X-ray data were collected at the ID14eh4 beamline of the ESRF. XDS[17] was used for data processing. Initial phases were obtained by the molecular replacement method with the model of the XoxI protein (3CNW) deposited in the Protein Data Bank using the program Phaser. Model building and refinement was carried out using the programs Coot and REFMAC[19].

Small angle X-ray scattering experiments. Small angle X-ray scattering (SAXS) data were collected at the BioSAXS station (ID14-3) of the ESRF (http://www.esrf.fr/UsersAndScience/Experiments/MX/About_our_beamlines/ID14-3). ID14-3 is a fixed-energy (13.32 keV, $\lambda = 0.931$ Å) station optimized for solution scattering from biological macromolecules. Samples are exposed using 30-μl volumes loaded into a 2-mm quartz capillary mounted in vacuum using an automated robotic system which enables the sample to be moved through the beam during exposure to minimize the effect of radiation damage. The measurement cell is cleaned (using a commercial detergent for quartz capillaries), rinsed (with distilled water) and dried using compressed air after each measurement using the automated system to minimize cross contamination and dilution of the samples. Two-dimensional scattering images were collected using the Vantec2000 gas-filled detector from Bruker at a distance of 1.745 m from the sample. Standard data collection time of 5 min was used for all samples split into ten 30-s time frames to assess and remove effects from radiation damage to the samples. Individual time frames are processed automatically and independently by the data collection software (BsxCUBE) developed by the BLISS team at the ESRF, yielding individual radially averaged curves of normalized intensity versus scattering angle ($s = 4\pi \mathrm{Sin}\theta/\lambda$). Time frames are combined, excluding any data points which are affected by aggregation induced by radiation damage, to give the average scattering cure for each measurement. The scattering from the buffer alone was measured before and after each sample measurement and the average of the scattering before and after each sample is used for background subtraction using the program PRIMUS[21] from the ATSAS package developed by EMBL Hamburg. A range of PYR1 protein concentrations (1.3 to 7.1 mg ml^{-1}) was measured to assess and remove any concentration-dependant inter-particle effects. PYR1 was measured with 1 mM ABA and excess ABA and no significant differences in the scattering were observed with increasing ABA concentration. The theoretical scattering from the different dimer configurations was fitted to the merged scattering curve (free from effects of radiation damage and concentration-dependant inter-particle effects) for PYR1 with 1 mM ABA using the program CRYSOL[22].

Isothermal titration calorimetry (ITC) experiments were performed as described[3] using a VP-ITC calorimeter equipped with the control, data acquisition and analysis software ORIGIN 7 (Microcal).

SEC–MALL experiments. Multi-angle laser light scattering (MALLS) coupled to size exclusion chromatography was performed as follows. Size-exclusion chromatography (SEC) was carried out with an S200 Superdex column (GE Healthcare) equilibrated with 20 mM Tris-HCl, 150 mM NaCl and 1 mM β-mercaptoethanol buffer. On-line MALLS detection was performed with a DAWN-EOS detector (Wyatt Technology Corp.) using a laser emitting at 690 nm and by refractive index measurement using an RI2000 detector (Schambeck SFD). Weight-averaged molar masses (M_w) were calculated using the ASTRA software (Wyatt Technology Corp.)[12].

Supplemental Table 1. **Summary of crystallographic analysis**

Data Collection	
Space group	P1
Unit cell a,b,c, (Å)	49.81, 61.28, 72.5
α,ß,γ	105.6, 102.2, 89.9
Resolution	2.0 (2.1 - 2.0)
Rsym (%)[a]	4.0 (26.4)
Completeness	93.5(86.9)
I/σ(I)	24.19 (5.16)
Refinement	
Resolution Range (Å)	25.0 - 2.0
No Reflections	196,698
No Unique refl.	50,927
Rwork(%)[b]	20.0 (23.0)
Rfree(%)[b]	23.4 (28.7)
No Atoms	
Protein	5,637
Ligand	38
Solvent	555
R.m.s. deviations	
Bond Length	0.010
Angles	1.48

Numbers in parenthesis refer to the highest resolution shell
[a] As defined by XDS[17]
[b] As defined by REFMAC[19]

The crystallographic asymmetric unit contains two PYR1 dimmers.

The refined crystallographic model of PYR1 contains 4 monomers in the asymmetric unit indicated as A to D in supplemental figure S2A. Analytical size exclusion chromatography in combination with multi-angle laser light scattering (MALLS)[1] demonstrates that PYR1 is a dimer in solution (see supplemental figure S1). In accordance with this, monomers A and B show extensive intermolecular contacts grouped in a single continuous area of over 700 $Å^2$ per

monomer. These contacts involve the side chains of both polar and hydrophobic residues (see supplemental figure S3), both surfaces are rugged and show spatial complementary. Moreover, the ensemble AB can be superimposed on ensemble CD, through a single non- crystallographic two fold pseudo-symmetry axis, all of which strongly indicated that ensembles AB (or CD) correspond to the dimer observed in solution. However, in addition to the molecular interactions described above, a second extensive interaction region can be observed between molecules B and D in the crystal. This involves in large part hydrogen bonds between backbone atoms generated by the apposition of strand ß1 from both molecules to form an anti-parallel beta sheet (see supplemental figure S2A). The nature of the intermolecular contacts in the BD ensemble and the fact that no single operator can superimpose it into the remaining subunits AC suggested this interaction is caused by the crystallization process. To determine unambiguously which of the ensembles corresponds to the PYR1 dimer, we performed Small Angle X-ray scattering experiments (SAXS) with PYR1 in solution at various concentrations in the presence of 1mM (+) ABA and compared it with the expected scattering curves for all possible ensembles (see Methods section). As shown in supplemental figure S2B, the computed theoretical scattering curve for ensemble AB yields a satisfactory fit (χ=0.72) to the experimental data, while ensembles BD and AC diverge significantly (χ=1.15 and χ=4.02 respectively), hence we conclude that PYR1 is a dimer in solution that corresponds to ensemble AB (and CD) in the crystal structure.

The P88S mutation in the ß3-ß4 loop of PYR1 destabilizes the ABA-PYR1^{P88S}-PP2C ternary complex.

It has been shown that point mutations P88S shows a reduced capacity to both interact and inhibit the protein phosphatase HAB1, while it seems to retain it's capacity to bind ABA[2]. We determined ABA binding affinities for wild type PYR1 and PYR1^{P88S} in the presence and absence of the catalytic core of the protein phosphatase HAB1 (ΔNHAB1). As shown in supplemental table 1 the affinity of PYR1 for ABA falls below the measurable range (due to limited solubility of ABA at the pH of the assay) while it shows a Kd of 20 nM in the presence of ΔNHAB1. This increase in affinity is in agreement with previous findings for other members of the family[3,4]**.** The mutant PYR1^{P88S} shows an affinity for ABA of 48.0 µM, however, in the presence of ΔNHAB1 the measured Kd is 0.47 µM, more than 20 times higher than that of the wt-PYR1/ΔNHAB1 pair. These results confirm that the mutant PYR1^{P88S} is capable of binding ABA, even with moderately higher affinity than the wild type PYR1, and that its reduced capacity to interact with the phosphatase shown previously[2] correlates with a decrease in the stability of the ternary complex ABA-PYR1^{P88S}-PP2C. This suggests that the loop ß3-ß4, is not only important for the stabilisation of the ligand, as the PYR1 structure suggests, but that it is also involved in interactions with the PP2Cs.

Supplemental Table 2. **ABA binding affinities of wt and P88S mutant Pyr1 proteins**.

	Ka (μM^{-1})	Kd (μM)	*ΔH* (kcal/mol)	*ΔS* (cal/mol·K)	N
PYR1	Not detected	Not detected	Not detected	Not detected	Not deteced
$PYR1^{P88S}$	0.0204±0.0015	48.9 ±1.73	3.117 ± 0.854	9.21± 3.3	1.076± 0.13
PYR1+ΔNHAB1	47.61±0.325	0.021±0.009	-10.330± 1.823	-4.41± 1.2	0.7215± 0.08
$PYR1^{P88S}$+ΔNHAB1	2.09± 0.35	0.478± 0.028	-11.355± 0.615	-8.81± 2.33	0.7755± 0.16

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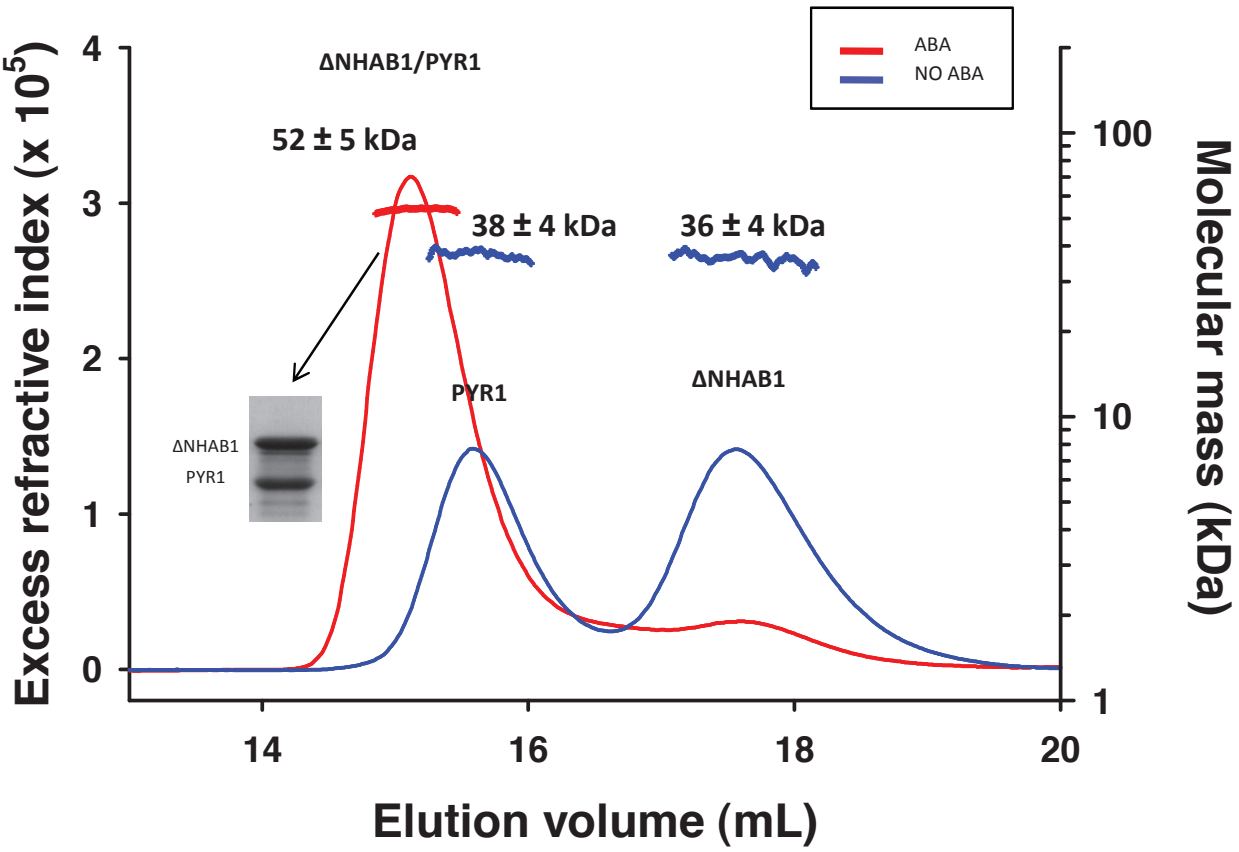

Supplemental Figure S1: Analysis of the interaction between PYR1 and HAB1 by SEC-MALLS. Size exclusion chromatography (SEC) combined with multi-angle laser light scattering (MALLS) and refractometry (RI) is a powerful method for measuring the absolute molecular mass of macromolecules and macromolecular complexes [1,2]. The determination of the molecular mass from light scattering intensity is absolute and thus completely independent of the migration on the SEC column. The separation by SEC eliminates small amounts of aggregates and allows analyzing mixtures of molecules as long as they can be separated on the column. The determination of the molecular mass variation across the chromatographic peak also provides an estimate of the dispersity of the compound. Size-exclusion chromatography (SEC) was performed with an S200 Superdex column (GE Healthcare) equilibrated with buffer containing

20mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM $MnCl_2$ and 1mM β-mercaptoethanol. On-line Multi-Angle Laser Light Scattering (MALLS) detection was performed with a DAWN-EOS detector (Wyatt Technology Corp., Santa Barbara, CA) using a laser emitting at 690 nm and by refractive index measurement using an RI2000 detector (Schambeck SFD)[2]. Weight-averaged molar masses (M_w) were calculated using the ASTRA software (Wyatt Technology Corp., Santa Barbara, CA). The chromatographic elution profile of an equimolar mixture of PYR1 and HAB1 (blue line) indicates that the proteins do not interact in the absence of ABA. PYR1 elutes at 15.8 mL and HAB1 at 17.6 mL. The blue crosses show the molecular masses of the two proteins, indicating that PYR1 is dimeric, whereas HAB1 is monomeric (expected masses of the monomeric forms are 21.5 kd and 37.4 kd respectively) . This experiment nicely highlights the power of the SEC/MALLS methods, since the proteins have similar molecular masses although they elute very differently from the SEC column. Addition of 1 mM (+) ABA to the mixture induces the formation of a complex between the two proteins, which elutes as a single peak at 15.1 mL (red line), the presence of the two proteins in the complex is demonstrated by PAGE-SDS analysis of the peak fractions and blue coomassie staining (inset). The molecular mass of 52 ± 4 kDa (red crosses) indicates a 1:1 complex between PYR1 and HAB1, thus revealing that, in the presence of ABA, binding of HAB1 induces the dissociation of the PYR1 dimers.

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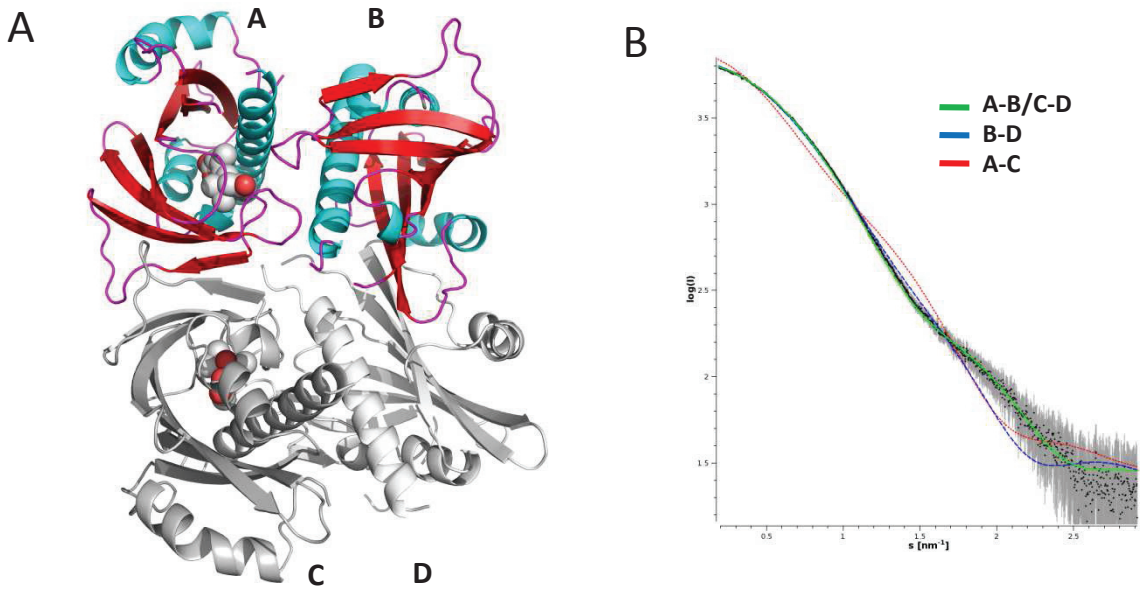
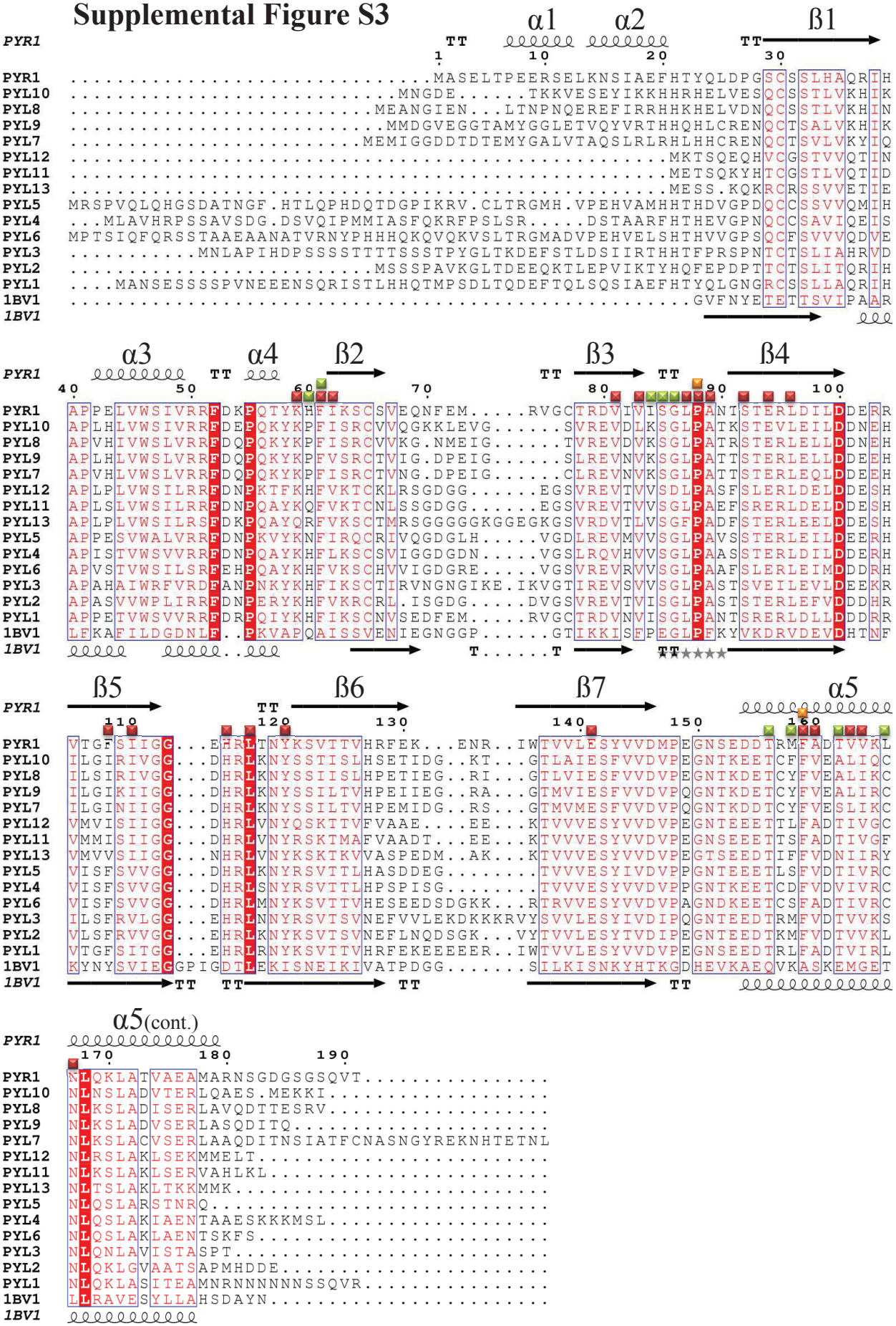


Supplemental Figure S2: The crystallographic asymmetric unit contains two PYR1 dimmers. **A,** PYR1 crystals contain four molecules in the asymmetric unit. The ensemble A-B is coloured according to secondary structure (strands in red, helices in cyan and loops in magenta). The subunits C and D are shown in gray and light gray. ABA molecules are shown with atoms represented as spheres with oxygen atoms in red. **B**, Experimental X-ray scattering data (black dots) with error bars (gray) of the PYR1 protein in solution in the presence of 1mM (+) ABA. The theoretical scattering curves calculated for possible ensembles AB/CD (green), BD (blue) and AC (red) are shown. Only the curve for ensembles AB/CD produced a good fit to the experimental data (χ=0.72) demonstrating that ensemble AB corresponds to the one found in solution.

Supplemental Figure S3



Supplemental Figure S3: Multiple Sequence alignment of the *Arabidopsis thaliana* PYR1/PYL/RCAR proteins. The sequence of the related Bet v I protein is included. The secondary structure of PYR1(top) and Bet v I are indicated. Aminoacids involved in interactions with the Abscisic acid (red squares) and dimerization (green squares) are indicated. The orange squares indicate aminoacids involved in contacts with Leu 87 of the unliganded dimer.

5. RESULTS: APPENDIX CHAPTER 2

Data published in:

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

Plant Physiology (2011) **156**, 106–116 (doi: 10.1104/pp.110.170894)

Dupeux F[*], Antoni R[*], Betz K[*], Santiago J[*], Gonzalez-Guzman M, Rodriguez L, Rubio S, Park SY, Cutler SR, Rodriguez PL and Márquez JA.

([*]) These authors contributed equally to this work

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

Comparative structural analysis between the PYR1 ABA-bound and ABA-free subunits (Santiago et al., 2009B) has revealed key interactions between the hormone and the receptor, as well as induced conformational changes upon ABA binding. These conformational changes are induced in the β3-β4 (S_{85}GLPA_{89}), β5-β6 (H_{115}RLT_{118}) and β7-α5 (M_{147}PEGNSEDDTRM_{158}) loops that surround the upper part of the cavity. In the ABA-bound subunit, the β3-β4 and β5-β6 loops function as gates, adopting a closed conformation and trapping the ligand inside the cavity. The β7-α5 loop is also implicated in stabilizing the closed conformation (Santiago et al., 2009B). In addition, these loops not only stabilized the hormone inside the pocket, but they seemed to be implicated in the interaction with the PP2C (Park et al., 2009; Santiago et al., 2009B). This prediction has been later on corroborated by the resolution of the ternary complex PYR1-ABA-HAB1 (Dupeux et al., 2011) and by analogy with other ternary complex structures resolved such as PYL1-ABA-ABI1 (Miyazono et al., 2009; Yin et al., 2009) and PYL2-ABA-HAB1 (Melcher et al., 2009). To further study the biological relevance of ABA interactions observed in the PYR1 structure, and the role of the surrounding loops in the interaction with the HAB1 PP2C, we performed a mutational analysis on the ABA receptor PYR1.

In order to test the importance of ABA binding for the PYR1 inhibitory capacity, we mutated amino acids that are directly involved in ABA binding such as Glu94Lys, Glu141Lys and Tyr120Ala. The side chains of these residues establish interactions with the hydroxyl and carboxyl groups of the ABA molecule through water molecules, stabilizing the hormone inside the cavity (Santiago et al., 2009B). According to the structure, mutation of these residues would strongly impair the binding of the molecule.

Selection of residues from the surrounding loops was first based on the shift movements upon binding of the hormone, which could promote interactions with the PP2C, and the establishment of interactions with the ABA molecule. Mutations are listed below (see Table 1).

PYR1 mutations were first tested for their capacity to interact with HAB1, through yeast two hybrid assays (Y2H), and to inhibit HAB1 phosphatase activity by *in vitro* phosphatase activity assays (Figure 1 and 2). As it can be seen in figure 1A,

PYR1 mutations either completely abolished or strongly impaired the ABA-mediated interaction with HAB1 as compared to the wild type. Similarly, PYR1 mutations had lost or reduced their capacity to inhibit HAB1 phosphatase activity in an ABA-dependent manner (Figure 1B, 2). The only exception was the PYR1^{R157H} mutation, located and the N-terminal of the α5 helix. Although this mutation was isolated in a pyrabactin (a seed ABA-agonist) resistance screening and its interaction with HAB1 was abolished in the presence of pyrabactin as compared to the wild type (Park et al., 2009); here, its phenotype resembled the one from the PYR1 wild type in the presence of ABA, showing very little effect in the interaction assay and in the reduction of its inhibitory capacity (Figure 1A, 1B and 2).

Table 1. Mutacions in PYR1 receptor

Location	Mutations
Binding pocket	Glu94Lys, Glu141Lys,Tyr120Ala
In the β3-β4 loop	Ser85Ala, Leu87Ala, Pro88Ser
In the β5-β6 loop	Arg116Ala
In the β7-α5 loop	Ser152Leu, Arg157His

PYR1 mutations were also tested for their effect on OST1 kinase activity (Figure 1C and 1D). In order to test this, we reproduced the system described by Fujii and co-workers (2009) in which they achieved reconstitution of the ABA signaling pathway *in vitro* by combining PYR1, PP2C, SnRK2.6/OST1 and ABF2. In this system, OST1 activity is measured both by auto-phosphorylation and phosphorylation of its downstream target ABF2. Figure 1C shows that HAB1 dephosphorylates OST1, inhibiting its kinase activity (see lanes 2 and 3), and therefore no ABF2 phosphorylation is detected (Figure 1D lane 2 and 3). Similarly, when we add PYR1 in the absence of ABA, HAB1 is still able to dephosphorylate OST1 being completely insensitive to the presence of the receptor (see lane 4). However, when PYR1 and ABA are added, the receptor is now able to inhibit HAB1 inactivating it. As a result, OST1 activity is recovered (see lane 5), leading to ABF2 phosphorylation (Figure 1D lane 5). In the case of the PYR1 mutants assayed, except for PYR1^{R157H}, they showed a significant reduced capacity to inactivate HAB1 to prevent OST1 dephosphorylation, having lost

their capacity to accomplish ABA-dependent recovery of OST1 kinase activity as compared to the PYR1 wild type.

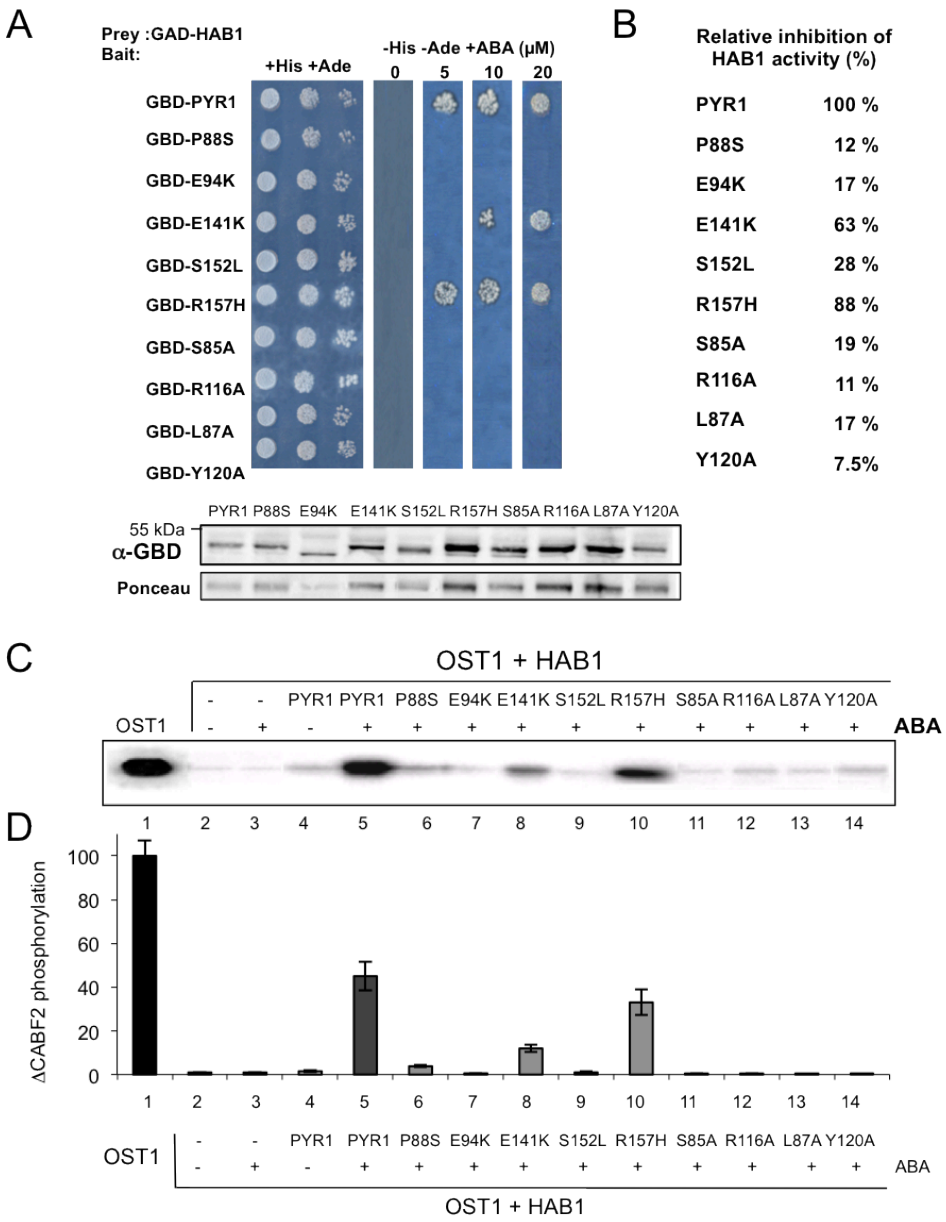


Figure 1. Analysis of the PYR1 mutations and their effect on the HAB1-dependent inhibition of OST1 activity. A, Interaction between HAB1 and PYR1 variants was analysed by the yeast-two-hybrid (Y2H) growth assays on medium lacking His and Ade in the presence of 5, 10 or 20 µM (+) ABA. Immunoblot analysis using antibody against the Gal4 binding domain (GBD) verifies the expression of the different fusion proteins in the Y2H assay. Ponceau staining from 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 wild type PYR1 (100%; SD was below 7%). **C,** OST1 *in vitro* kinase activity assay in the presence of HAB1, PYR1 wild type and mutated versions, ΔCABF2 and 10 µM ABA, when indicated. The autorradiography shows the levels of auto-phosphorylation of OST1. **D,** Quantification of ΔCABF2 phosphorylation levels of the assay shown in C, using the phosphoimager Image Gauge V.4.0. Standar error measurements are shown (*n=3*). Data published in *Plant Physiology ,* Dupeux et al., 2011.

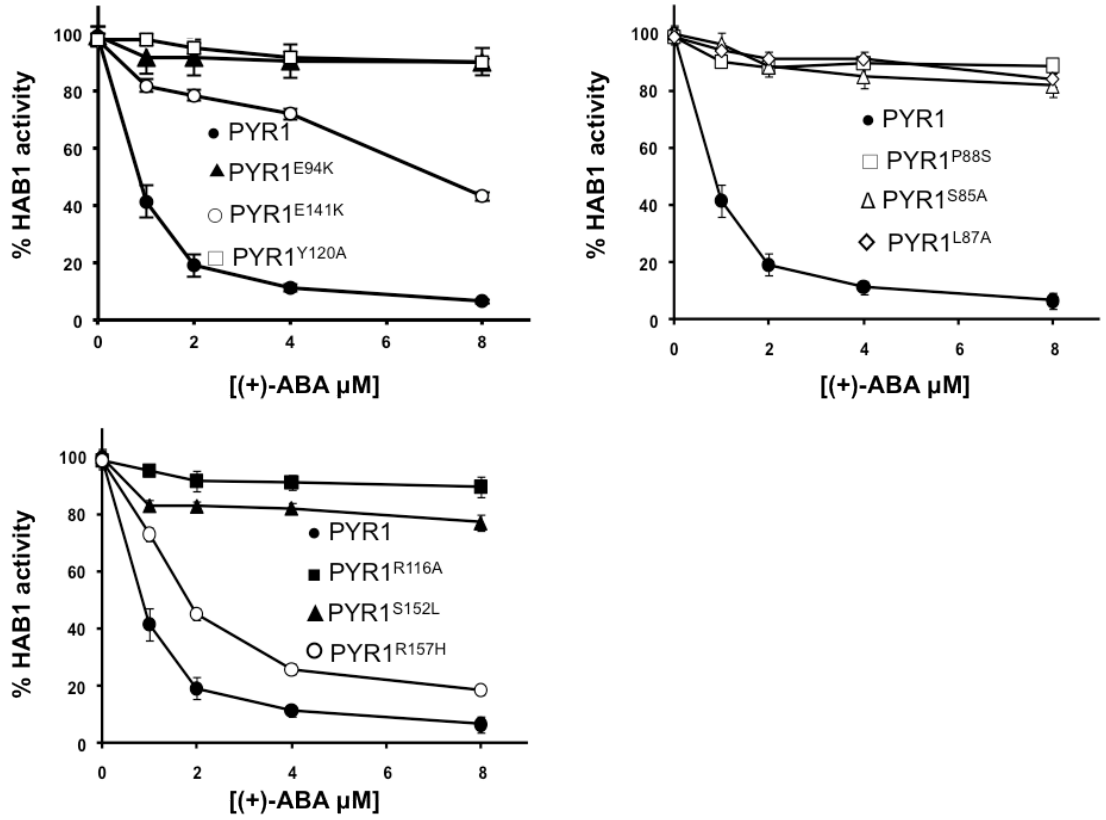


Figure 2. Comparison of the ABA-dependent inhibitory effect of PYR1 wild type and PYR1 mutant proteins, on HAB1 activity. Phosphatase activity was measured using the Ser/Thr Phosphatase assay system (Promega) and the RRA(phosphoT)VA peptide as substrate. Data are averages ± SE from three independent experiments. The HIS_6-HAB1 and HIS_6-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 HIS_6-HAB1 in the absence of ABA (100 % activity) was 4.6±0.3 nmoles Pi/min · mg. Data published in *Plant Physiology ,* Dupeux et al., 2011.

Biochemical characterization of hab1^{G246D}

In the PYR1 structure the residue Ser85 (equivalent to Ser89 in PYL2 and Ser112 in PYL1) completely flipped upon ABA binding, being exposed to the solvent and prone to interact with the PP2C. Resolution of PYL1-ABA-ABI1 (Miyazono et al., 2009; Yin et al., 2009) and PYL2-ABA-HAB1 (Melcher et al., 2009) ternary complexes, showed that PYL2 Ser89 and PYL1 Ser112, located at the β3-β4 loop, were establishing hydrogen bonds with the active site residues Gly246 and Glu203 of HAB1 and Gly180 and Glu142 of ABI1, respectively. A similar situation has been later on described in the case of PYR1-ABA-HAB1 complex (Dupeux et al., 2011), where interactions between Ser85 and HAB1 Gly246 and Glu203 have been reported. To further study the biological relevance of these interactions we selected the HAB1

Gly246 to Asp mutation, similar to *abi1-1D* and *abi2-1D* mutations, whose expression *in planta* leads to a dominant ABA-insensitive phenotype (Robert et al., 2006) and is still able to dephosphorylate OST1 (Vlad et al., 2009).

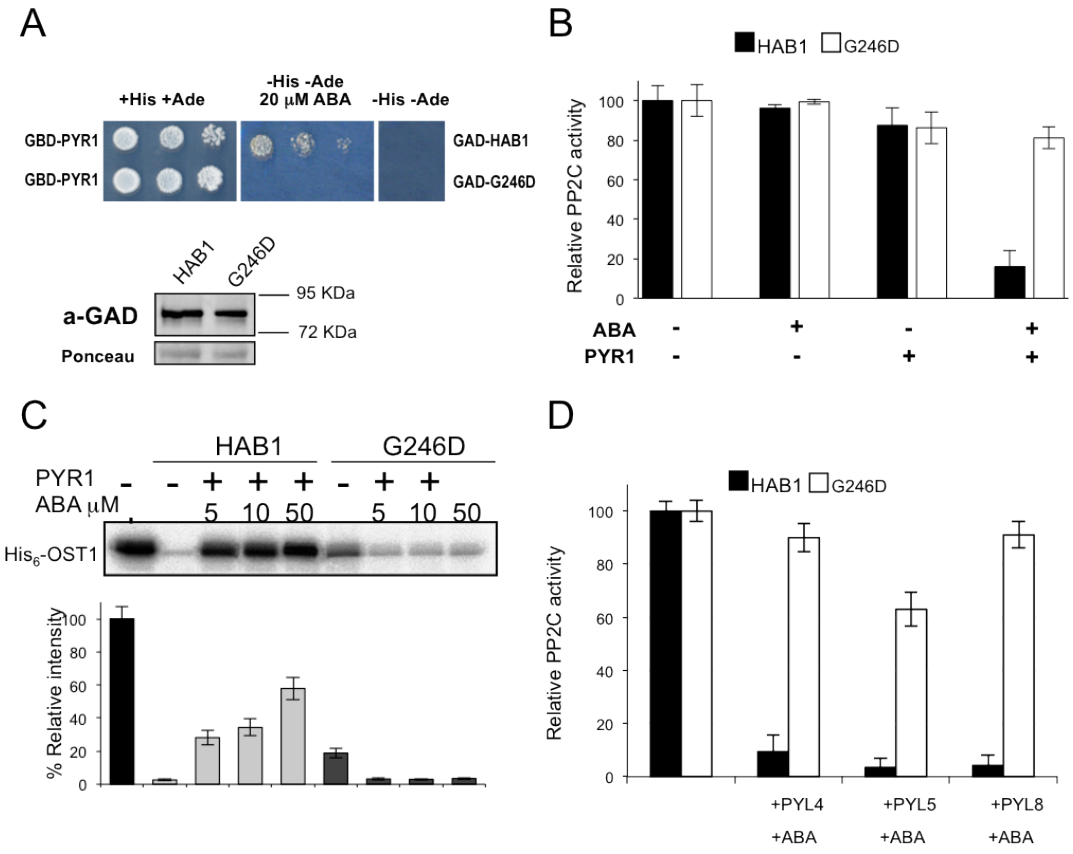


Figure 3. hab1^{G246D} is refractory to inhibition by PYR1 and dephosphorylates OST1 in the presence of ABA and PYR1. **A,** The HAB1 mutation Gly246Asp abolishes the interaction of the PP2C and PYR1 in a Y2H assay. Immunoblot analysis using antibody against the Gal4 activation domain (GAD) is shown 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 and hab1^{G246D} proteins was measured *in vitro* using p-nitrophenyl phosphate as substrate in the absence and presence of PYR1 and ABA, as indicated. Assays were performed in a 100 µl reaction volume containing 2 µM phosphatase and, when indicated, 4µM 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 HAB1 wild type, hab1^{G246D} , PYR1 and ABA, as indicated. The autoradiography shows the level of autophosphorylation of OST1 in each reaction. The graph shows the quantitative analysis of the autoradiogram. **D,** hab1^{G246D} is resistant to ABA-mediated inhibition by different PYR/PYLs. The assay was performed as described in B. Data published in *Plant Physiology ,* Dupeux et al., 2011.

We first tested the capacity of hab1^{G246D} to interact with PYR1 by Y2H. As it can be seen in figure 3A, the ABA-dependent interaction between hab1^{G246D} and PYR1 is completely abolished as compared to wild type HAB1. Similarly, and opposite of what it happens to HAB1 wild type, hab1^{G246D} is refractory to ABA-dependent inhibition by

PYR1 and other PYLs such as PYL4, PYL5 or PYL8 (figure 3B and 3D). Moreover, we also analyzed the hab1^{G246D} *vs* HAB1 wild type effect on the control of OST1 kinase activity (Figure 3C), in a similar system as described above. While HAB1 was susceptible to be inhibited by PYR1 in the presence of ABA, leading to the recovery of OST1 kinase activity, hab1^{G246D} was still able to dephosphorylate OST1 under the same experimental conditions. These results indicate that hab1^{G246D} qualifies as a hypermorphic (Wilkie, 1994) mutant compared to wild type HAB1 in the presence of ABA and PYR/PYL/RCAR proteins. However, in the absence of PYR1 and ABA, hab1^{G246D} is initially less effective in dephosphorylating OST1 than the wild type (figure 3C) (also reported in Vlad et al., 2009). This could be explained because this mutation is located in the proximity of the catalytic site of the PP2C. Indeed, when measuring hab1^{G246D} activity, using p-nitrophenol as substrate, showed 4 times lower specific activity than HAB1 wild type (4.86 ± 0.43 and 18.76 ± 2.13 nmoles Pi/min•mg, respectively).

DISCUSSION

In summary, mutational analysis of PYR1 gives support to the model suggested by the structural analysis in which the β3-β4 and β5-β6 loops provide the surface for the interaction with the PP2C, inhibiting its activity. Moreover, the mutational analysis also illustrates that ABA binding is crucial for conferring PYR1 its inhibitory capacity, since mutations located inside the cavity, which are presumably not affecting the gating loops, severely impair or abolish the interaction and inhibition of the phosphatase. Mutations in PYR's loops, Pro88Ser and His115Ala, are capable of abolishing ABA-mediated PP2C inhibition without disrupting ABA binding (Park et al., 2009; Santiago et al., 2009B, this work), suggesting that both events can be uncoupled. However, structural and mutational analyses reveal that they are closely linked, and suggest that the hormone perception signal is transferred through the conformational changes induced in the receptor upon binding of ABA.

In addition, the results obtained for the PP2C mutant hab1^{G246D} demonstrate that specific mutations in the phosphatase can be insensitive to the ABA-mediated inhibition of PYR/PYLs proteins. In addition, they also provide an explanation for the strong ABA-insensitive phenotype of the 35S:hab1^{G246D} plants (Robert et al., 2006), in which we can presume that the phosphatase escapes from the inhibition of PYR/PYL/RCAR receptors. Moreover, these results reassert the model proposed in

Vlad et al. (2009) in which it is explained the negative regulation of OST1 activity and ABA responses by HAB1, which remains constitutively active in the case of $hab1^{G246D}$.

EXPERIMENTAL PROCEDURES

Construction of plasmids

Plasmids pETM11 or pET28a were used to generate N-terminal His_6-tagged recombinant proteins. The cloning of 6xHis-□NHAB1 (lacking residues 1-178), PYR1, PYL4, PYL5 and PYL8 constructs was previously described (Santiago et al., 2009A, Experimental procedures Results: Chapter 1). 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, 2009B). 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.

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 (1 µM). When indicated, PYR-PYL recombinant proteins (4 µM) 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 wild type 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). Wild type and mutant versions of PYR1 and HAB1 were fused to the GAL4 DNA-binding domain using vector pGBKT7 (GBD) and the GAL4 activation domain (GAD) using the pGADT7 vectors respectively. Interactions were determined by growth assay on medium lacking His and Ade, either in the absence or presence of 5, 10 and 20 µM (+)-ABA. Dilutions (10^{-1}, 10^{-2}, 10^{-3}) of saturated cultures we spotted onto the plates, and photographs were taken after 5 days. In order to verify the expression of the different protein fusions, Yeast protein extracts from co-transformed cells were separate by PAGE-SDS, transferred onto Immobilon-P membranes (Millipore) and stained with Ponceau to analyse protein loading or subjected to Immunoblot analysis with anti-GAD and anti-GBD primary antibodies (Clontech) and ECL anti-mouse-peroxidase (GE Healthcare) as secondary antibody to determine the expression level of the fusion

proteins. Detection was performed using the ECL advance western blotting detection kit (GE Healthcare).

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6. RESULTS: CHAPTER 3

Manuscript submitted to EMBO:

A thermodynamic switch modulates abscisic acid receptor sensitivity

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A thermodynamic switch modulates abscisic acid receptor sensitivity

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Key words: hormone receptor / protein phosphatase 2 C PP2C / PYR1-PYL-RCAR / START domain / plant stress response / sensing / PYL5 / PYL6 / PYL8 / HAB1

Subject categories: Plant Biology, Structural Biology

Running title: A thermodynamic switch in the ABA pathway

Abstract

The plant hormone abscisic acid (ABA) plays crucial roles in abiotic stress responses and development. ABA activates stress signalling by binding to the soluble PYR/PYL/RCAR class of ABA receptors, which stabilizes a conformational change that allows the activated receptors to bind and inhibit type 2C protein phosphatases (PP2Cs); this in turn triggers numerous downstream cellular responses. The ABA receptor family contains 14 genes in Arabidopsis and is currently the largest plant hormone receptor family known; however, it is unclear what functional differentiation exists between receptors. Here we demonstrate that a major difference between ABA receptors is their oligomeric state (monomeric vs. dimeric) in their unactivated, apo forms. We identified a key residue in PYR1, H60 that is variable between family members and plays a key role in determining oligomeric state. Moreover, the formation of homodimers is disfavourable for ABA and PP2C binding. To investigate the implications of these biochemical observations, we constructed a mathematical model of pathway activation, which suggests that monomeric receptors are preferentially activated by ABA when the two receptor types coexist in the same cell. This could potentially lead to differential cellular responses over the physiological range of ABA concentrations. More generally, our work illustrates how receptor oligomerization can modulate a signalling system's sensitivity.

INTRODUCTION

The plant hormone Abscisic acid (ABA) is an important regulator of plant growth and development and plays a key role in control of the plant stress response (Cutler et al, 2010; Verslues & Zhu, 2007). Under adverse environmental conditions, particularly drought and salinity the levels of ABA increases in the plant triggering a series of adaptive responses required for plant survival (Buchanan, 2000; Lee et al., 2006 Nambara and Marion-Poll, 2005). Recently a family of intracellular ABA receptors, named PYR/PYL (pyrabactin resistance / PYR1-like) (Park et al., 2009) or RCAR (Regulatory Component of Abscisic acid Receptor) (Ma et al., 2009) have been shown to play a crucial role in this response. The PYR/PYL/RCAR proteins are able to bind to ABA and regulate the activity of clade A of serine/threonine protein phosphatases 2 C (PP2Cs) including ABI1, ABI2 and HAB1 (Leung et al., 1994; Meyer et al., 1994; Saez et al., 2004). This in turn regulates phosphorylation of serine/threonine protein kinases in the sucrose non-fermenting1-related subfamily 2 (SnRK2) (Mustilli et al., 2002;

Yoshida et al., 2002). Under un-activated conditions, clade A PP2Cs dephosphorylate SnRK2 protein kinases, keeping them in an inactive state (Umezawa et al., 2009; Vlad et al., 2009). Under conditions of stress, ABA levels increase and induce the formation of a stable complex between PYR/PYL/RCARs and PP2Cs, which inactivates PP2C phosphatase activity and enables accumulation of active, phosphorylated SnRK2s (Cutler et al., 2010). Once activated, SnRK2s phosphorylate key mediators of stress adaptation such as the SLAC1 ion channel, involved in stomatal closure, and the ABF2 transcription factor, required for transcriptional activation of stress responsive genes. (Fujii et al., 2009; Fujita et al., 2009; Geiger et al., 2009 ; Lee et al., 2009).

Recent structural analyses of PYR/PYL/RCAR proteins alone and in complex with ABA and PP2Cs have provided significant insight into the molecular mechanism of ABA signalling (Dupeux et al., 2011; Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009) (For review see Cutler et al., 2010; Melcher et a., 2010; Weiner et al., 2010). ABA binds inside a conserved hydrophobic cavity, which is surrounded by two flexible loops, the β3/β4, and β5/β6 loops (called the gate and latch), that control the access to the ABA-binding pocket. In the ABA-free form, these loops adopt an open conformation leaving a free passage into the cavity, however once the ABA is bound, these loops close over the hormone stabilising it inside the cavity. These ABA-induced induced conformational changes promote the formation of a stable complex between ligand-bound receptor and catalytic domain of clade A PP2Cs. In this complex the β3/β4 gate loop inserts in the PP2C catalytic site and inhibits activity, acting as a competitive inhibitor that blocks substrate access (Dupeux et al., 2011; Melcher et al., 2009). At the same time a conserved tryptophan residue in the flap subdomain of clade A PP2C inserts between the gating loops, stabilizing them in the closed conformation and trapping the hormone inside. These interactions explain the increased stability of the ternary receptor-hormone-phosphatase complex as compared to that of the receptor-hormone complex alone.

Despite these molecular level details, major questions still remain poorly understood. For example PYR/PYL/RCAR proteins form multigene families in plants. Microarray datasets suggest that several different receptor proteins are often co-expressed in a single cell (Kilian et al., 2007), but it is still unclear whether they carry out fully redundant or specialized functions. Also molecular details of the receptor activation process are still poorly understood; for example, although the receptors studied so far crystallographically, including PYR1, PYL1 and PYL2 are dimeric, the receptor-ABA phosphatase complexes form show 1:1:1 stoichiometries, which implies that dissociation of the receptor dimer is necessary for receptor activation and

signalling (Melcher et al, 2009; Miyazono et al, 2009; Nishimura et al, 2009; Santiago et al, 2009B; Yin et al, 2009). Moreover, the receptors and PP2Cs form stable complexes with affinities for ABA in the low nanomolar range, however ABA levels *in vivo* have been observed to vary over 3 orders of magnitude (from nanomolar to micromolar) (Harris, 1988; McCourt and Creelman, 2008). This raises the question whether the proposed mechanism for ABA perception, i.e. a low-nanomolar sensing system, is enough to account for the perception of changes across the whole range of physiological ABA concentrations (McCourt and Creelman, 2008). Interestingly when hormone binding is measured for the PYRL/PYL/RCAR proteins alone, the affinity for ABA is not only lower than that measured in the presence of PP2Cs, but the values of the Kd vary significantly among different receptor proteins. For example, PYL1 and PYL2 have Kds for ABA of 52 μM and 59 μM respectively (Miyazono et al., 2009; Yin et al., 2009), while PYL5, and PYL8 have affinities of 1.0 μM and 0.9 μM respectively (Santiago et al., 2009A; Szostkiewicz et al., 2010). These differences in affinity are not likely to be explained by differences in the ABA binding pocket, since critical amino acids involved in hormone stabilization and in the gating loops are highly conserved among PYR/PYL/RCAR proteins. Here we present a detailed analysis of several members of the PYRL/PYL/RCAR family and provide new insight into the receptor activation mechanisms. Moreover, the biochemical and structural characteristics of these proteins suggest the existence of two distinct classes of ABA receptors characterized by the oligomeric states of their apo forms. The differential properties of these two types of ABA receptors might lead to distinct responses when they are considered in the context of the plant.

RESULTS

ABA induces dimer dissociation of PYR1

The PYR/PYL/RCAR proteins studied at a structural level to date (PYR1, PYL1, and PYL2) are homodimeric in the absence of ABA, but form 1:1 monomeric complexes with PP2Cs after ABA binding (Melcher et al, 2009; Miyazono et al, 2009; Nishimura et al, 2009; Santiago et al, 2009B; Yin et al, 2009). Interestingly, the homodimerization and PP2C-binding interfaces of these receptors are largely overlapping (Figure 1). This structural organization suggests that homodimerization and PP2C binding are in competition with one another and that dimerization, together with the conformational changes in the gating loops, block receptor– PP2C interactions

for unactivated dimeric receptors, presumably functioning to lower basal pathway activation. However, these conclusions are based largely on crystallographic observations of receptors at high concentration that shift equilibria towards dimer formation (Nishimura et al., 2009; Santiago et al., 2009B).

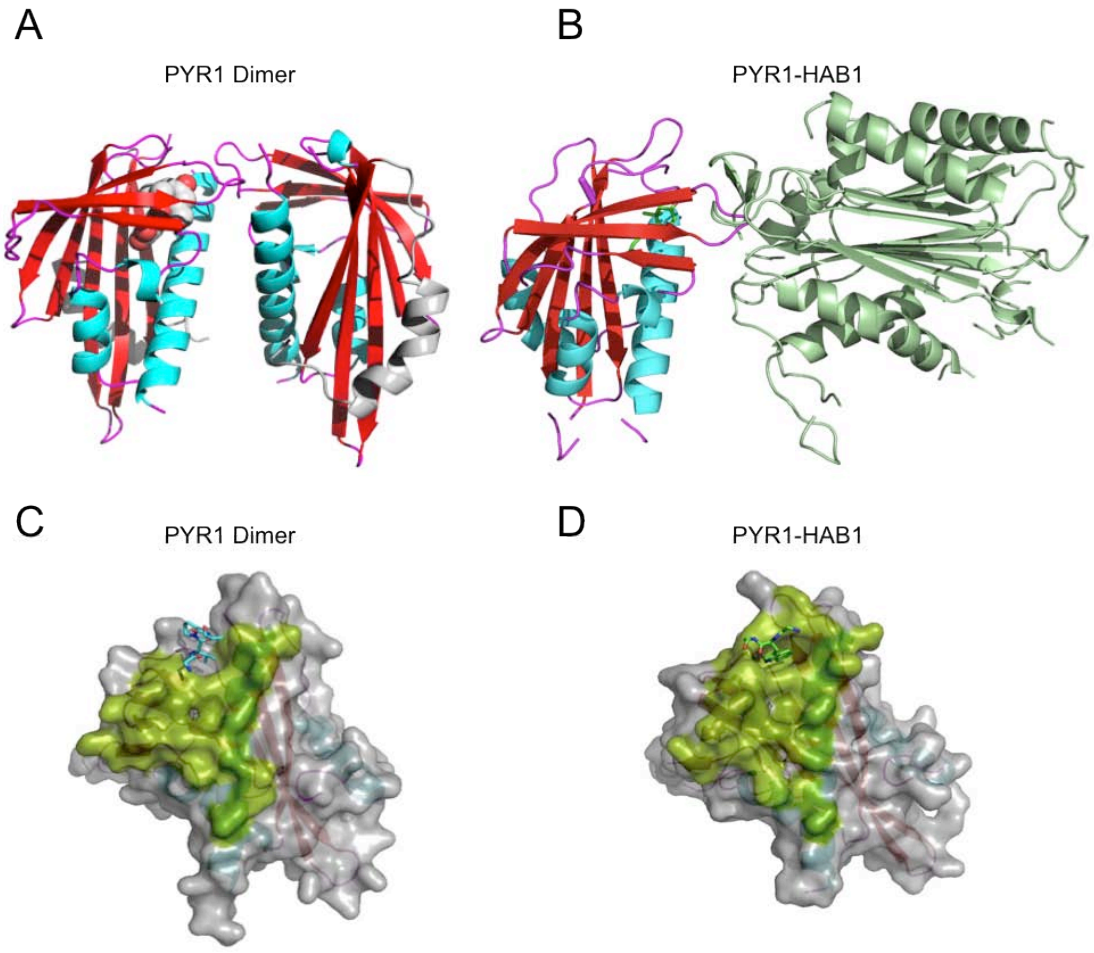

Figure 1. The PYR/PYL/RCAR dimerization and phosphatase interaction regions. The structures of the PYR1 dimer (**A**) and the PYR1-ABA-ΔNHAB1 ternary complex (**B**) are shown. The PYR1 molecular surfaces involved in dimerization (**C**) and ΔNHAB1 interaction (**D**) are also shown (in green). The large degree of overlap between the two surfaces shows that the PP2C interaction region is occluded in the PYR1 dimer, and that receptor dissociation would be required for the formation of the ternary complex.

To address this, we analyzed the oligomeric state of several PYR/PYL/RCAR proteins by Size Exclusion Chromatography (SEC) coupled to Multiple Angle Laser Light Scattering (MALLS). In this approach SEC followed by PAGE-SDS analysis of the eluted fractions reveals the formation of complexes, while the MALLS analysis provides an independent and highly accurate determination of the molecular mass of the eluting species which, unlike SEC, is not affected by deviations from globularity. Additionally,

with this technique it is possible to work at moderately low protein concentrations (in the low μM range).

Using this approach we studied the oligomeric state, PP2C interactions and effects of ABA for 5 members of the receptor family: PYR1, PYL1, PYL5, PYL6 and PYL8. The analyses of these proteins in the absence of added ABA indicate that PYR1 and PYL1 are dimeric proteins (apparent Mw of 39 kDa and 50 kDa; predicted 44 kDa and 51 kDa respectively (Figure 2 and supplementary Figure 1). Unexpectedly, PYL5, PYL6 and PYL8 are monomeric proteins (Figure 2 and supplementary Figure 1). When the same experiments are performed in the presence of ABA, PYR1 eluted at higher volumes (indicative of a decrease in molecular size) and the apparent molecular mass was 21 kDa, which coincides with that of a PYR1 monomer (predicted = 22 kDa). On the contrary, analysis of the $PYR1^{Y120A}$, a mutant defective in ABA binding (Dupeux et al., 2011), indicates that this mutant does not become monomeric in response to ABA (Figure 2). Together, these results indicate that ABA binding is both necessary and sufficient for dissociation of the PYR1 dimer. Similarly, PYL1 showed a reduction of its apparent Mr in the presence of ABA (Supplementary Figure 1), although it was less completely dissociated by ABA as PYR1. As expected, the apparent masses for monomeric receptors PYL5, PYL6 and PYL8 did not shift in the presence of ABA (Figure 2 and supplementary Figure 1). Thus, ABA receptors can exist in either monomeric or dimeric forms and ABA promotes dissociation of the dimeric receptors.

We also examined interactions between receptors and PP2C in the absence of ABA, which revealed differences in basal PP2C interactions between monomeric and dimeric receptors. In agreement with crystallographic studies, all receptors tested form stable complexes with PP2C in the presence of ABA at 1:1 stoichiometry, as indicated by the apparent Mr of the complexes (right panels in Figure 2 and supplementary Figure 1). Importantly, PYR1 and PYL1 do not interact with PP2C in the absence of ABA, while PYL5, PYL6 and PYL8 show partial interactions (Figure 2 and supplementary Figure 1), and MALLS analyses show intermediate molecular masses, indicative of a fast exchange between complexed and uncomplexed forms. These results indicate that monomeric receptors are capable of establishing weak, but clearly detectable interactions with HAB1 *in vitro* in the absence of ABA and suggest that a key function of dimerization in receptors like PYR1 and PYL1 is to prevent basal interactions with PP2Cs in the absence of ABA.

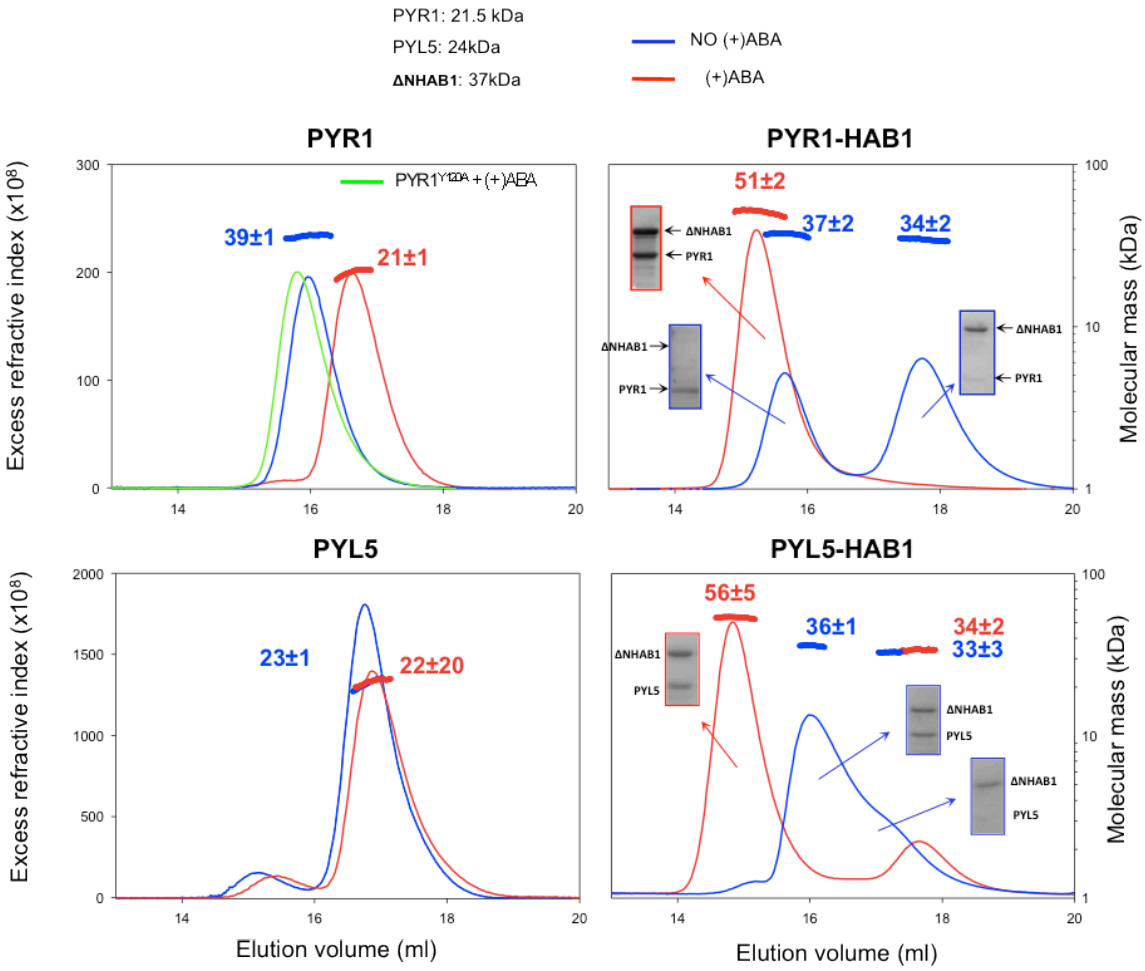

Figure 2. ABA receptors exist in dimeric and monomeric forms. SEC-MALLS analysis of PYR1 (top) and PYL5 (bottom) alone (left) and in the presence of the ΔNHAB1 (right). The experiments were done in the absence (blue) and presence (red) of 1mM (+)ABA. PAGE-SDS analysis of eluted fractions is shown (boxes). Both the SEC elution profiles and the molecular size calculated by MALLS indicate dissociation of the PYR1 dimer in the presence of ABA (expected Mw 42 kDa and 21 kDa for dimers and momoners respectively). The PYR1^{Y120A} mutant, defective in ABA binding, remains as a dimer in the presence of ABA (green line). PYL5 is monomeric (expected Mw 24 kDa) and is not affected by the presence of (+)ABA. Both proteins form 1:1 complexes when combined with ΔNHAB1 in the presence of ABA (right panels), however while PYR1 does not interact with ΔNHAB1 in the absence of ABA the interaction between PYL5 and ΔNHAB1 in the same conditions is revealed by a decrease in the height of the peak corresponding to monomeric ΔNHAB1 and the appearance of a peak containing both PYL5 and ΔNHAB1. See supplementary figure 1 for similar experiments with PYL1, PYL6, and PYL8.

Monomeric and dimeric receptors have different intrinsic affinities for ABA

The ABA dissociation constants for PYL1, PYL2, PYL5 and PYL8 have been previously determined (Miyazono et al., 2009; Santiago et al., 2009A; Szostkiewicz et al., 2010; Yin et al., 2009) and are shown in Table 1. We carried out Isothermal

Titration Calorimetry (ITC) experiments to measure the ABA binding affinity of PYL6 and PYR1. The Kd for PYL6 was 1.1 µM (± 0.01) with a negative enthalpy indicating an exothermic binding process (see Table 1). Under the same conditions, PYR1 did not produce significant heat signals indicating a much lower affinity. When PYR1 was assayed, at higher protein concentrations (200 µM), an endothermic binding curve was obtained. Due to the low binding affinity, the exact value of the Kd could not be precisely determined, however given the experimental conditions used it can be estimated to be greater than 50 µM. NMR experiments in which binding was followed through ABA-induced resonance shifts on an $^{15}N^{13}C$ labelled PYR1 sample were used to obtain an independent estimation of the dissociation constant. The value of the Kd obtained was 97 ± 0.36 µM (data not shown). Thus, the dimeric receptor PYR1 has an affinity for ABA that is almost 2 orders of magnitude less than that of the monomeric receptors.

The oligomeric state and affinities for ABA of the PYR/PYL/RCAR receptors studied here and elsewhere are summarized in Table 1. Comparison of these parameters indicates a strong correlation between the ABA affinities and the oligomeric state of the apo form of the receptor, with monomeric receptors showing Kds in the range of 1 µM and negative enthalpies, and dimeric receptors showing much lower affinities and positive binding enthalpies for ABA. To determine whether these correlations are directly associated with receptor oligomerization, we identified and characterized a PYR1 point mutation, H60P, that destabilizes the PYR1 dimer. The H60P mutant was isolated in a screen of PCR-mutagenized PYR1 genes that display improved binding to the PP2C HAB1 using a yeast two-hybrid based screen.

Table 1. Affinities and oligomeric state of PYR/PYL/RCAR receptors

	Kd (µM)	***ΔH* (kcal/mol)**	**Oligomeric state**	**Reference**
PYR1	97 (±0.36)	Endothermic	Dimer	This study
$PYR1^{H60P}$	3.0 (±0.26)	-2.5 (±0.035)	Monomer	This study
PYL1	52.0	+ 1.4	Dimer	Miyazono et al. and this study
PYL2	59.1 (± 2.5)	Endothermic	Dimer	Yin et al.
PYL5	1.0 (± 0.06)	- 7.9 (± 0.2)	Monomer	Santiago et al.
PYL6	1.1(± 0.01)	-3.573 (± 0.1)	Monomer	This study
PYL8	0.9 (± 0.15)	- 10.4 (± 0.3)	Monomer	Szostkiewicz et al. and this study

A Histidine 60 to Proline substitution destabilizes the PYR1 dimer and leads to increased affinity for ABA binding

As shown above, monomeric receptors have a certain capacity to interact with HAB1 in the absence of ABA while dimeric receptors do not. This observation is in agreement with the strong ABA-dependence of PYR1 and PYL1-HAB1 interactions observed previously in a yeast two-hybrid (Y2H) assay system and the ABA-independent interactions observed for PYL5, PYL6 and PYL8 (Park et al., 2009; Santiago et al., 2009A). Using a previously described error prone PCR mutagenized PYR1 library (Peterson et al. 2010), we screened yeast cells for PYR1 mutants that enable interaction with HAB1 in the absence of added ABA, which led to the identification of a missense mutant in which histidine 60 was replaced by proline.

The SEC-MALLS analysis of the H60P mutant produced a single elution peak with an apparent mass of 30 kDa (Figure 3B), which is between those expected for the dimer and monomer species. This elution peak is wide and asymmetric, indicative of polydispersity. In contrast, in the presence of ABA $PYR1^{H60P}$ elutes as a single symmetric peak with an apparent mass of 22 kDa, as expected for the monomeric form. This indicates that in the absence of ABA, $PYR1^{H60P}$ behaves as a mixture of dimeric and monomeric species in rapid exchange. In order to confirm this, we measured dimer dissociation rates by ITC with wt and mutant PYR1. For this purpose, concentrated solutions of either protein were injected into the ITC cell filled with buffer. In this way, the sudden dilution of the protein promotes dissociation and the associated heat can be measured. As it can be observed (Figure 3C) the thermogram corresponding to $PYR1^{H60P}$ shows a profile typical of protein dissociation, with endothermic peaks whose area decreases as the protein concentration in the ITC cell increases. The calculated dissociation constant is 39µM. In contrast, wt PYR1 produced much weaker signals that disappeared after a few injections indicating that limited dissociation occurs only in extremely diluted conditions.

We next tested whether the $PYR1^{H60P}$ mutant could interact *in vitro* with HAB1 in the absence of ABA *in vitro* as is the case for monomeric receptors. For this purpose $PYR1^{H60P}$ and HAB1 were mixed in equimolar amounts and analysed by SEC-MALLS. As shown in Fig 3D, wt PYR1 does not interact with HAB1 in the absence of ABA, however, $PYR1^{H60P}$ shows partial interaction with HAB1, similar to monomeric receptors (Figure 2), and confirming the constitutive interactions detected by the Y2H assay (Figure 3A).

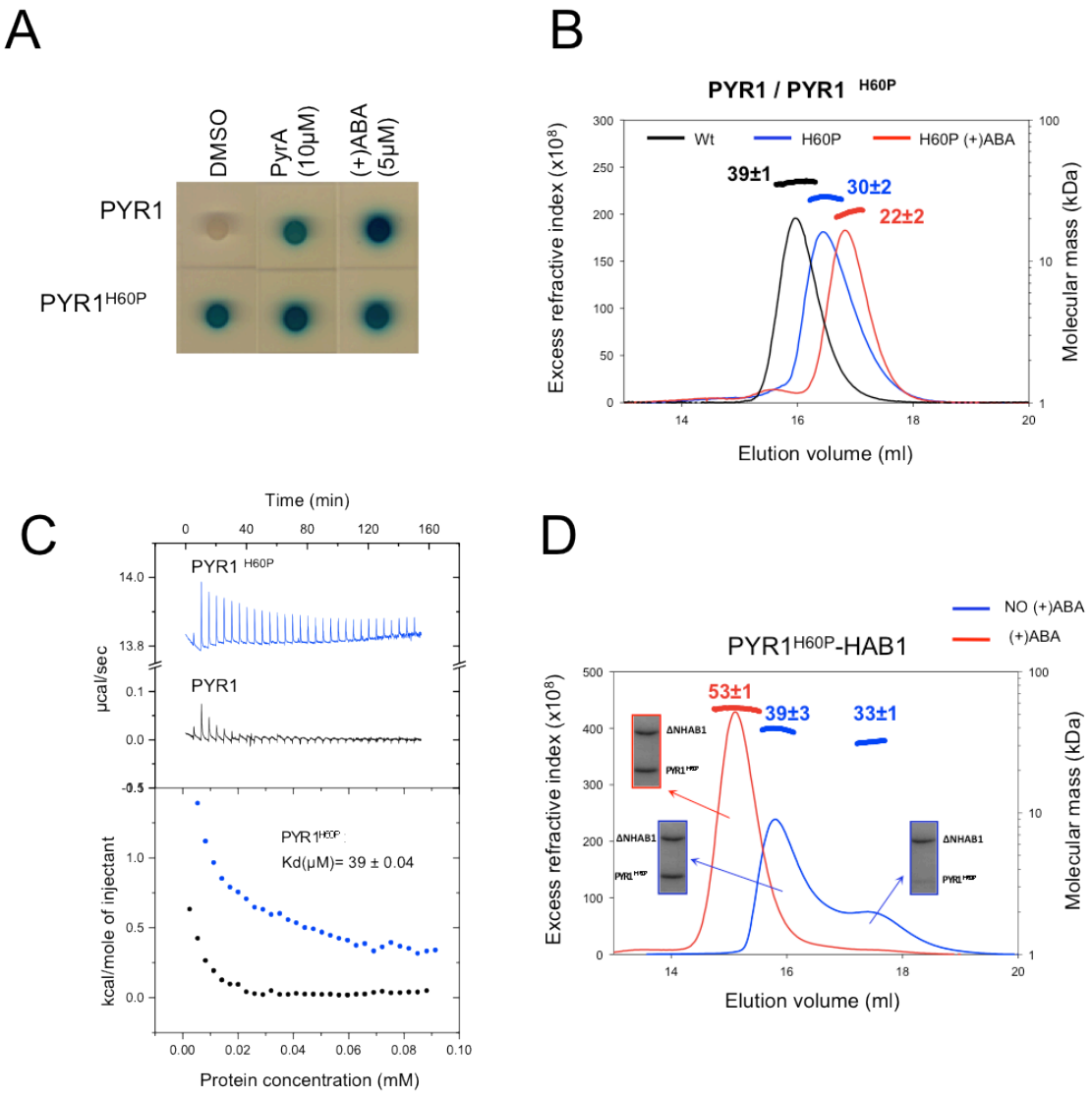

Figure 3. **The PYR1 His60Pro mutation favours both dimer dissociation and constitutive interaction with HAB1**. **A,** Y2H assay showing constitutive interaction between PYR1^{H60P} and ΔNHAB1. This interaction is strictly dependent on ABA or its analog, Pyrabactin for wt PYR1. **B,** SEC-MALLS analysis of wt PYR1 (black) and PYR1^{H60P} in the absence (blue) and presence (red) of 1 mM (+)ABA. The shift towards lower apparent molecular size in SEC and MALLS is indicative of an equilibrium between monomeric and dimeric forms. PYR1^{H60P} dissociates completely into monomeric species in the presence of 1mM ABA (red curves). **C,** Comparison of the dissociation rates of PYR1 and PYR1^{H60P} measured by ITC. Concentrated solutions of both proteins were repeatedly injected into the ITC cell filled with buffer and the dissociation thermograms were recorded. The estimated Kd for PYR1^{H60P} is indicated. **D,** SEC-MALLS analysis showing constitutive interactions between ΔNHAB1 and PYR1^{H60P}. Experiments were conducted by mixing equimolecular concentrations (80 µM) of both proteins before injection, in the absence (blue) and in the presence (red) of 1 mM (+)ABA. The composition of the SEC peaks was analyzed by PAGE-SDS (boxes). PYR1^{H60P} shows partial constitutive interaction with ΔNHAB1.

Then, we measured the affinity and enthalpy for ABA binding of the PYR1^{H60P} mutant and compared it with that of wt PYR1 (Figure 4A and 4B).

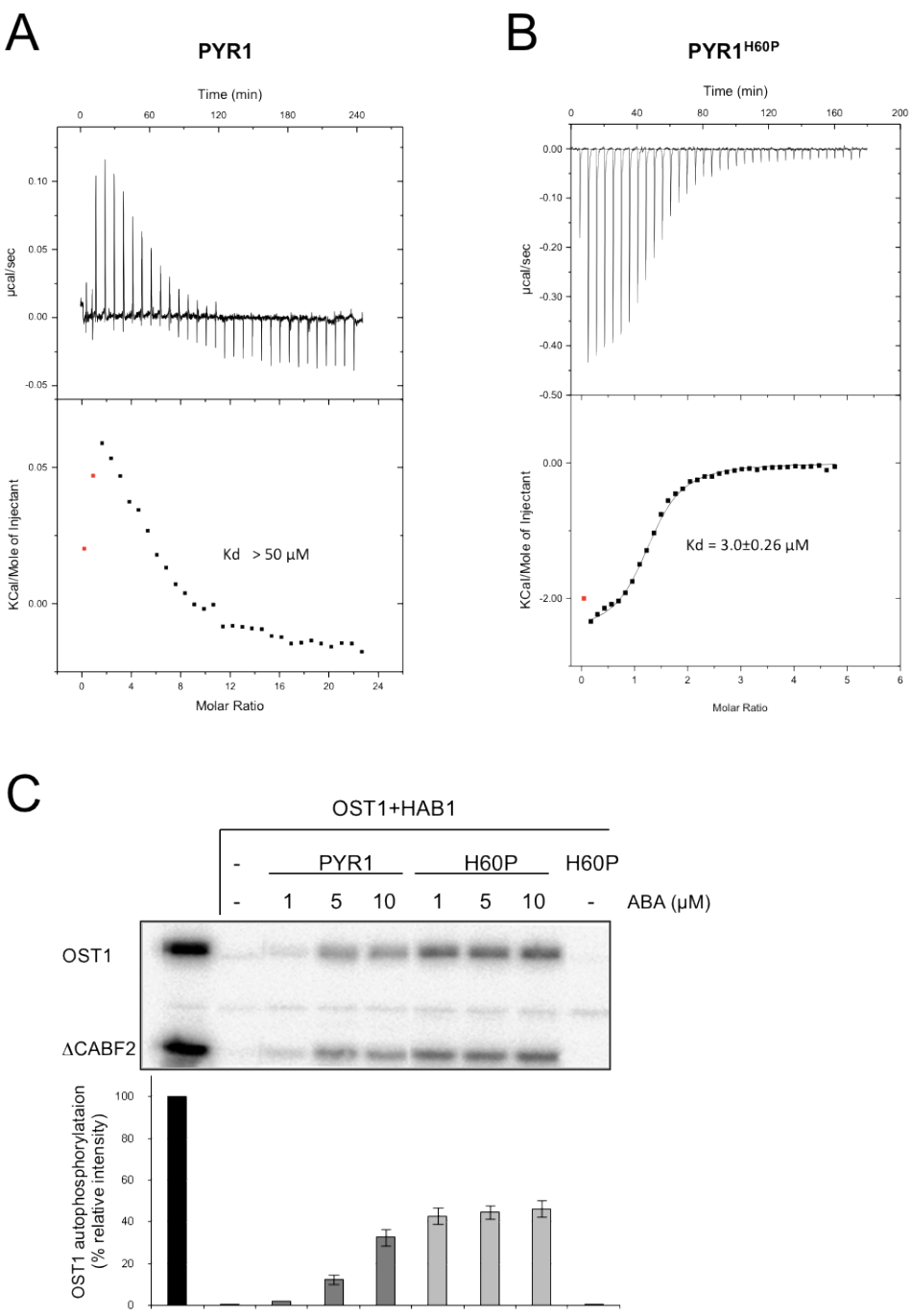

Figure 4. **The PYR1 His60Pro mutation leads to increased affinity for ABA.** Typical thermograms resulting from isothermal titration calorimetry experiments with wt PYR1 (**A**) and PYR^{H60P} (**B**) (the concentration of PYR1 is 7 times higher than that of PYR1^{H60P}). The estimated Kds for ABA binding are indicated. The increase in affinity and the change in the sign of the enthalpy, endothermic for PYR1 and exothermic PYR^{H60P} , can be appreciated. **C**, *in vitro* OST1 kinase activity assays in the presence of wt HAB1, wt PYR1 and the mutant PYR1^{H60P} . The experiment shows an enhanced recovery of OST1 activity in the presence of PYR1^{H60P} at low ABA concentrations, as compared to wt PYR1. The top panel shows a representative autoradiogram from a protein kinase assay. The bottom panel shows the quantification of the activity based on relative intensity of bands corresponding to OST1 phosphorylation products, using a phosphoimager Image Gauge V.4.0. Quantification of ΔCABF2 was also performed obtaining similar results. Standard error measurements are shown (*n=3*).

The introduction of this mutation leads to a significant increase in ABA binding affinity with a dissociation constant of 3 µM and a negative enthalpy, similar to those of monomeric receptors. This enhanced ABA binding affinity was confirmed in a functional assay based on the *in vitro* reconstitution of the ABA signaling cascade (Fujii et al., 2009). In this assay, $PYR1^{H60P}$ was able to recover OST1 activity at lower ABA concentrations than wt PYR1 (Figure 4C). This result is in agreement with the measured increase in ABA binding affinity and indicates that a monomeric variant of PYR1 is more effective than the dimeric PYR1 for the inhibition of the phosphatase activity of HAB1 at lower ABA concentrations.

Taken together, these results demonstrate that the His60Pro mutation produces a destabilisation of the PYR1 dimer and that this renders the mutant PYR1 capable of establishing weak, but clearly detectable interactions with HAB1 in the absence of the hormone, supporting the notion that dimerization contributes to prevent the interaction between receptor and phosphatase in the absence of ABA by occluding the phosphatase docking region on the surface of the receptor. Moreover they demonstrate that the differences in affinity between monomeric and dimeric receptors are produced by a negative contribution of dimer dissociation to the receptor activation process.

DISCUSSION

The data presented here provide novel mechanistic insight into the process of activation of PYR/PYL/RCAR Abscisic acid receptors and show how the capacity of the apo form of the receptor to dimerize or not divides this protein family in two groups with distinctive properties. These differential properties may have important consequences for the activation of the ABA response pathway when they are considered in the cellular context.

Our analysis indicates that PYR/PYL/RCAR proteins can be separated in two distinct sub-classes: one corresponding to dimeric receptors, to which PYR1, PYL1 and PYL2 belong, and another corresponding to monomeric proteins, including at least PYL5, PYL6 and PYL8. Dimeric receptors display lower apparent affinities for ABA and have a strict requirement on ABA for interaction with PP2Cs, while monomeric receptors have a significantly higher intrinsic affinity for ABA and can form low affinity complexes with PP2Cs in the absence of ABA. These distinctive properties do not result from differences in the ABA binding pocket. Instead, they are the consequence of

the oligomeric state of the apo form of the receptor. In dimeric receptor the PP2C interaction surface is occluded in the absence of ABA and ABA binding is required to both promote the closed conformation of the gating loops and induce dimer dissociation, which releases the PP2C-interaction region on the surface of the receptor making it competent for interaction with a phosphatase. The lower ABA affinity characteristic of dimeric receptors is the consequence of the thermodynamic penalty imposed by dimer dissociation to the receptor activation process. For dimeric receptors, the heat released by hormone binding is reabsorbed by the process of dimer dissociation, which is endothermic, resulting in net positive enthalpies and a less favourable ΔG for the overall receptor activation process, decreasing in turn the apparent ABA binding affinity. The activation of monomeric receptors does not involve this unfavourable energetic contribution which explains their intrinsic affinity for ABA, but on the other hand, in their ABA-free form the PP2C interaction region is not occluded leading to a certain capacity to establish weak interactions with the receptor. The distinctive properties of monomeric and dimeric receptors are particularly well illustrated by the PYR1 wt and PYR1^{H60P} proteins. Even though their ABA binding pocket and PP2C interaction regions are virtually identical, the different oligomeric state of their apo forms entails a different ABA sensitivity and ability to activate OST1.

The analysis of the ABA binding regions in the PYR/PYL/RCAR family indicates that for dimeric receptors like PYR1, PYL1 and PYL2, the residues involved in dimerization are surrounded or adjacent to residues involved in ABA binding (see supplemental Figure 2), which helps understand how ABA binding could result in changes in the dimerization interface. Indeed, by comparing the structures of apo and ABA-bound PLY2, Yin and co-workers had already noted a significant weakening of the dimer interface upon ABA binding (Yin et al., 2009). This weakening is mostly due to the closing of the gating loops induced by ABA, which in the apo form are open and establishing both polar and hydrophobic contacts with the neighbouring subunit, and to a slight change in the relative orientation of the two protomers, all of which results in a reduction in the number of Van der Waals contacts and hydrogen bonds between the two subunits and a reduction of the dimer interaction surface. Similar differences can be observed between the ABA-bound and free forms of PYL1, PYL2 and PYR1.

The thermodynamic disadvantage of dimeric receptors will be offset by the formation of the new receptor-phosphatase protein interface in the ternary complex, which explains why both monomeric and dimeric receptors show increased affinities for ABA in the presence of clade A PP2Cs. However, our results indicate that if both types of receptors are equally expressed in a single cell, monomeric receptors would be

preferentially activated. For example during the early stages of the stress response, or when intracellular ABA levels are low, monomeric receptors are likely to play a major role in the control of the activity of Clade A PP2Cs, while dimeric receptors might only be fully activated under more severe stress conditions or at a later stage in the response (Figure 5).

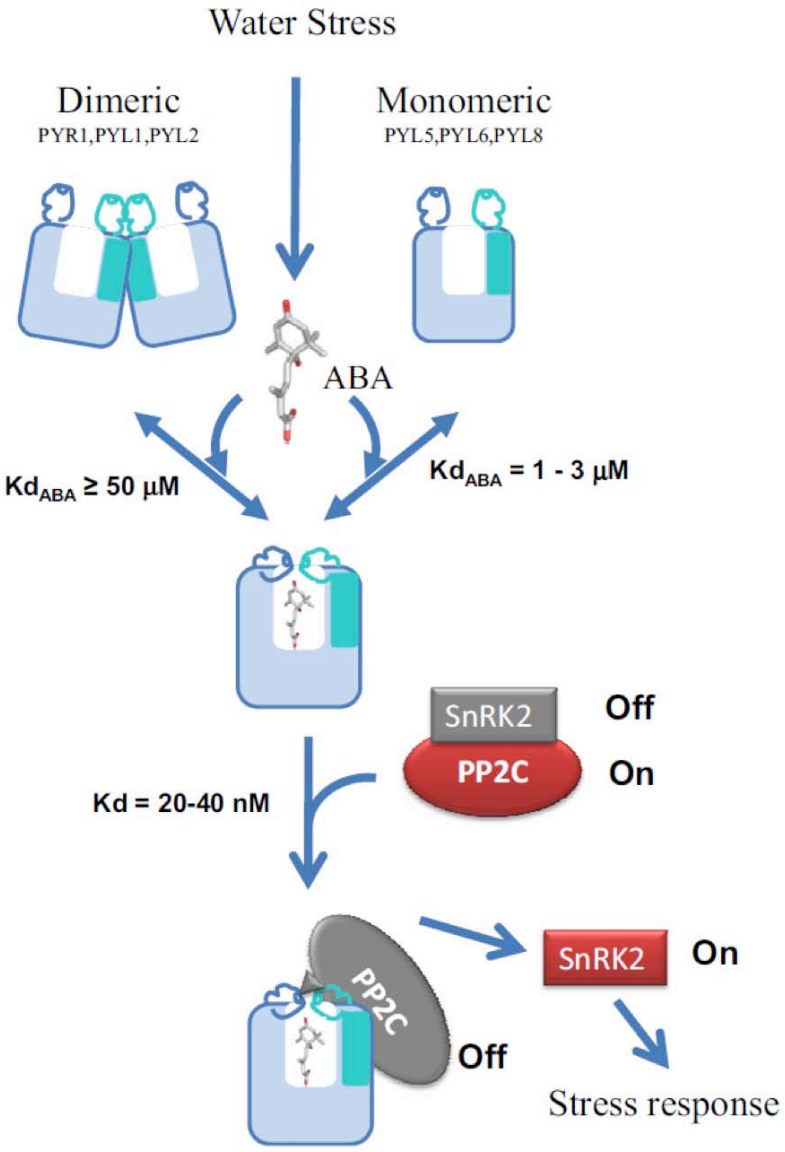

Figure 5. Model of the Abcisic Acid receptor activation process. Binding of ABA to dimeric receptors provokes closing of the gating loops and dissociation of the receptor dimer, releasing its PP2C interaction region. The process of dimer dissociation imposes a thermodynamic penalty on ABA binding (see text), and results in lower affinities for the hormone as compared to monomeric receptors. Once bound to ABA and in monomeric state, the receptor is competent for binding to PP2Cs resulting in the activation of specific protein kinases, including members of the SnRK2 family, and the stress response.

This mechanism might contribute to modulate the dose-response curve to ABA by making it proportional in a wider range of ABA concentrations but it also suggests that the two types of PYR/PYL/RCAR proteins might actually have specialized functions and contribute differentially to ABA signalling. Indeed, microarray studies indicate that the transcriptional response of the plant to ABA does not involve a simple "on" and "off" mechanism but a complex one with at least three distinct groups of ABA-induced genes: one showing an early and transient induction, another showing a late response and a third one with a rapid and persistent induction (Huang et al., 2007; Seki et al., 2002). This indicates that changes in the expression profile of PYR/PYL/RCAR proteins may also influence the response to ABA and offers a potential mechanism through which the plant could differentially modulate the response to stress in specific organs or tissues or under particular physiological conditions.

The PYR/PYL/RCAR family contains 14 members in Arabidosis, only a number of which have been characterized so far. According to the data presented here, the affinity for ABA could be used to predict the oligomeric state of yet uncharacterized receptors. On the other hand, analysis of the PYR^{H60P} mutant indicates that a Proline at position 60 is unfavourable for homodimerization. Similar conformational changes are found at equivalent positions in the PYL1 and PYL2 dimers. This suggest that proteins like PYL7, PYL9 and PYL10 that contain a proline at the position equivalent to that of His60 in PYR1, are likely to be monomeric. This is indeed the case for PYL8 as we show here. Other proteins, like PYL5 and PYL6 seem to acquire a monomeric structure through a different mechanism involving other amino acid variations at the dimerization interface. Further structural characterization of members of the PYRL/PYL/RCAR family should contribute to understand these mechanisms.

Finally, our results indicate that monomeric ABA receptors are able to interact with the HAB1 phosphatase in the absence of ABA *in vitro*. This suggests that although the hormone plays a critical role in the stabilization of the ternary complex a less stable complex can be formed between the phosphatase and monomeric receptors. Constitutive interactions between monomeric PYR/PYL/RCAR/ receptors and PP2Cs have been observed in yeast two hybrid assays (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A) and also in plants (Nishimura et al., 2010). However, under those experimental conditions it is difficult to exclude completely the presence of small amounts of ABA. Nevertheless, the biochemical data hereby provided suggest the existence of similar constitutive interactions *in vivo*, which might contribute a certain basal activation of the components of the ABA pathway. Additionally, monomeric receptors would efficiently compete with dimeric receptors for low endogenous ABA

levels that might be necessary for the regulation of certain developmental processes not related to stress. Indeed, both severe ABA-deficient and ABA-insensitive mutants are severely impaired in growth and reproduction even when they are grown under high humidity conditions (Barrero et al., 2005; Cheng et al., 2002; Fujii et al., 2009). Alternatively, the activity of the apo form of some monomeric receptors might be modulated through interaction with yet unidentified non-receptor proteins, leading to the formation of heteromeric complexes, rather than homodimers.

The results presented here reveal an unexpected level of complexity in the molecular mechanisms governing the activity of the PYR/PYL/RCAR receptors and provide a novel framework for the understanding of the ABA signalling pathway and the activation of the stress response in plants. More generally this work illustrates how receptor oligomerization can modulate ligand binding affinity by influencing the thermodynamics of the overall activation reaction, which implies that a high degree of amino acid sequence conservation at a ligand binding site does not necessarily lead to similar binding properties.

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MATERIALS AND METHODS

Construction of plasmids, expression and purification of 6xHIS fusion proteins

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 and PYL5 constructs were previously described (Santiago et al, 2009A; Santiago et al,

2009B). Full length PYL1, PYL6, and PYL8 were cloned in pETM11. PYR1(Y120A) mutants was produced using the Overlap extension procedure (Ho et al, 1989) and cloned into pET11. The coding sequence of OST1 and a C-terminal deletion of ABF2 (ΔCABF2, amino acids 1-173) were cloned into pET28a.

The H60P mutation was obtained in a screen designed to identify altered PYR1-PP2C interactions, the coding sequence for PYR1 was mutated by error-prone PCR and the mutagenized library was transformed into *S. cerevisiae* strain Y190 containing pAD-HAB1 and $1x10^6$ colonies were screened. Y190 contains a galactose inducible HIS3 nutritional reporter that allows positive selection for PYR1-HAB1 interactions in the absence of exogenously added ABA by growth on media containing 3-amino triazole. Once identified, the H60P mutation was introduced into PYR1 using the QuickChange site directed mutagenesis protocol.

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 were purified as described in (Santiago et al, 2009B). Briefly, cell pellets were re-suspended in lysis buffer (30mM Tris pH7.5, 500mM NaCl, 15mM Imidazole, 1mM $MnCl_2$, 1mM βmercaptoethanol and Protease cocktail inhibitor), lysed with a microfluidizer (Microfuildics) and purified by Ni-affinity. For proteins produced from pETM11 vectors the 6xhis-tag was excised by digestion with the TEV protease. In the case of PYR1, PYL1, PYL6 and PYL8, an additional step of gel filtration using an S200 column (GE) was carried out.

Size-exclusion chromatography-Multi-angle laser light scattering (SEC-MALLS)

Size-exclusion chromatography (SEC) was performed using a S200 Superdex column (GE Healthcare) equilibrated with 20mM Tris pH7.5, 150mM NaCl and 1mM β-mercaptoethanol. For the experiments with ABA, either 1 or 5mM ABA was included in the equilibration buffer. For the analysis of receptor-PP2C complexes equimolecular concentrations of both proteins were mixed before injection in the presence or absence of 1mM (+)ABA. The experiments were carried out at 20°C with a flow rate of 0.5 mL.min-1 as described previously (Gerard et al, 2007).

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using a VP-ITC calorimeter. All solutions were centrifuged, degassed, and equilibrated to the corresponding temperature prior to each experiment. Experiments were carried out at pH 7.5 and 25ºC. The titrations were typically carried out with 30 injections of 7 µl each and with varying concentrations of protein and ligand. The heat of dilution obtained from independent titration experiments injecting ABA into buffer was subtracted to the experimental data. For wt PYR1, an independent determination of the ABA binding affinity was carried out by NMR experiments were done at 37°C on a sample of ^{15}N labelled PYR1 containing 150mM NaCl and 20m M tris pH 7.5. ^{1}H-^{15}N HSQC spectra were acquired at a ^{1}H resonance frequency of 600 or 800 MHz. An estimation of the dissociation constant, Kd, of the PYR1:ABA complex was obtained by titration of ABA into an ^{15}N labelled sample of PYR1. Measurements were done at 10 different ABA/PYR1 concentration ratio ranging from 0 to 2.59. For each step of the titration a ^{1}H-^{15}N HSQC was recorded. Dissociation experiments were carried out by serial injections of protein solutions of either wt or His_6PYR1(H60P) into the cell containing the same buffer. Binding isotherms were analyzed by non-linear least squares fitting with the ORIGIN 7 software (OriginLab Corporation, Northampton, MA). The dissociation data was analyzed with the dissociation model provided by Dr. Alan Cooper's (http://www.chem.gla.ac.uk/staff/alanc/service1.htm). Data shown are the average of three independent experiments ± SD.

OST1 in vitro activity assays

Phosphorylation assays were done as described previously (Belin et al, 2006; Vlad et al, 2009). Radioactivity was detected using a Phosphoimage system (FLA5100, Fujifilm). Quantification of activity based on relative intensity of phosphorylated bands was performed using a phosphoimager Image Gauge V.4.0. The data presented are averages of at least three independent experiments.

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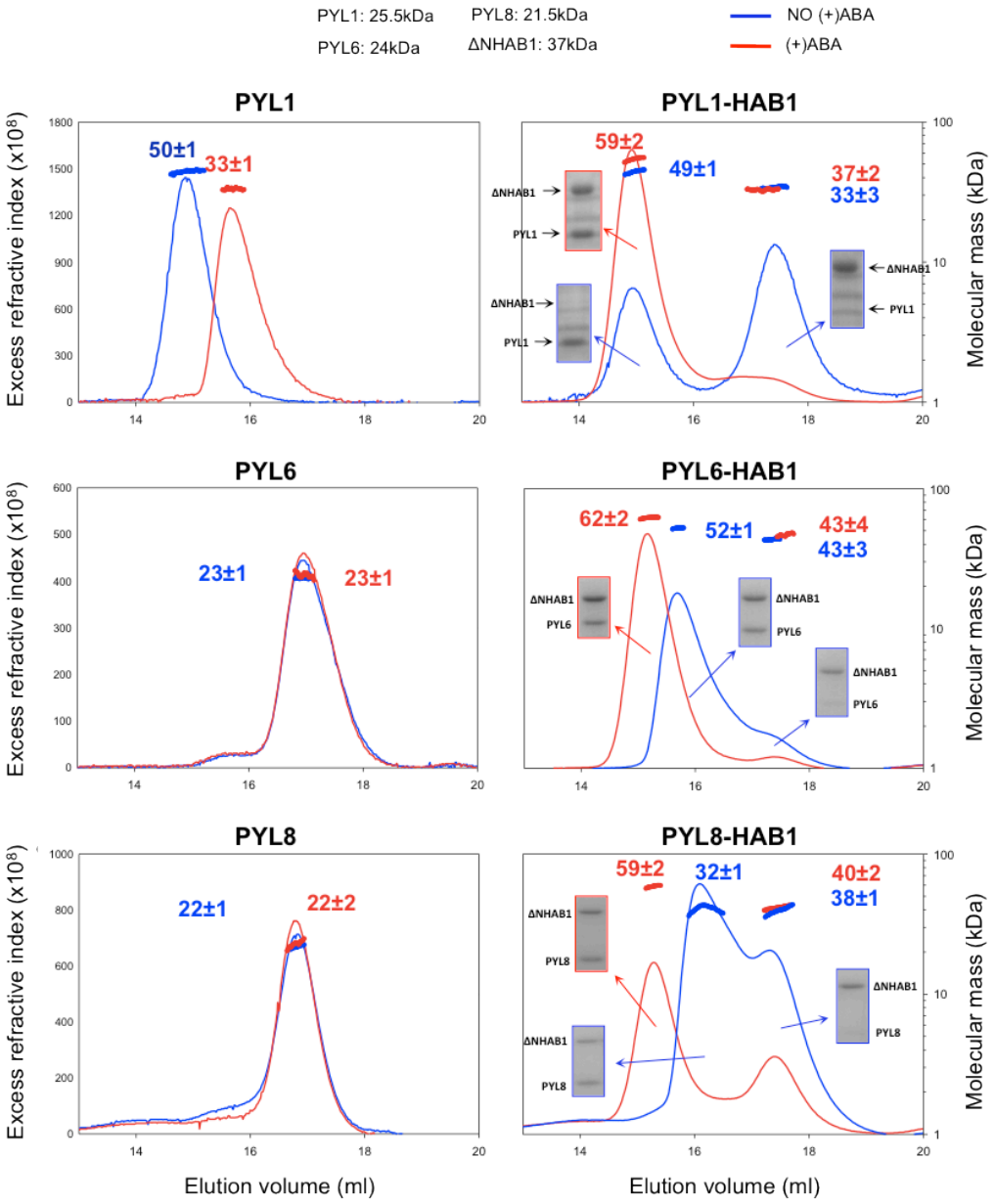
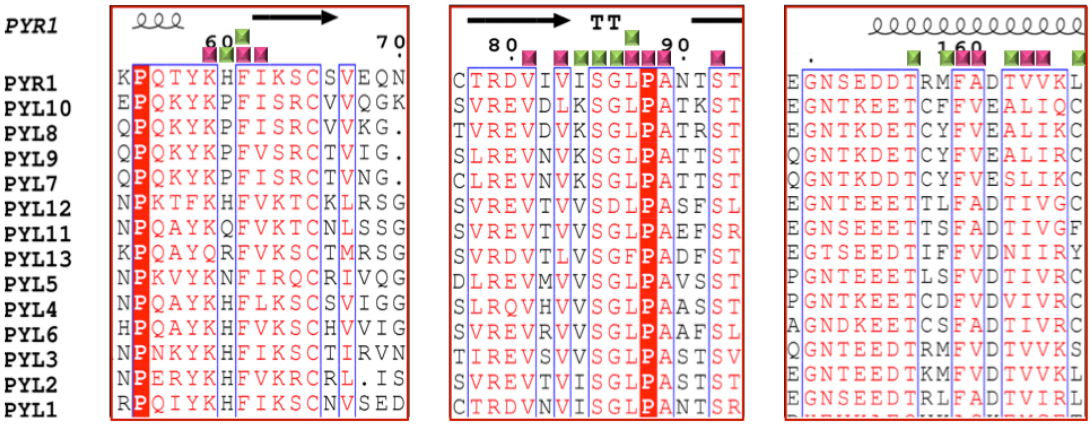
SUPPLEMENTAL MATERIAL



Supplemental Figure 1. ABA receptors exist in dimeric and monomeric forms. SEC-MALL analysis of PYL1, PYL6 and PYL8 alone (left panels) and in the presence of ΔNHAB1 (right panels). The experiments were done in the absence (blue) and presence (red) of 1mM (PYL6 & PYL8) or 5mM (PYL1) (+)ABA. The apparent size of PYL6 and PYL8 indicate that they are monomeric, both in the presence and absence of (+)ABA. PYL1 is dimeric in the absence of ABA and addition of 5mM ABA produces partial dissociation. All receptor proteins tested in this study form 1:1 complexes when combined with ΔNHAB1 in the presence of (+)ABA (right panels). However, while dimeric proteins including PYL1 and PYR1 (see figure 2 in main text) do not interact with ΔNHAB1 in the absence of (+)ABA, the formation of less stable complexes between monomeric receptors PYL6, PYL8 and ΔNHAB1 in the same conditions is revealed by a decrease in the height of the peaks corresponding to monomeric ΔNHAB1, and the appearance of new peaks containing both receptor proteins and ΔNHAB1 (figure 2 in main text shows similar experiments for PYR1 and PYL5).



Supplemental Figure 2. Amino acids involved in dimerization and ABA binding in the PYR/PYL/RCAR family. Multiple sequence and secondary structure alignment of selected regions containing amino acids involved in (+)ABA binding (pink squares) and receptor dimer formation (green). As it can be appreciated residues involved in dimerization are surrounded by or next to amino acids involved in ABA binding. PYR1, PYL1 and PYL2 are dimeric proteins while PYL5, PYL6 and PYL8 (containing a Proline at position 60) are monomeric.

7. RESULTS: CHAPTER 4

The ABA-PYR/PYL/RCAR-PP2C signalling pathway is conserved in cultivated plants

The ABA-PYR/PYL/RCAR-PP2C signaling pathway is conserved in cultivated plants

INTRODUCTION

Water scarcity, typically accompanied by increasing salinity, is one of the major causes of poor plant performance and limited crop yield worldwide (Buchanan et al., 2000), particularly in developing countries where crop growth and productivity mainly depend on rainfall patterns. Global climate change, which is increasing temperatures worldwide and changing rainfall patterns, has exacerbated regional droughts, creating a general drying trend which has been proved to have a clear detrimental effect on biomass yields (Zhao and Running, 2010) and therefore in agriculture. Improvements in the abiotic stress tolerance capacity of plants through biotechnological approaches would represent a great promise for overcoming these problems.

Oryza sativa (rice) is one of the most important staple crops globally, providing the 21% of the global human energy per capita (Maclean et al., 2002; FAO: International Year of Rice, 2004). Rice is a high-demanding water crop, so it is regularly grown in flooded rice paddies and its production covers 9% of the earth's arable land (Maclean et al., 2002). In addition to its agronomic importance, *O. sativa* is also an important biological model species for monocots plants. All these arguments make rice a suitable crop to be improved to enhance its abiotic stress tolerance, particularly to short water conditions.

The phytohormone abscisic acid, besides controlling various aspects of plant growth and development (Finkelstein et al., 2002; Himmelbach et al., 2003), plays a key role in regulating the adaptive responses of plants to environmental stresses such as drought and salinity (Zhu, 2002; Verslues et al., 2006). In the past two years several important findings have provided a big step in understanding the ABA signaling pathway in *Arabidopsis*. One of them has been the identification of the core elements of the ABA signaling cascade (Fujii et al., 2009), together with the identification of the intracellular ABA receptors PYR/PYL/RCAR family (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A). This core pathway provides a four-step linear cascade that goes from ABA perception to ABA-regulated gene expression, whose products would ultimately confer drought tolerance to the plant. The PYR/PYL/RCAR receptors function at the apex of this core signaling cascade, regulating the ABA-dependent SnK2s kinase activity by interacting and inhibiting the negative regulators of the

pathway, the clade A PP2Cs. Accumulation of active phosphorylated SnRK2s leads to the activation of downstream AREB/ABF transcription factors, which would ultimately result in the activation of the ABA adaptive responses (Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Moreover, the structure resolution of these receptors alone and in complex with the following elements of the pathway, the PP2Cs, have revealed how ABA ultimately inhibits PP2C activity by converting the PYR/PYL/RCAR receptors into PP2C inhibitors (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009; Dupeux et al., 2011). This transformation occurs when ABA binds to the PYR/PYL/RCAR receptors inducing conformational changes in the called 'gating loops' (SGLPA and HRL) located at the upper part of the ligand cavity. This gates motion generates a surface that facilitates the PP2C docking on to the PYR/PYL/RCAR receptors through a conserved Trp. The β3-β4 SGLPA loop of the ABA-bound receptors then inserts into the PP2C catalytic site, blocking it through the interaction of the receptor's Ser with two well conserved Gly and Glu residues of the PP2C active site.

These core elements have already been proven to represent a potential biotechnological tool to enhance plant drought tolerance in *Arabidopsis*. The first approach was carried in the work of Saez et al., (2006), through the combined inactivation of two main AtPP2Cs, HAB1 and ABI1. The double knockout mutants resulted to present significant drought resistance as compared to the wild type. Later on, in the work of Rubio et al., (2009), a similar approach was used combining the inactivation of pairs of PP2Cs with similar results. Besides the inactivation of negative regulators of the pathway, overexpression of positive regulators has also been carried out with successful results. For instance, overexpression PYR/PYL/RCAR receptor members, PYL5 or RCAR1/PYL9, or the AREB/ABF transcription factors, have also resulted in drought-avoidant plants (Kang et al., 2002; Fujita et al., 2005; Ma et al., 2009; Santiago et al., 2009A).

This ABA-signaling core structure appears to be conserved in rice, making it susceptible to be modified in order to generate plants with higher tolerance to drought stress. Some of these core elements in rice have been well characterized to be involved in ABA signaling such as the SAPK kinases, SAPK8, SAPK9 and SAPK10, orthologs to the *Arabidopsi*s ABA-activated SnRK2s (Kobayashi et al., 2004; Kobayashi et al., 2005), or the rice AREB/ABF transcription factors family (Lu et al. 2009; Xiang et al. 2008; Zou et al. 2008; Hossain et al., 2010A; Hossain et al., 2010B). Functional characterization of some of these transcription factor members such OsABF1, OsABF2, OsABI5, OsbZIP23 and OsbZIP72 have revealed them as ABA

positive regulators, capable of binding to the *cis* ABRE element (ABA responsive element) and activating the expression of downstream ABA regulated genes, that would ultimately confer drought tolerance to the plant. The overexpression approach of some of these elements, particularly of OsbZIP23 and OsbZIP72, has already been documented resulting in plants more tolerant to salinity and drought (Lu et al. 2009; Xiang et al. 2008). In the case of the OsPP2Cs, a comparative genome-wide overview of the PP2C family of *Arabidopsis* and rice, has revealed that both species have a similar number of genes encoding for PP2C proteins, as well as a similar number of PP2Cs classified as clade A, 9 and 10 respectively (Xue et al., 2008) (Figure 1A). Moreover, these OsPP2Cs seem to also share roles with AtPP2CAs since they appear to be up-regulated in response to ABA and salt, similarly as AtPP2CAs (Xue et al., 2008), suggesting that OsPP2Cs might also be involved in ABA signaling. When looking for PYR/PYL/RCAR homologues in rice, 14 high identity genes popped up (Figure 1B). These eleven OsPYR/PYL/RCAR members seem to present high homology in sequence amino acid and secondary structure, presenting a similar pattern of seven β-sheets sandwiched by two main α-helices (Figure 2). Important to highlight in these putative OsPYR/PYL/RCAR ABA receptors is that sequences encoding for the 'gating loops', 'SGLPA' (between β3 and β4) and 'HRL' (between β5 and β6) that confer to the receptors their inhibitory capacity when binding ABA, are well conserved. This provides additional support to the idea of these putative OsPYR/PYL/RCAR proteins behaving as ABA receptors and turning into PP2C inhibitors upon binding to the hormone.

Even though the ABA signaling pathway has not been experimentally reconstituted using these rice core elements, they seem to present similar properties and share similar roles with the *Arabidopsis* ones, suggesting that the ABA signaling pathway that goes from hormone perception to gene expression would be highly conserved in rice.

The work here presented was based on the biotechnological approach suggested by the works of Saez et al. (2006) and Rubio et al. (2009), in which enhanced drought tolerance in plants was conferred by the inactivation of two genes encoding for PP2CAs. So, we wondered if we could extrapolate the same approach in rice plants.

In spite of the identification, by sequence homology, of the OsPP2C members, not phenotypic characterization of this family was yet carried out. Here we report the identification of two *Oryza sativa* PP2CA T-DNA mutants that are homologues to the *Arabidopsis* ABI1, ABI2, HAB1 and HAB2, and the characterization of their response to

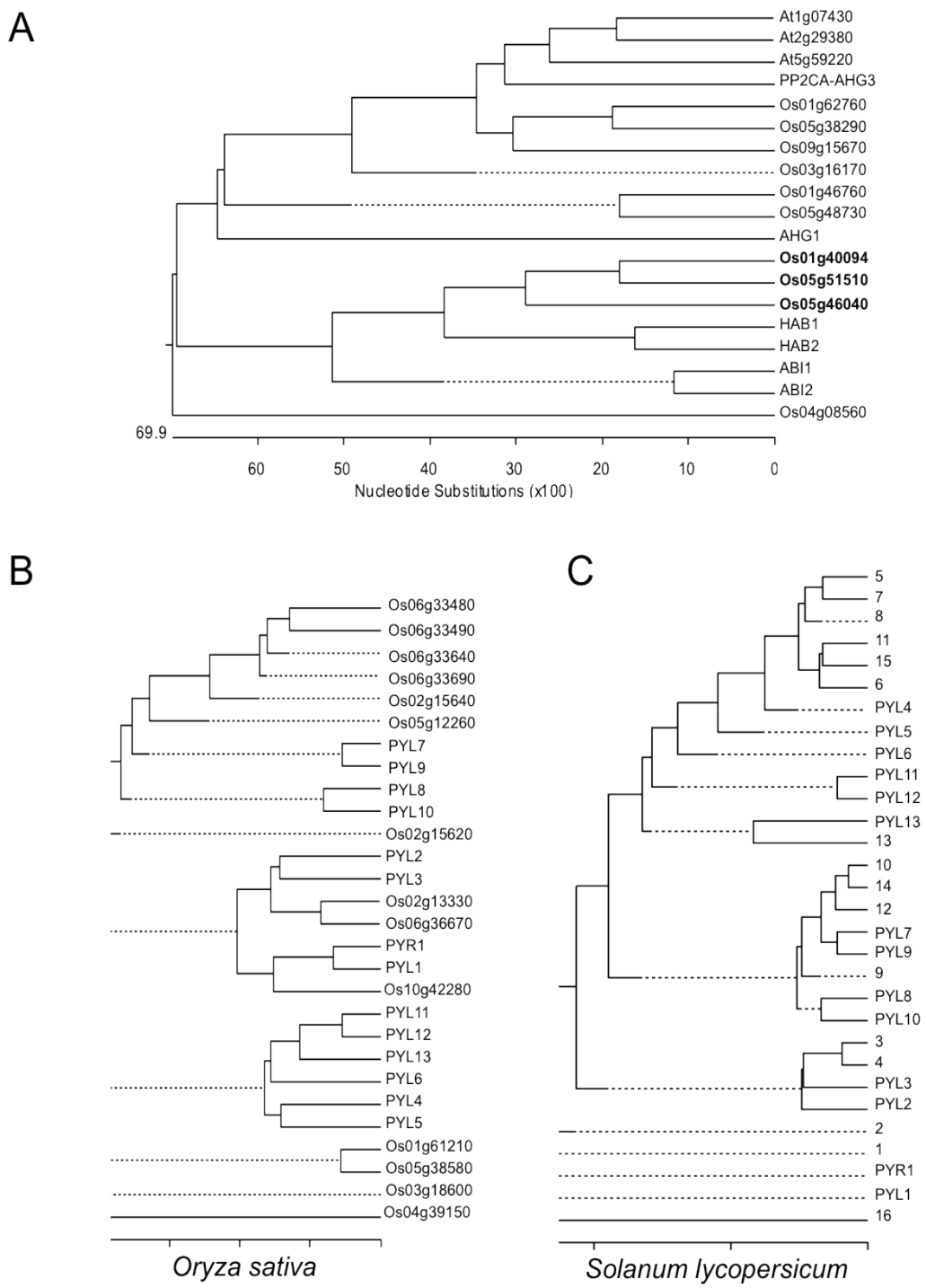


Figure 1. A, Clade A PP2Cs and PYR/PYL/RCAR family in *Arabidopsis*, *Oryza sativa* and *Solanum lycopersicum*. Cladogram based on sequence alignment of *Arabidopsis* clade A type-2C phosphatases and *Oryza sativa* homologues. In bold the *Oryza sativa* closest homologues to the *Arabidopsis* ABI1, ABI2, HAB1 and HAB2. **B** and **C,** Cladograms based on sequence alignment between PYR/PYL/RCAR family in *Arabidopsis* and their closest homologues in *Oryza sativa* and *Solanum lycopersicum*, respectively. Phylogenetic analysis reveal a similar 4 group distribution between *Arabidopsis* and tomato, whereas in rice we can only see 3. This might be due to a problem in annotation of some of the rice members. (*Solanum lycopersicum* information was gently and confidentially provided by the group of Granell, A.).

ABA and drought. As a result, single loss-of-function mutants did not show significant phenotypic differences as compared to the wild type, suggesting a potential functional redundancy among the different members of the family. In order to overcome this potential functional redundancy, both parental mutants were crossed to generate a double mutant. The double mutant was screened in the F2 progeny without much success, probably due to a segregation distortion event. Therefore generation of the next self-pollinated progeny (F3) was carried out in order to increase the chances for the double mutant identification and characterization.

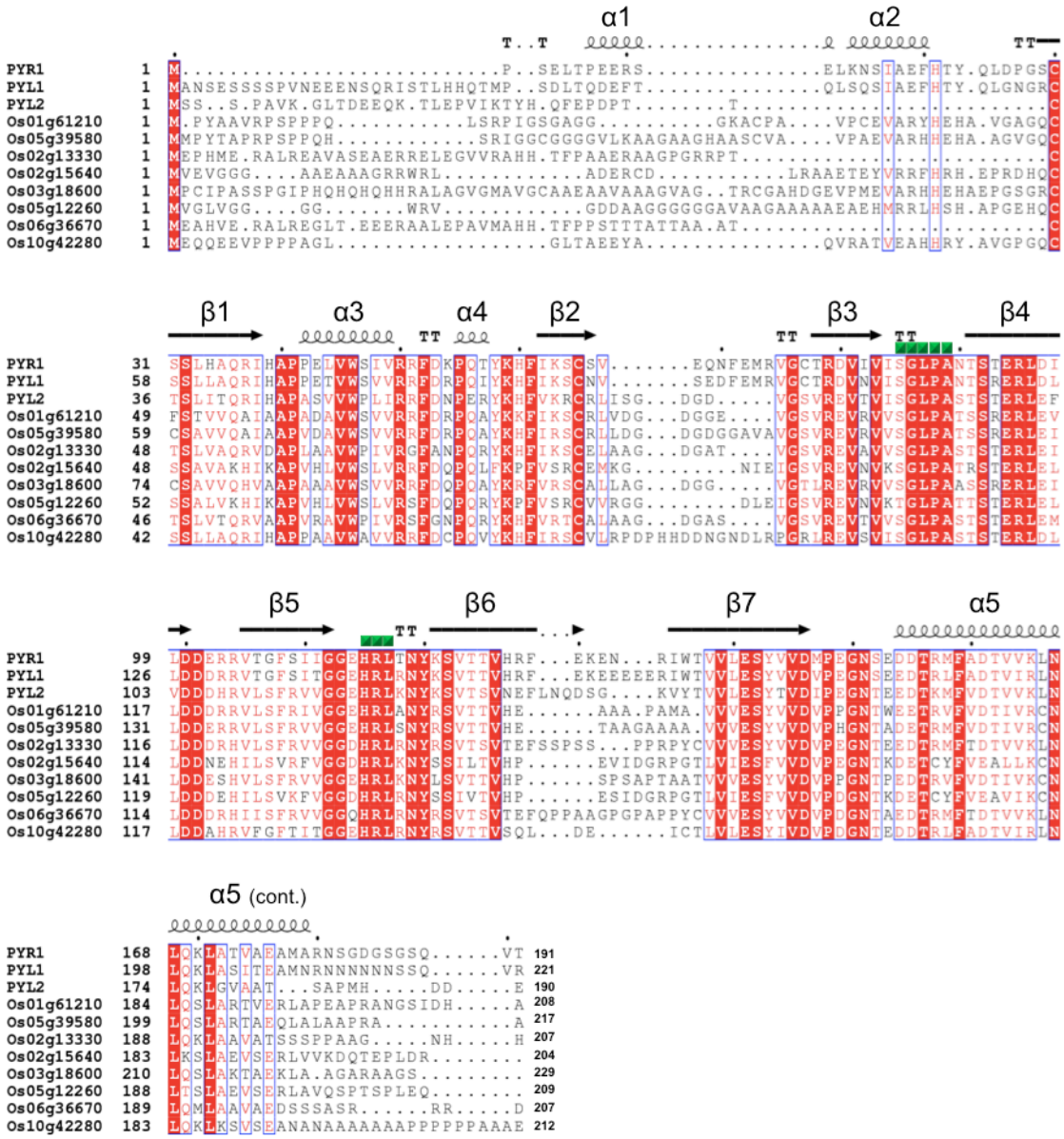


Figure 2. Amino acid sequence and secondary structure alignment of *Arabidopsis* PYR/PYL/RCAR ABA receptors with their closest homologues in *Oryza sativa*. In green squares marked the amino acid residues corresponding to the gating loops. Figure generated with ESPript 2.2 (Gouet *et al.,* 1999).

RESULTS

Identification of T-DNA insertion mutants of *LOC_Os5g46040* and *LOC_Os1g40094*

Phylogenetic and comparative analysis of AtPP2CAs and its homologues in rice, illustrates a similar distribution in two main branches. One of the branches comprises ABI1, ABI2, HAB1 and HAB2, and the other one includes AHG1 and PP2CA/AHG3 (Figure 1A). Additionally, comparative analysis of amino acid sequence and secondary structure of *Arabidopsis* ABI1, ABI2, HAB1 and HAB2 phosphatases with their closest homologues in rice, reveal that they share a secondary structure pattern and a well conserved catalytic site (Figure 3). So, we selected the loci *LOC_Os5g46040*, *LOC_Os1g40094* and *LOC_Os5g51510* for being the closest homologues to the *Arabidopsis* ABI1, ABI2, HAB1 and HAB2 phosphatases. T-DNA insertion mutants were found for the loci *LOC_Os5g46040* and *LOC_Os1g40094* in the SIGnAL RiceGE collection of T-DNA lines, which correspond to donor stock numbers PGF_4A-01569.L and PGF_1A-08436.R*,* respectively (Figure 4A). The genotype background was *Oryza sativa* L. *japonica* genotype Dongjin. In order to indentify homozygous individuals for both T-DNA insertion lines we first performed a selection of plants in hygromycin antibiotic. Genomic DNA was extracted from hygromycin-resistant plants and submitted to PCR analysis for each locus to identify knockout individuals. Primer pairs F601g46040/ R960g46040 and F1056g40094/R1344g40094 were used to analyze individuals from PGF_4A-01569.L and PGF_1A-08436.R lines, respectively. One homozygous individual was identified for the PGF_4A-01569.L line, named *4a1,* and two for the PGF_1A-08436.R line, named *1a2* and *1a6* (Figure 4B). Sequencing of the T-DNA flanking region in *1a2* and *4a1* showed that the insertion was localized 620 and 242 nucleotides downstream from the ATG start codon, respectively (Figure 4A). These loss-of-function mutants were used for further characterization and to generate the double mutant *1a2 4a.*

ABA response characterization of *OsPP2C* single mutants in germination and early seedling growth

Single mutants of the *Arabidopsis HAB1* and *ABI1* have been reported to show ABA-hypersensitivity inhibition of germination, among other responses (Leonhardt et al., 2004; Saez et al., 2004; Saez et al., 2006). Based on gene homology and the results from these studies, we wanted to characterize the germination and early seedling growth response of our rice PP2C loss-of-function mutants, *1a2* and *4a1*,

under ABA conditions. Since we had not work with the rice system before, we had to first establish the window of ABA concentrations in which the wild type was sensitive but still allowed us to see differences in responses when assaying the single mutants.

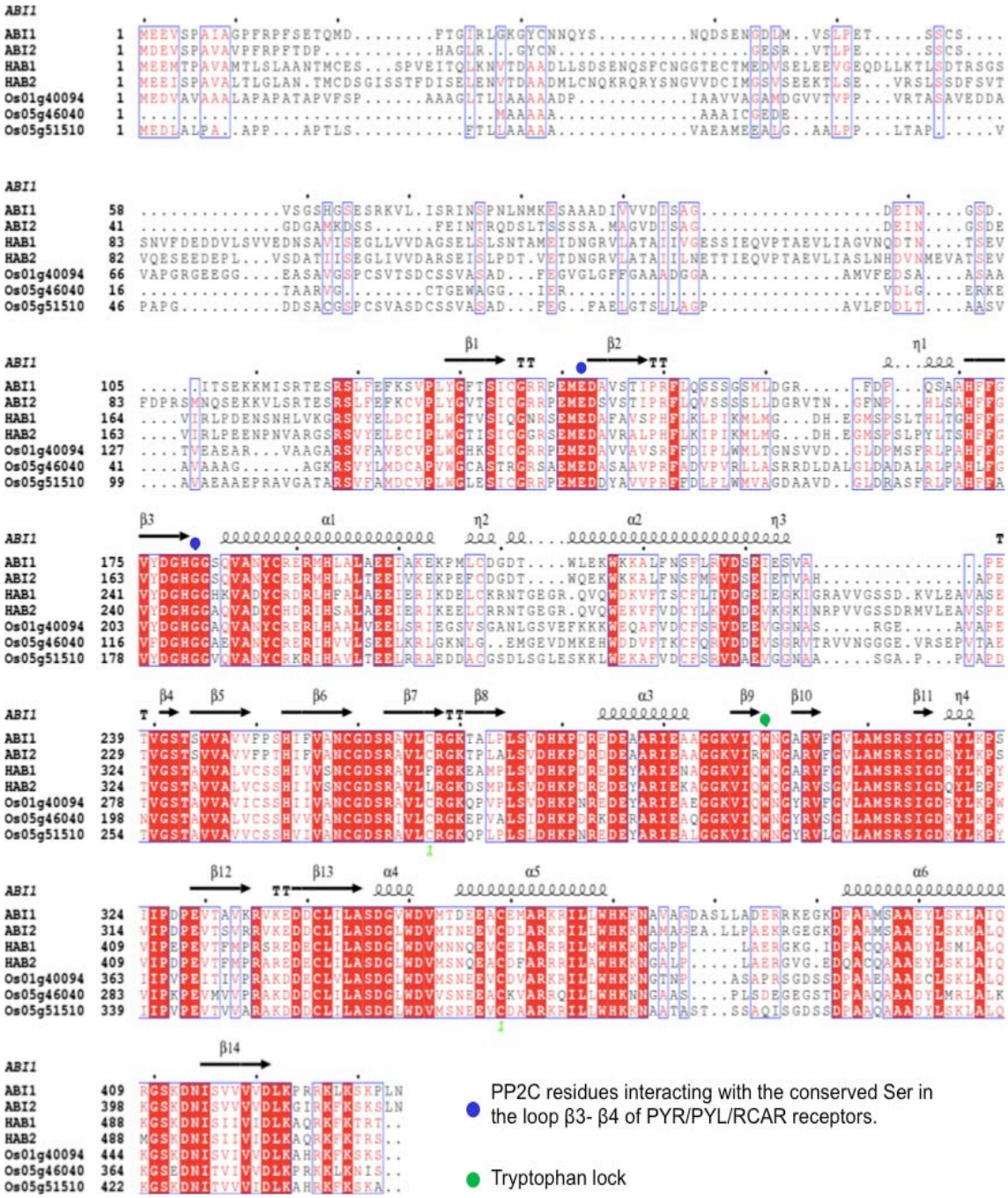


Figure 3. Amino acid sequence and secondary structure alignment of *Arabidopsis* clade A PP2Cs with their closest homologues in *Oryza sativa*. Colour codes indicate the amino acid residues involved in the interaction with PYR/PYL/RCAR ABA receptors that are responsible for the inhibition of the phosphatase. Figure generated with ESPript 2.2 (Gouet *et al.,* 1999).

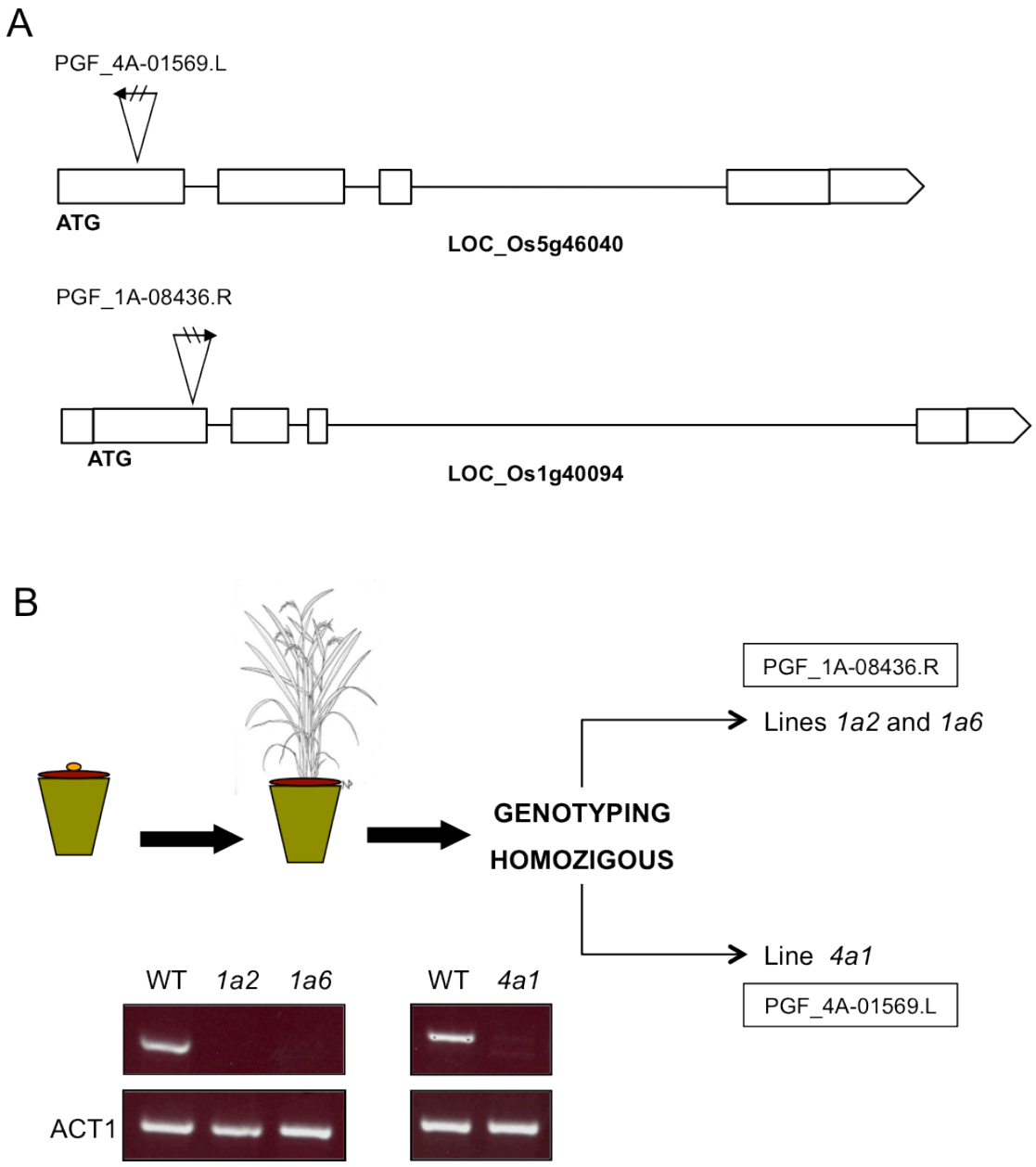


Figure 4. Identification of loss-of-function mutants of *Oryza sativa* clade A PP2C members. A, Schematic representation of the *LOC_Os5g46040* and *LOC_Os1g40094* genes. T-DNA insertions are indicated and orientation of the left-border sequence of the respective T-DNAs is represented by broken arrows. **B,** Schematic representation of the steps followed to identify the PP2C knockout mutants and PCR analysis. PCR reactions were performed with primers F1056/R1344 for lines *1a2* and *1a6* and F601/R960 for the line *4a1*. ACT amplification was used as control (see primers in Experimental procedures).

We performed a preliminary assay with the rice wild type in MS liquid medium and MS supplemented with 1, 2, 4 and 10 µM ABA. After 6 days, seeds had germinated in 1, 2 and 4 µM ABA (Figure 5, 6 days after sowing, wild type (WT) line), showing different degrees of tolerance to the hormone, whereas germination was completely arrested under 10 µM ABA (data not shown). Effects on early growth could

be appreciated among the different concentrations 12 days after sowing, being significantly inhibited in 4 µM ABA (Figure 5, 12 days after sowing, WT line). Since we could see different degrees of sensitivity to ABA in the wild type from 1 to 4 µM ABA, we decided to use this window of concentrations to analyze the germination and early seedling growth response of the *1a2* and *4a1* mutants.

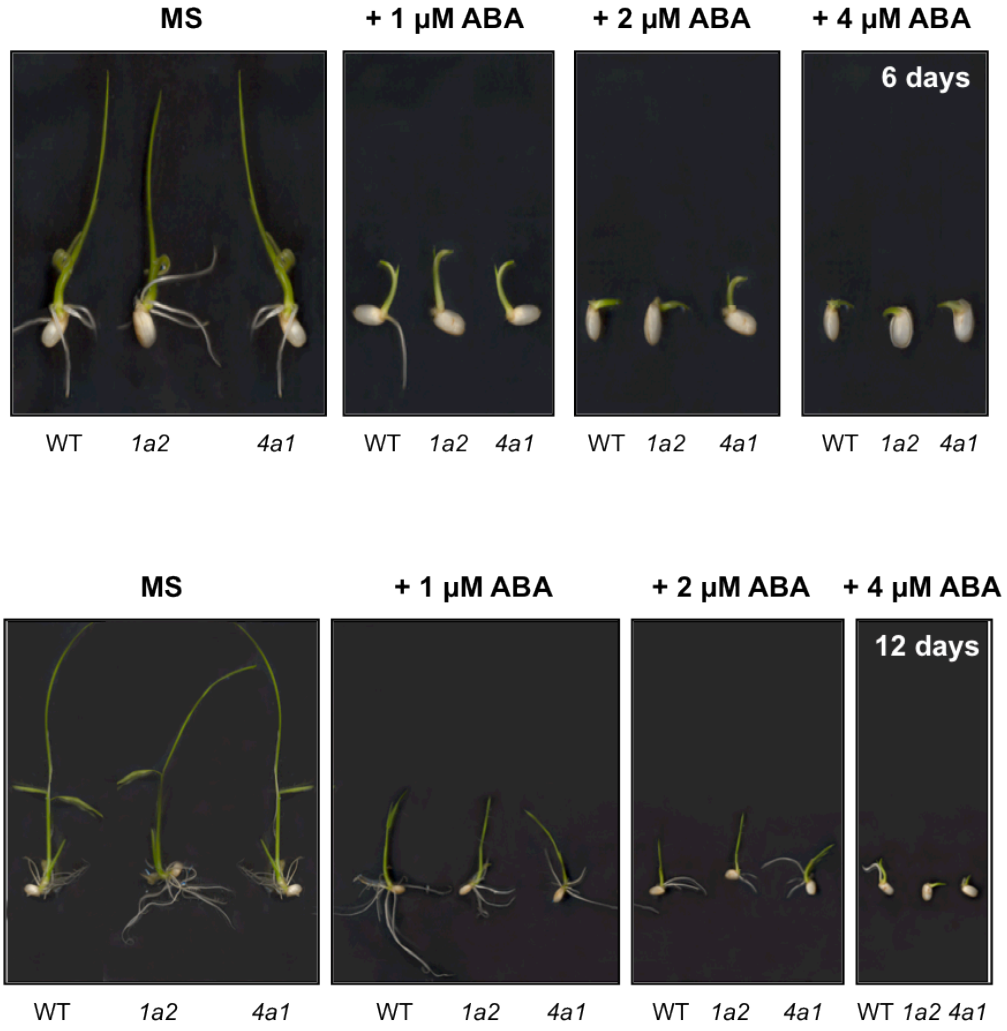


Figure 5. ABA response characterization of *Oryza sativa* PP2C single mutants in germination and early seedling growth. A. Rice seeds were sterilized and stratified during 1 day. Seeds were sown in MS liquid medium without sucrose and supplemented with ABA when indicated. Representative plants were selected and photographs were taken 6 and 12 days after sowing.

We performed a similar assay including *1a2* and *4a1* mutants and data were recorded 6 and 12 days after sowing. As we can see in figure 5, neither of the mutants showed differences in germination or early seedling growth with respect to the wild type, in the presence of exogenous ABA. An explanation for that could be functional redundancy among the OsPP2C members. Sequence analysis among OsPP2C family

members reveal that the closest branch to *Arabidopsis* ABI1, ABI2, HAB1 and HAB2, is composed by three members: Os01g40094, Os05g51510 and Os05g46040 (Figure 1A), suggesting that probably it would be necessary to inactivate several phosphatases to reveal a phenotype.

Growth characterization of OsPP2C single mutants in response to ABA

To further characterized *1a2* and *4a1* mutants we also analyzed their shoot and root growth phenotype in the presence of the hormone. *Arabidopsis HAB1* and *ABI* single mutants had also been reported to exhibit growth inhibition in the presence of ABA (Saez et al., 2006; Rubio et al., 2009) so we wondered if any of our rice single mutants would show ABA-mediated growth inhibition as compared to the wild type. Similarly as before, we had first to establish the experimental conditions. A preliminary assay was performed with wild type rice seedlings (Figure 6A, WT line). 6-days old plants, grown in MS medium, were transferred to plates containing 10 and 30 µM ABA. After 8 days we could see that the wild type was sensitive to these hormone concentrations, both in shoot and root growth, and that there was still a window to identify ABA hypersensitivity. We then performed the assay with *1a2* and *4a1* but both mutants presented the same growth impairment in shoot and root as the wild type (Figure 6A, B and C). These results supported the idea, above presented, of functional redundancy among the members of the family.

Water loss characterization of OsPP2C single mutants *1a2* and *4a1*

In response to drought stress, ABA signaling plays a critical role in reducing water loss through transpiration by regulating stomatal aperture. To evaluate drought responses in wild type and the different mutant backgrounds in terms of water loss, we performed a short-term drought assay in which reduction of fresh weight of detached leaves was evaluated (Verslues et al., 2006). Similarly to the other ABA-responses, loss-of-function *1a2* and *4a1* mutants did not exhibit significant differences in the transpiration rate of detached leaves compared to wild type (Figure 6D). Similar results had been reported for single *Arabidopsis* PP2CA mutants. Phenotypes of reduced water loss in detached-leaf assays were reported when combining inactivation of two or more PP2CAs (Saez et al., 2006; Rubio et al., 2009). These results, together with the above presented, strongly suggested the need to inactivate several OsPP2Cs to be able to characterize the response to ABA of these rice PP2Cs.

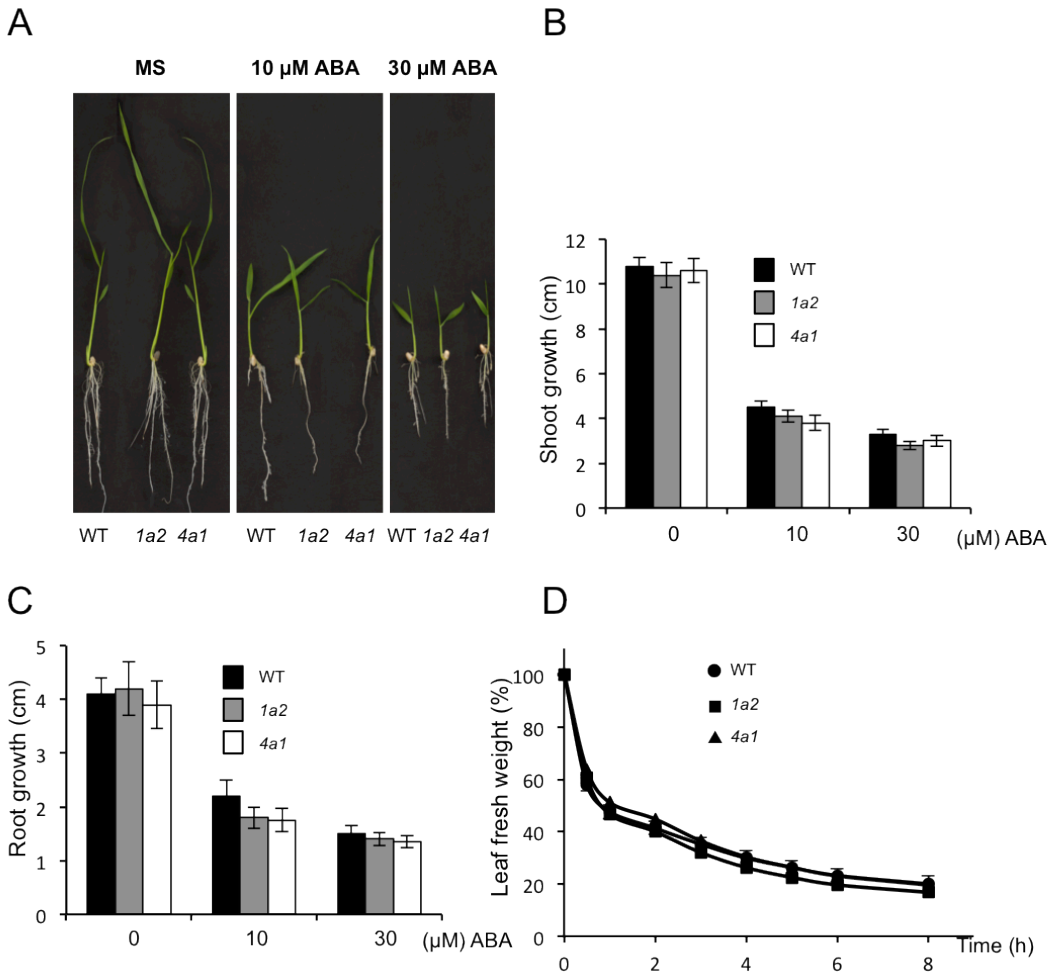


Figure 6. Growth characterization of *Oryza sativa* PP2C single mutants in response to ABA and water loss performance A, Rice seeds were sterilized and stratified during 1 day, and sown in MS-agar plates. 6-day old seedlings were transferred to plates lacking or supplemented with 10 and 30 µM ABA. Representative seedlings were selected and photographs were taken 8 days after the transfer. **B** and **C,** Shoot and root growth quantification, respectively, of *1a2* and *4a1* mutants vs. WT. Data are averages ± SD from three independent experiments (*n=20*). **D,** Water loss assay measured in detached leaves of single mutants *1a2* and *4a1* vs. WT. Values are averages from 3 independent experiments (*n=5*). SD values were lower than 8%.

Generation and identification of the OsPP2C double mutant *1a2 4a1*

As mentioned before the rice closest branch to *Arabidopsis* ABI1, ABI2, HAB1 and HAB2, is composed by three members. In addition, analysis of single loss-of-function mutants had revealed no differences in ABA-mediated responses as compared to the wild type, suggesting a possible functional redundancy among the members of these phosphatases. To unravel this potential functional redundancy, we decided to generate a double mutant containing the knockout alleles of both genes. To this end we crossed *1a2* mutant with *4a1* (Figure 7A). After 3 months, 6 F1 embryos

were obtained. To verify the success of the crossing, we analyzed the F1 individuals for the presence of both T-DNA insertions by PCR, obtaining 5 out of 6 positive results (Figure 7B). The F1 individuals were transferred to the greenhouse and the F2 progeny was collected 6 months later. According to the prediction of the double mutant presenting an ABA hypersensitive phenotype, we decided to perform a phenotypic screening, in the presence of ABA, to select the most hypersensitive F2 individuals in order to enrich the population that would be further analyzed by PCR. The Mendelian proportion expected would be 1/16, so we screened 40 seeds of two F2 progenies using 1 µM ABA in comparison to the wild type (Figure 7C). After 6 and 12 days we could not identify clear phenotypic differences between the potential double mutant and the wild type (data not shown). So, 15 individuals from each F2 progeny assayed (30 individuals in total) were picked and genotyped for both knockout genes. Genotyping did not provide any double knockout mutant. PCR analysis of F2 individuals resulted in the amplification of the targeted genes. However, F2 individuals also resulted to have at least one copy of each T-DNA insertion. This seemed to indicate that our F2 population was rich in heterozygotic individuals, having lost the Mendelian proportion. An explanation for that could be segregation distortion, which can be defined as a deviation from the expected Mendelian proportion of individuals in a given genotypic class within a segregating population (Lyttle, 1991). This phenomena has been frequently observed in rice and particularly in progenies derived from *indica-japonica* crosses (between different varieties) (Xu et al., 1997; Jamil et al., 2009), but it has also been documented in *japonica* crosses (Redona and Mackill, 1996; Yamagishi et al., 2010). Segregation distortion is often linked with segregation-distortion factors, such as hybrid sterility (*S*) genes (Sano 1990) and gametophyte competition (*ga*) genes (Nakaghara 1972), but distortion has also been observed in other chromosomal regions where no corresponding gametophyte or sterility genes have previously been reported (Xu et al., 1997). In addition, one of the distortions effects observed in F2 populations is an excess of the heterozytic class (Xu et al., 1997), which could fit to our case. In order to overcome this possible problem we decided to obtain another self-pollinated generation, F3, to increase our homozygosis ratio and also analyze a larger number of individuals, 5 times more than for a normal Mendelian proportion (in between 150-200 individuals), in order to increase our chances to identify the double mutant. Due to technical limitations we could only obtain one rice generation per year, so the F3 was generated but we could not proceed to further analysis.

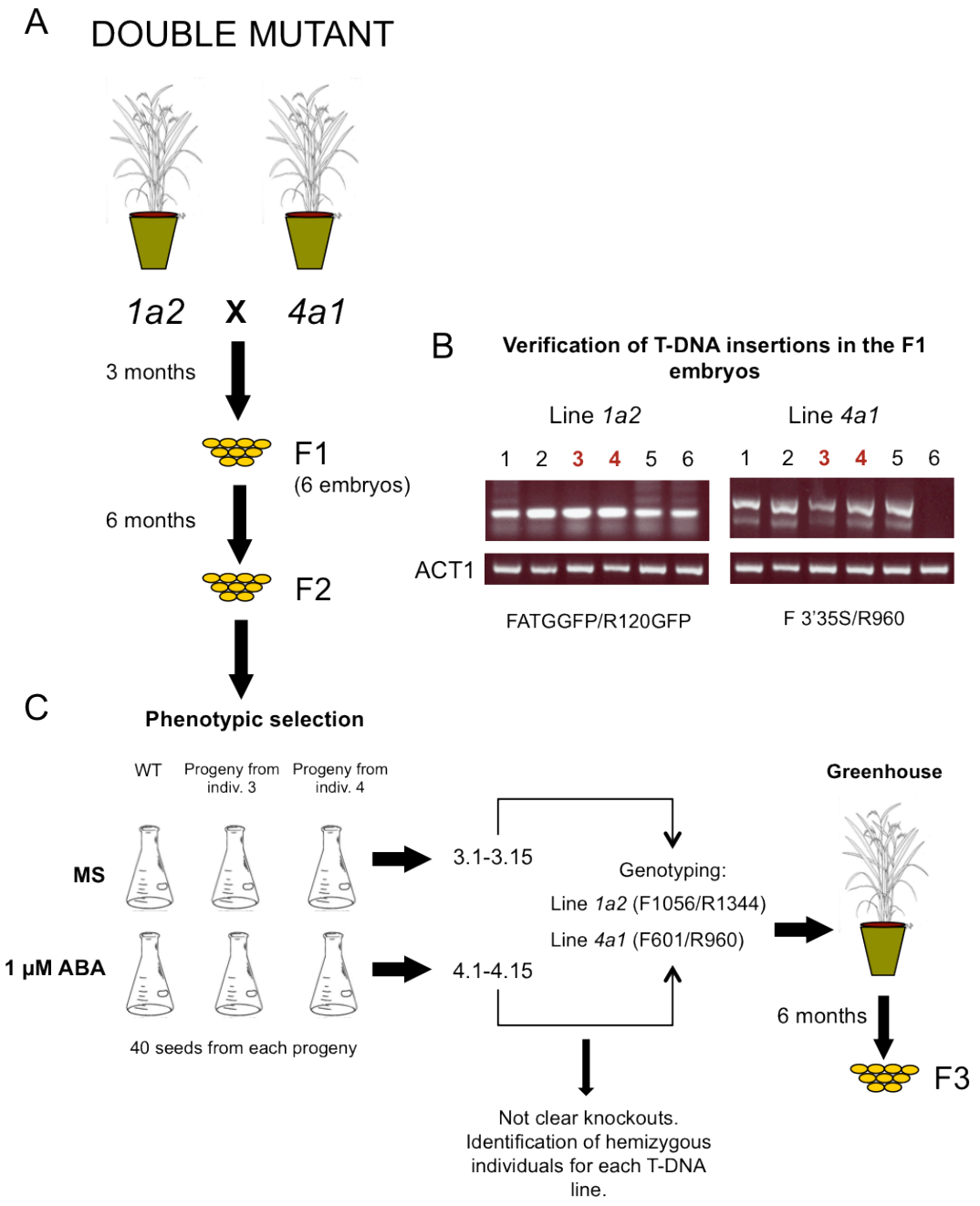


Figure 7. Schematic representation of the steps followed for generating and identifying the rice double-knockout mutant of the PP2CAs *1a2 4a1*. A, Crossing of the two parental mutant lines and generation of the following progenies**. B,** Verification of the presence of the T-DNA insertions in the F1 embryos. **C,** Identification strategy of the double mutant *1a2 4a1*.

DISCUSSION

The first objective of this work was to characterize the OsPP2C family in response to ABA and drought, to further use them as a potential biotechnological tool to enhance drought tolerance in rice. To this end we first identified and selected the

putative candidates. Phylogenetic and sequence homology analysis revealed 10 rice homologues to AtPP2CAs that were similarly distributed in two main branches (Figure 1A). Os01g40094, Os05g51510 and Os05g46040 loci were selected for being the closest homologues to the *Arabidopsis* ABI1, ABI2, HAB1 and HAB2. Besides their sequence homology, a clue of their implication in ABA signaling was provided by analyzing their expression in response to ABA (Figure 8A). Transcriptional data from public databases showed that these OsPP2Cs were up-regulated in response to drought. Identification and characterization of single loss-of-function mutants from two of these OsPP2Cs, *1a2* and *4a1*, showed no phenotypic differences when compared to the wild type in any of the ABA-mediated responses examined such as germination-early seedling growth or shoot and root growth. Since the branch was composed by three members, Os01g40094, Os05g51510 and Os05g46040, an explanation for these results could be functional redundancy among the different members of the family. So that, only by combined inactivation, a phenotypic response could be revealed. Single mutants of *Arabidopsis* PP2Cs exhibit a phenotype of hypersensitivity to ABA but partial overlapping functions have also been documented among them (Saez et al., 2004; Saez et al., 2006; Nishimura et al., 2007; Rubio et al., 2009). The results in the case of *1a2* and *4a1* rice mutants could mean that functional redundancy might not only be partial, in which case the experimental conditions used might also have not been suitable for detecting small phenotypic differences if any. Additionally, when we assayed the mutants in response to drought no differences were shown between the OsPP2C mutants and the wild type, probably due to the same explanation.

In order to unravel this functional redundancy, and to evaluate if we could extrapolate the same approach carried out in *Arabidopsis*, we initiated the generation of a double mutant *1a2 4a1*. To this end we crossed *1a2* and *4a1* mutants obtaining 5 F1 individuals carrying both T-DNA insertions (Figure 7A and B). The following F2 progeny was generated and collected to proceed to the identification and characterization of the double mutant *1a2 4a1*. Neither ABA-hypersensitive phenotypic screening nor PCR analysis of the F2 individuals analyzed, gave us any positive double mutant. However, F2 individuals resulted to have at least one copy of each T-DNA insertion, suggesting that our F2 progeny was rich in heterozygotic individuals, having lost the Mendelian proportion. This increase in heterozygotic individuals in rice F2 populations, has been qualified as a segregation distortion effect (Xu et al., 1997), and could explain our case. Segregation distortion effects have been frequently observed in progenies derived from rice *indica-japonica* crosses (Nakaghara 1972), but also it has been documented in intravarietal *japonica* crosses (Redona and Mackill,

1996). A solution to this problem would consist in increasing the homozygosis ratio by obtaining self-pollinated generations and analyzing larger numbers of individuals in order to increase the chances to indentify a double mutant. Due the fact that we could only obtained one generation per year, we could not proceed with the analysis of the F3 progeny in order to characterize this rice family of phosphatases in response to ABA and to evaluate if combined inactivation of OsPP2Cs would generate drought tolerant plants. Additionally, these results reflect the genetic difficulties that could be found when working with this crop.

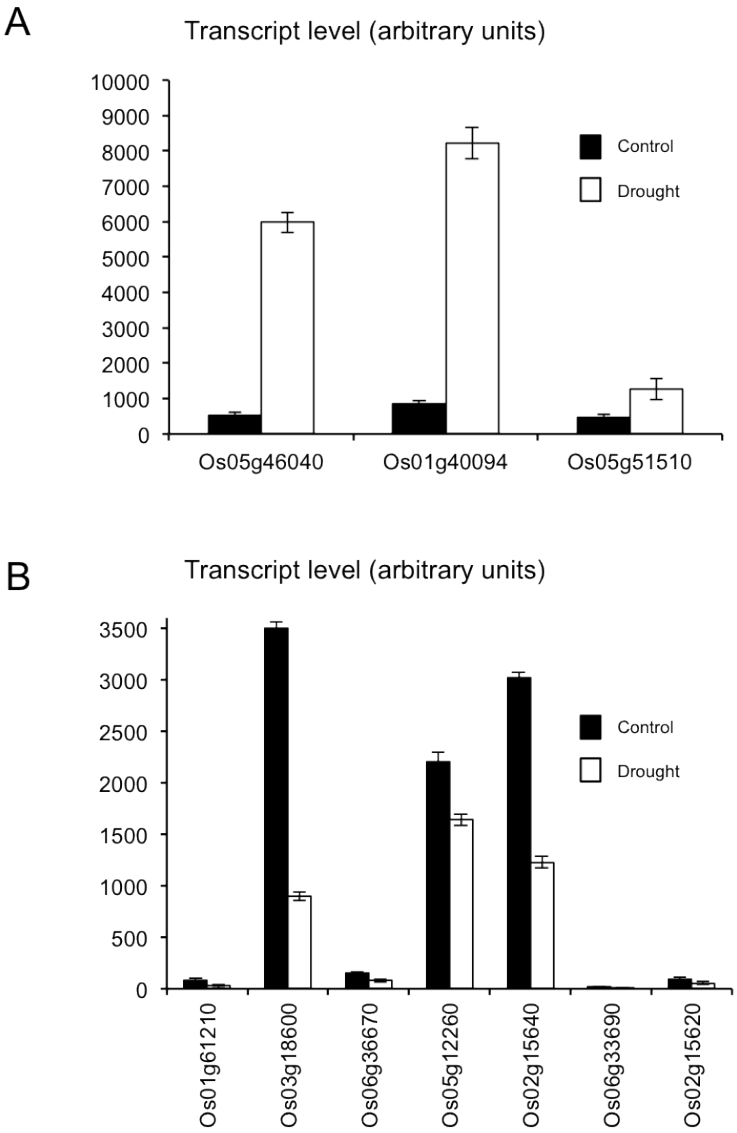


Figure 8. Expression data of *Oryza sativa* clade A PP2Cs and PYR/PYL/RCAR family members. A, Expression levels of PP2C family members in 7-day old seedlings in normal or under drought stress conditions. **B,** Expression levels of PYR/PYL/RCAR members in 7-day old seedlings under similar conditions as in (A). Data were obtained from de Bio-array Resource for *Arabidopsis* Functional Genomics (http://bar.toronto.ca).

This work was started before the identification of the PYR/PYL/RCAR receptors family. But as we have described before, when looking for putative OsPYR/PYL/RCAR receptors we could find 8 candidate members of high identity. Comparative sequence analysis with the PYR/PYL/RCAR receptors in *Arabidopsis* shows that these rice proteins have conserved the sequences that encode for the called 'gating loops' that are crucial for the interaction and inhibition of PP2Cs (Figure 2). Moreover, when analyzing the OsPP2Cs, we can also find conserved the residues through which the interaction ABA-PYR/PYL/RCAR-PP2C is established, such as the 'Trp lock' and the Gly and Glu residues at the active site (Figure 3), suggesting a similar mechanisms of inhibition. In addition, expression data analysis from public data bases shows that these rice PYR/PYL/RCAR members are down-regulated by drought, resulting in high ABA levels (Figure 8B), similarly as it happens with most of the *Arabidopsis* PYR/PYL/RCAR members (Santiago et al., 2009A; Figure 5A Introduction).

These putative rice receptors could also be susceptible of modification with the aim of generating drought tolerant plants. A possible approach would be the generation of overexpressing lines of these positive regulators, similarly as done in *Arabidopsis* (Santiago et al., 2009A). However, another option would be the modulation of their function by taking advantage of their role as hormone receptors. Structural and mutational analyses of PYR/PYL/RCAR members in *Arabidopsis* with the first ABA agonist pyrabactin, have revealed subtle changes in the binding pockets of these proteins that can explain ABA agonist or antagonist effects (Hao et al., 2010; Peterson et al., 2010; Melcher et al., 2010; Yuan et al., 2010). This information provides a solid framework for the design or identification of new ABA activators that would allow the manipulation of ABA responses in plants, in order to improve crop yield under drought conditions.

EXPERIMENTAL PROCEDURES

Phylogenetic analysis

To identify *Arabidopsis* PP2CAs homologues in *Oryza sativa*, a BLASTp was performed in Gramene (http://www.gramene.org/) using *Arabidopsis* HAB1 and ABI1 as templates. In the case of the homologues to the PYR/PYL/RCAR family, a similar procedure was followed using *Arabidopsis* PYR1 and PYL5 as templates. Sequence

alignments by ClustalW method and phylogenetic trees were performed using Lasergene MegAlign software.

Plant material

T-DNA insertional mutant lines of *LOC_Os5g46040* and *LOC_Os1g40094* were identified from SIGnAL RiceGE collection of T-DNA lines, with donor stock numbers of PGF_4A-01569.L and PGF_1A-08436.R, respectively. To identify individuals homozygous for the T-DNA insertions, genomic DNA was extracted from hygromycin-resistant seedlings and submitted to PCR genotyping using T-DNA specific primers and the following gene-specific primer pairs:

F601g46040:5'-ATCTTGGGGAGAGGAAGGAG-3'

R960g46040:5'-CACGGAATCGGTCAAAATCGTGAGCTTAGG-3'

F1056g40094:5'-GTCGACGGCCTCGACCCCATG-3'

R1344g40094:5'-TAGCACCGGACACAGACCCCT-3'

For in vitro culture assays, seeds from the wild type rice (*Oryza sativa* L. *japonica* genotype Dongjin), *1a2* and *4a1* mutants, were surface sterilized with 70% ethanol during 2 minutes and then transferred to a solution containing 50% commercial bleach + 0.05% Tween-20 for 30 minutes in constant shaking. Seeds were washed 3 times in sterile distilled water, during 5, 10 and 15 minutes to remove the bleach. Seeds were then immersed in distilled water for 1 day at 4ºC in the dark for stratification and then sowed in Murashige-Skoog (MS) liquid medium. Plant germination and growth was carried out in a growth chamber with controlled environmental conditions (23ºC, 16/8 h day/night photoperiod).

ABA sensitivity tests: germination-early seedling growth and root/shoot growth

To perform the ABA sensitivity test at the germination and early seedling growth stage, seeds surfaces were sterilized and stratified as above indicated. Seeds were then sowed in MS liquid medium without sucrose, lacking and supplemented with different concentrations of ABA (1, 2, 4 and 10 μM). The experiment was performed using 30 seeds of each genotype. Photographs were taken 6 and 12 days after sowing.

For the root/shoot growth assay, sterile seeds were germinated on solid MS medium without sucrose and 1% agar. 6-days old seedlings were transferred to MS solid medium plates lacking or supplemented with 10 and 30 μM ABA. Shoot and root lengths were measured 8 days after the transfer and representative seedlings were photographed. Data are averages ± SD from three independent experiments (*n=20*).

Water loss assay

Seeds from the different genotypes were sterilized and stratified as described before. Rice plants were grown hydroponically in MS medium for 3 weeks. Five leaves per genotype were excised and fresh weight was determined by submitting the leaves to the drying atmosphere of a flow laminar hood for 8 h. Data are averages ± SD from three independent experiments (*n=5*).

Double mutant generation and identification

To generate the *1a2 4a1* double mutant, we performed the crossing of *1a2* and *4a1* parental lines in both directions, using *1a2* as a male and *4a1* as a female and the other way round. Crossing was performed by cutting the flower stamens from a panicle from the mutant used as the female parent, in order to prevent self-pollination. For pollen transfer, a panicle from the male parent was attached to the one from the female. Crossing panicles were knotted carefully with paper bags to prevent pollen contamination, and each individual rice seedling crossed was labeled with a tag to prevent seed contamination. The F1 embryos were analyzed by PCR, using T-DNA specific primers, to verify the presence of both T-DNA insertions. 5 out from 6 F1 embryos were sowed in the greenhouse and after 6 months the F2 progeny was ready to be collected. In the F2 the initial double mutant ratio should be 1/16. In order to indentify the double mutant, a phenotypic screening strategy was selected to enrich the double mutant population that would be later on analyzed by PCR. Forty F2 seeds coming from F1 individuals 3 and 4 were grown in parallel in MS liquid medium and MS liquid medium supplemented with 1 μM ABA. The phenotypic screening to select individuals by being more hypersensitive to ABA than the wild type was not very clear, therefore, 15 F2 individuals from each F1 parent were picked according to exhibiting some sensitivity to the hormone. Genotyping analysis by PCR was carried out for the presence of the double mutant using the primer pairs F601g46040/R960g46040 and F1056g40094/R1344g40094. Not double knockouts were identified. We then identified 15 individuals from each F2 progeny that would have at least one copy for each T-DNA

insertion line and they were transferred to the greenhouse in order to obtain the F3. Six months later the F3 progeny was collected.

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8. GENERAL DISCUSSION

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8.1 HORMONE SENSING AND PERCEPTION: a critical overview on the ABA receptors

Understanding how ABA was perceived in the cell and how was the signal transduced, was a critical missing part to better comprehend the ABA signalling pathway. Up to now, a few intracellular and plasma membrane candidates had been proposed, some of them raising some questions about their implication in ABA signalling.

Here we are going to contrast and discuss the ABA receptors described up to now with the so-called new family of ABA-intracellular receptor PYR/PYL/RCAR family.

FCA

FCA was the first ABA receptor described (Razem et al., 2006). Its identification was based on sequence similarity to ABAP1; a barley ABA binding protein isolated using a polyclonal anti-idiotypic ABA antibody (Razem et al., 2004). Unfortunately, the role of FCA as an ABA receptor was called into question, when FCA binding to ABA could not be reproduced in other hands using a similar [^{3}H]-(+)ABA binding assay (Risk et al., 2008, 2009). Risk et al., (2008) reported that the described ability of FCA to bind ABA was probably due to poor-quality protein, combined with low sensitivity of the ABA-binding assay used and poor data analysis. All this would have led Razem et al., to erroneous conclusions. Additionally, another group tried to reproduce the FCA receptor model proposed by Razem et al. (2006) in rice with its functional homologue OsFCA, again with no success (Jang et al., 2008). Based on these results, the authors finally retracted the paper (Razem et al., 2008). Later on, Risk et al. (2009) also tried to reproduce ABA binding assays with ABAP1, using the same protocol as in Razem et al. (2004). They could not obtain protein of sufficient purity and stability to perform the analysis, suggesting that maybe protein used in the original assay was of uncertain quality and that this might have contributed to the observed binding (Razem et al., 2004). This raised questions of whether ABAP1 was binding ABA in the first place (Risk et al., 2009).

CHLH/ABAR

CHLH/ABAR was defined as an ABA receptor residing in chloroplast and mediating ABA responses in seed germination, post-germination growth and stomatal movement. In addition, the expression of ABA responsive genes was also affected by

changes in CHLH expression (Shen et al., 2006). This receptor was identified as a homologue of the putative ABA-binding receptor (ABAR) from broad bean (Zhang et al., 2002). CHLH was identified as a subunit of the magnesium protoporphyrin IX chelatase (Mg-chelatase), with a known function in mediating plastid-to-nucleus signalling (Mochizuki et al., 2001). Isolation of the ABA-binding protein from broad bean, and corroboration of CHLH/ABAR binding to ABA, was carried out using an ABA affinity matrix. This matrix was constructed by coupling ABA to a shepharose affinity-chromatography column through its carboxylate group (Zhang et al., 2002; Wu et al., 2009). This avenue was raising the first question mark of this ABA receptor. According to this approach the COOH group from ABA would be completely cancelled to establish any interaction with the protein. This presents some discrepancies with the fact that the carboxylate group from ABA had already been reported to be critical for its bioactivity (Milborrow, 1974). Additionally, structure resolution of the intracellular ABA-receptors PYR/PYL/RCAR in complex with ABA, has revealed the important role of this chemical group in the binding of ABA to the receptor (further discussed in the following point of the General discussion; Melcher et al., 2009; Nishimura et al., 2009; Dupeux et al., 2011. The binding affinity of CHLH was also examined by Shen et al. (2006), obtaining a specific Kd for the (+)-ABA form of 32 nM.

In addition to the concerns about the *Arabidopsis* CHLH/ABAR binding ABA, barley's CHLH (named XanF) was unable to bind ABA and its loss-of-function mutants showed normal ABA responsiveness. This suggested that barley's CHLH did not function as an ABA receptor or that CHLH proteins played a different role, with respect to ABA binding and signalling, between monocot and dicot plants (Müller and Hansson, 2009).

Another open question for this receptor was the mechanism by which the ABA signal was transduced. Shang et al., (2010) proposed a model in which CHLH/ABAR was connecting with the ABA signalling pathway, by eventually regulating the expression of ABA responsive transcription factors such as ABI4, ABI5, ABF4 and MYB2, through the interaction with a group of WRKY transcription factors (WRKY40, WRKY18 and WRKY60). They proposed a signal transduction model, connecting the following elements: ABA-ABAR-WRKYs-ABI5. According to it, ABA stimulated the interaction between ABAR and WRK40, also promoting the movement of this transcription factor from the nucleus to the cytoplasm. They described these WRKY transcription factors as negative regulators of the ABA signalling pathway, acting as repressors of ABA-responsive transcription factors. Therefore, in the presence of ABA, ABAR was highjacking WRKY40, which is an ABA repressor (Shang et al., 2010).

However, despite describing a model linking the primary signalling events to ABA downstream gene expression, this model presented some inconsistencies and open questions. One of the inconsistencies lied on the function of these ABAR-interacting WRKY proteins. Characterization of CHLH loss-of-function mutants and RNAi lines, by themselves and other independent groups, seemed to reveal that CHLH had a role in stomatal regulation (Shen et al., 2006; Legnaioli et al., 2009; Tsuzuki et al., 2011). However, despite both proteins being connected in the model proposed by Sheng et al. (2010), none of the *WRKY* single or combined loss-of-function mutants presented phenotypes in stomata (Chen et al., 2010; Shang et al., 2010) Moreover, Shang et al. (2010) had reported these WRKY proteins as transcriptional repressors, while genetic analyses from another group, Chen et al. (2010), had revealed two of these proteins, WRKY18 and WRKY60, as transcriptional activators rather than repressors. Moreover, under the model proposed by Shang et al. (2010), ABA also repressed the expression of *WRKY40*, which seemed to be mediated by ABAR signalling, and it was suggested to be an additional way for releasing the break of ABA responsive genes. However, in the work of Chen et al. (2010), ABA played the opposite role, activating *WRKY40* expression. So, these contradictory results made difficult to simply accept the model proposed. Even though, the results presented by Shang et al., (2010) suggested that CHLH/ABAR played a role in ABA signalling, there were still open questions about the hormone transduction mechanism and its role as an ABA receptor. For instance: how and where, in the ABAR protein, would ABA bind? Or, by which mechanism would ABAR recruit WRK40 from the nucleus to the cytosol to interact?

A recent work has brought some light to some of these questions, providing evidence of CHLH playing a role in ABA-signalling, particularly involved in stomatal movements, but not being an ABA receptor itself (Tsuzuki et al., 2011). In this work CHLH was genetically identified as *rtl1* (rapid transpiration mutant-1). The missensed mutant presented a quick change in weight. This rapid water loss was likely due to insensitivity to ABA since it did not show ABA-induced stomatal closure. However, other ABA responses such as seed germination or root growth exhibited normal ABA sensitivities. After being identified as CHLH, and since it had already been proposed to be an ABA receptor, Tsuzuki et al. (2011) examined the ABA-binding ability of recombinant CHLH protein using a [^{3}H]-labeled ABA assay. The PYR1 receptor was used as a positive control this time. Even though binding was not detected for any of the proteins alone, binding was accounted, in the case of PYR1, in the presence of the phosphatase (Tsuzuki et al., 2011). An explanation for this could be the assay limitations to detect affinity constants in the range of high micromolar. PYR1 has been

determined to have a Kd over 50 μM (Results Chapter 3 of this work). This is a feasible explanation, since ABA binding was accounted in the presence of the PP2C, which detection range happens to be in the range of nanomolar (Tsuzuki et al., 2011). These results suggested that CHLH was not acting as an ABA receptor. However, genetic evidences from different groups supported the role of CHLH in ABA-related stomatal regulation (Sheng et al., 2006; Legnaioli et al., 2009; Sheng et al., 2010; Tsuzuki et al., 2011). Its role in stomatal guard cells is still not clear, but some experimental evidences suggest that it might be involved in Ca^{2+} mobilization (Tsuzuki et al., 2011), a well known ABA-secondary messenger (Kim et al., 2010). Moreover, CHLH might also be a connection node between ABA signalling and the circadian clock (Legnaioli et al., 2009).

G-protein-coupled receptors (GPCRs)

In the case of GCR2, it was identified through *in silico* approaches as a putative candidate to function as an extracellular ABA receptor (Liu et al., 2007). Since null mutants in components of the heterometric G-protein complex (*gpa1* and *agb1*), had ABA-response phenotypes (Pandey et al., 2006), it was predicted that a G protein-coupled receptor (GPCR) might function as an ABA receptor. GCR2 seemed to fit all the requirements, since it was predicted to be a membrane protein with seven transmembrane helices, the *gcr2* mutant was shown to exhibit ABA insensitive phenotypes in all known ABA responses and it was reported to interact with the Gα-subunit protein GPA1. However, four other works published between 2007 and 2009 have raised questions about the function of GCR2 in ABA signalling (Gao et al., 2007; Johnston et al., 2007; Guo et al., 2008; Risk et al., 2009). Apparently, GCR2 is not a seven-transmembrane protein as predicted. The prediction programs used by Liu et al. (2007) were known to be prone to false positives without incorporating a proper filter to avoid this problem, which was integrated in the newer version of the DAS software. A new prediction of GCR2, using this filter, did not recognize it as a 7TM protein. Moreover, amino acid sequence analysis revealed that GCR2 was a member of the LanC protein superfamily, as it shared 40% sequence identity with human LANCL1 and LANCL2, which are similar to bacterial lanthionine synthetases (Gao et al., 2007; Johnston et al., 2007). In support to this finding Johnston et al., (2007) modelled GCR2 based on the crystal structure of the LanC family member nisin cyclase, giving a valid model with robust statistical confidence. This suggested that GCR2 was a peripheral membrane protein, rather than a transmembrane protein (Bauer et al., 2000). Similarly, the ease of the protein purification method used by Liu et al., (2007) to obtain pure and

soluble GCR2 protein, disagrees with the arduous protocols normally required to purify GPCR proteins with 7TM helices (Sarramegna et al., 2003).

In the work of Liu et al. (2007) GCR2 binding to ABA was examined by using the same binding assay as for FCA. Moreover, the experiment was performed using FCA (ultimately retracted) as a positive control. Out from this experiment GCR2 was concluded to only bind the (+)-ABA form, with a Kd of 20,1 nM. Risk et al. (2009), similarly as done with FCA, tried to reproduce ABA binding to GCR2 but no binding was accounted. They noted again, that poor-quality protein and data processing might have given a false positive result; also questioning the ABA-binding method used to identify the ABA receptors.

In addition, it appears that *gcr2* mutants, and mutants in two related genes, *gcr2-like1* and *gcr2-like2,* together with the various double and triple mutant combinations all failed to exhibit the ABA insensitive phenotypes in stomatal regulation, seed germination or early seedling development reported by Liu et al. (2007). Similarly, transcription levels of ABA markers genes were comparable to those in wild type (Gao et al., 2007; Guo et al., 2008). Taken together, these results indicate that GCR2 is not required for ABA responses in these processes.

In the ABA signalling model proposed by Liu et al. (2007), GRC2 constitutively binds GPA1. Upon ABA binding to GCR2, GPA1 gets released from the heterotrimeric complex, transducing the signal through the activation of downstream ABA effectors that would finally trigger the ABA responses. According to this model, both *GCR2* and *GPA1* should be genetically coupled, showing similar ABA phenotypes. However, while *gcr2* mutants seem to present ABA insensitivity (Liu et al., 2007), *gpa1* mutants do show hypersensitivity to ABA (Pandey et al., 2006), which is inconsistent with the model proposed.

All this reported results contradict the findings of Liu et al. (2007), setting controversy about GCR2 acting as an ABA extracellular receptor.

Another two novel GPCR proteins, GTG1 and GTG2, were also identified using bioinformatic approaches (Pandey et al., 2009). Consistent with their proposed role in ABA signalling, Arabidopsis *gtg1gtg2* double mutant showed insensitivity to ABA in germination, growth, stomatal closure and a compromised expression of ABA-responsive genes. Identification of GTG1 and GTG2 as GPCRs relied in part on their similarity with GPR89/GPHR (68% amino acid similarity). GPR89/GPHR is a human protein that was reported to be an orphan GPCR (Pandey et al., 2009). However,

GPHR (Golgi pH regulator) has been demonstrated to actually function as an anion channel that modulates Golgi functions through regulation of acidification (Maeda et al., 2008). Sequence motifs to identify GPCR proteins are poorly defined (Davies et al., 2011), so evidence that GTG proteins were functional GPCRs mainly came from their interaction with the Gα-subunit protein GPA1 and their ability to bind ABA. Interaction of GTG1 and GTG2 with GPA1 was demonstrated using the split-ubiquitin Y2H system and by coimmunoprecipitation assays. Apparently, these methods have been described to lead to erroneous conclusions, especially when using overexpressed membrane-localized proteins, so that further validation is desirable in order to corroborate their results (Müller and Johnsson, 2008). GPCRs proteins are arduous to purify (Sarramegna et al., 2003), which makes in vitro experiments quite difficult. In the case of Pandey et al. (2009), they performed the ABA-binding experiments with purified protein, but they reported that only 1% of the purified GTG proteins were able to bind ABA. According to the assay both proteins were described as (+)-ABA specific with apparent Kd (dissociation constant) values of around 40nM. ABA binding is a critical point when describing a receptor and this results should be taken cautiously since the stoichiometry of the experiment is very low, approximately 0.01 mol of ABA per mol of protein. The authors suggest that further optimization of protein purification methods are required to allow a more detailed analysis of ABA binding (Pandey et al., 2009). In order to support a direct role of these GTG proteins in ABA binding, identification of the ABA-binding pocket would provide strong endorsement.

The function and role of these GTG proteins, described by Pandey et al. (2009), in ABA signalling is also quite intriguing. These GTG proteins were defined by the authors as a new GPCR-like G proteins, concluding that GTG1 and GTG2 bound ABA and executed the initial steps of GPCR-G-protein signalling cascade. Moreover, according to their model, the Gα-subunit, GPA1, did not typically work as a downstream-effectors modulator but as a negative regulator of the GPCR-like G system. This is quite surprising, as no other GPCR proteins have been described to both perceive the signal and carry out the initials steps of the transduction pathway (Risk et al., 2009). Intriguingly, these GTG proteins also bind GDP/GTP, and the bound nucleotide influences ABA signal transduction. In addition, and conversely to what it happens in conventional Gα proteins, it is the GDP-GTG form which binds ABA and initiates the ABA signalling cascade. So, in this scenario, the active form of GPA1 (GTP-GPA1) would act as a repressor of the ABA signalling, by promoting the GTP binding to GTGs and inhibiting GTGs GTPase activity. Both effects would shift the GTGs to their GTP-bound form, which does not bind ABA (Pandey et al., 2009).

The model proposed by Pandey et al. (2009) describes an unprecedented GPCR signalling system. Besides the identification of the GTGs ABA-binding pocket, future investigation should also be oriented in identifying the direct downstream targets of the GTGs and to connect them with other factors known to be involved in ABA signalling.

PYR/PYL/RCAR receptors

The PYR/PYL/RCAR proteins were identified in parallel by 4 independent groups using different approaches, and they were described as intracellular receptors since they localized both in cytosol and nucleus (Ma et al., 2009; Nishimura et al., 2010; Park et al., 2009; Santiago et al., 2009A). The intracellular localization of these ABA receptors highlighted the importance of ABA uptake into the cell, in order to the cellular signalling process to occur. A few months later of the identification of these receptors, it was reported that an ABC transporter was responsible for delivering ABA into de cell in a regulated fashion (Kang et al., 2010). The finding that ABA was actively delivered into the cell, and not only distributed by passive diffusion, nicely matches with the identification of intracellular receptors, and gives support to a model of intracellular hormone perception and signal transduction.

Biochemical and structural characterization of these proteins has provided definitive evidence of their function as ABA receptors. Specific and saturable ABA binding was shown using NMR and calorimetry assays (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009A). Another particularity of the PYR/PYL/RCAR proteins with respect to the rest of the ABA receptors above described, is that some of the family members were able to bind the (-)-ABA form and transduce the signal (Park et al., 2009; Santiago et al., 2009A). Even though the binding affinity was much lower than for the (+)-ABA form, this could explain the phenotypes and responses triggered by the unnatural form (Walker-Simmons et al,. 1994; Nambara et al., 2002; Lin et al., 2005; Huang et al., 2007). The fact that the (-)-ABA form is biologically active, is not so surprising taking into account the chemical structure of both enantiomers. In the ABA molecule a plane of symmetry can be drawn along the quiral carbon. So, if the (-)-ABA is rotated 180º, the structural differences between the two molecules reside in the methyl groups in positions C'2 and C'6 (Introduction: Figure 1B). Due to this plane of symmetry, the rest of the functional groups of the (-)-ABA molecule remain in the same position than in the (+)-ABA form, providing an explanation for the partial stereoselectivity of the PYR/PYL/RCAR receptors to both enantiomers. In support to the biochemical data, structural resolution of the PYR/PYL/RCAR binding pocket has

revealed that both forms can be accommodated and that is probably that change in the methyl groups in positions C'2 and C'6 in the (-)-ABA ring, what determines the selective binding to some of the PYR/PYL/RCAR members. This binding selectivity probably depends on steric constraints imposed by the hydrophobic residues that surround the upper part of the cavity of each receptor (Nishimura et al., 2009). Intriguingly, despite the chemical similarity of both enantiomers, the rest of the ABA receptors seem to present an extreme stereoespecificity for the (+)-ABA form.

The PYR/PYL/RCAR proteins are also well connected with the rest of the ABA-signalling pathway elements. They have been described as the apex elements of the core signalling cascade that connects hormone perception to gene expression (Fujii et al., 2009), as well as to regulate the non transcriptional response of the hormone, by controlling stomatal closure (Lee et al., 2009; Geiger et al., 2010). The PYR/PYL/RCAR proteins directly interact and inhibit clade A PP2Cs. Under stress conditions, ABA levels increase and induce the formation of a stable complex between PYR/PYL/RCAR and PP2Cs. This results in the inactivation of PP2Cs phosphatase activity, which would lead to activation of SnRK2s, either by autophosphorylation or by putative upstream activating kinases (UAKs). Once activated, SnRK2 kinases phosphorylate ABF2 transcription factor, which is required for transcriptional activation of stress responsive genes. Additionally, SnRK2s regulate stomatal closure by activating the ion channel SLAC1 and the NADPH oxidase AtrbohF, and inactivating the potassium channel KAT1, inhibiting, this way, the K+ uptake by the cell. In addition to SnRK2s, PYR/PYL/RCAR-ABA-PP2C complexes also regulate the activation of calcium-dependent protein kinases (CPKs), also involved in the regulation of ion fluxes (Fujii et al., 2009; Geiger et al., 2009; Sirichandra et al., 2009A; Sato et al., 2009; Geiger et al., 2010; Geiger et al., 2011).

Genetic evidence has also given support to the role of PYR/PYL/RCARs as ABA receptors and linked them with drought-stress response in plants. Triple and quadruple loss-of-function mutants (*pyr1pyl1pyl4* and *pyr1pyl1pyl2pyl4*, respectively) presented strong ABA-insensitve phenotypes in multiple ABA responses, such as germination and root growth, ABA-induced stomatal closure and induction of ABA responsive genes (Park et al., 2009; Nishimura et al., 2010). Conversely, overexpression of PYL9 and PYL5 members caused ABA hypersensitivity (Ma et al., 2009; Santiago et al., 2009A). The overexpression of PYL5 was demonstrated to confer drought resistance in *Arabidopsis*, clearly connecting this new receptor family to stress-tolerance physiology (Santiago et al., 2009A). In the quadruple mutant, the ABA-

induced activation of SnRK2s was severely impaired, which allowed to establish the so called core signalling pathway i.e. PYR/PYL/RCAR-PP2Cs-SnRK2s (Park et al., 2009).

Finally, structure resolution of some of the PYR/PYL/RCAR members alone and in complex with the hormone, together with the structure resolution of the ternary complex PYR/PYL/RCAR-ABA-PP2C, have provided the final proof of their role as ABA receptors. The structural characterization of these proteins has revealed the binding cavity of the hormone and the molecular mechanisms by which the ABA signal is transduced (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009; Dupeux et al., 2011). Similarly, elucidation of the structure and the ABA binding pocket of the rest of the claimed receptors would provide definitive evidence on their role as ABA receptors.

Further characterization of this receptor family has shown that they can be separated in two distinct sub-classes, depending on their oligomeric state (dimeric or monomeric). The different oligomeric state of the PYR/PYL/RCARs has revealed a versatile molecular mechanism that affects their ABA-binding affinity, allowing them to modulate the response to the hormone in a wider range of concentrations (Results: Chapter 3 of this work). This complex regulatory mechanism fits well with what it happens in the cellular context, since ABA levels *in vivo* have been observed to vary over several orders of magnitude (from nanomolar to micromolar) (Harris et al., 1988; McCourt and Creelman, 2008). This also suggests that the two types of PYR/PYL/RCAR proteins might have specialized functions and that they might contribute to ABA signalling differentially, maybe modulating the response to stress in specific organs or tissues, or under particular physiological conditions. This hypothesis also correlates with what it can be observed when having a look at the transcriptional response of the plant to ABA in microarray studies. The gene expression profiles do not simply involve activation or deactivation, but a rather complex mechanism involving at least three distinct groups of ABA-induced genes, presenting different expression profiles along the time response (Huang et al., 2007).

The identification and characterization of the intracellular receptors PYR/PYL/RCAR has represented a breakthrough in ABA signalling, but still there are major questions that remain poorly understood. For instance, even though the two sub-classes of PYR/PYL/RCAR proteins suggest distinct responses, public gene expression datasets illustrate that different receptors proteins are often co-expressed in a single cell (Introduction: Figure 5). Therefore, a more detailed analysis of PYR/PYL/RCAR tissue expression, as well as different combinations of loss-of-function

mutants might allow a better understanding of whether these proteins are functionally redundant or they present specialized functions. Similarly, it is still unclear if these receptors might be also modulated by other non-receptors proteins or if they might take part in the regulation of any other elements.

8.2 ARCHITECTURE AND FUNCTION OF THE PYR/PYL/RCAR RECEPTORS

ABA recognition and signal transduction

Structure resolution of the PYR1 receptor, together with other members of the family like PYL1 and PYL2, in their apo form and in complex with ABA, has been crucial for understanding the molecular mechanism underlying hormone perception and signal transduction. PYR/PYL/RCAR receptors behave as allosteric switches, changing their conformation upon ABA binding and turning into PP2C inhibitors (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009; Dupeux et al., 2011). Ultimately, this allosteric change becomes the vehicle of signal transduction to downstream elements of the pathway, the PP2Cs. According to this model, the monomeric subunits of these receptor proteins are structurally arranged to form a hydrophobic central cavity where the hormone accommodates. The walls of this cavity are formed by a curved 7-stranded β-sheet and a carboxy-terminal α-helix (α5), juxtaposed to the β-sheet. The cavity is closed in its bottom side by two small α-helices (α3-α4), while the upper part is surrounded by two flexible loops (β3-β4 and β5-β6), which are the ones adopting a different conformation upon ABA binding. In the apo form, the loops result to be in an open conformation, creating a passage into the cavity, whereas upon binding of the hormone, the loops close up over the cavity burying the hormone inside (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). This conformational change is driven by how ABA is recognized and oriented inside the pocket. ABA is directly coordinated in the pocket by highly conserved residues (identical in 13 out of the 14 PYR/PYL/RCAR members), suggesting a common mode of hormone recognition among the receptors family (Santiago et al., 2009B; Yin et al., 2009). The coordination of ABA by these residues is mediated by a combination of polar and hydrophobic interactions that match the different functional groups of the hormone (Figure 1A). ABA is an amphipathic molecule containing polar and hydrophobic regions. The polar groups are composed by the ketone and hydroxyl groups of the clyclohexene ring, and the carboxylate; while the hydrophobic regions are formed by the cyclohexene ring (including methyl groups) and the isoprene moiety.

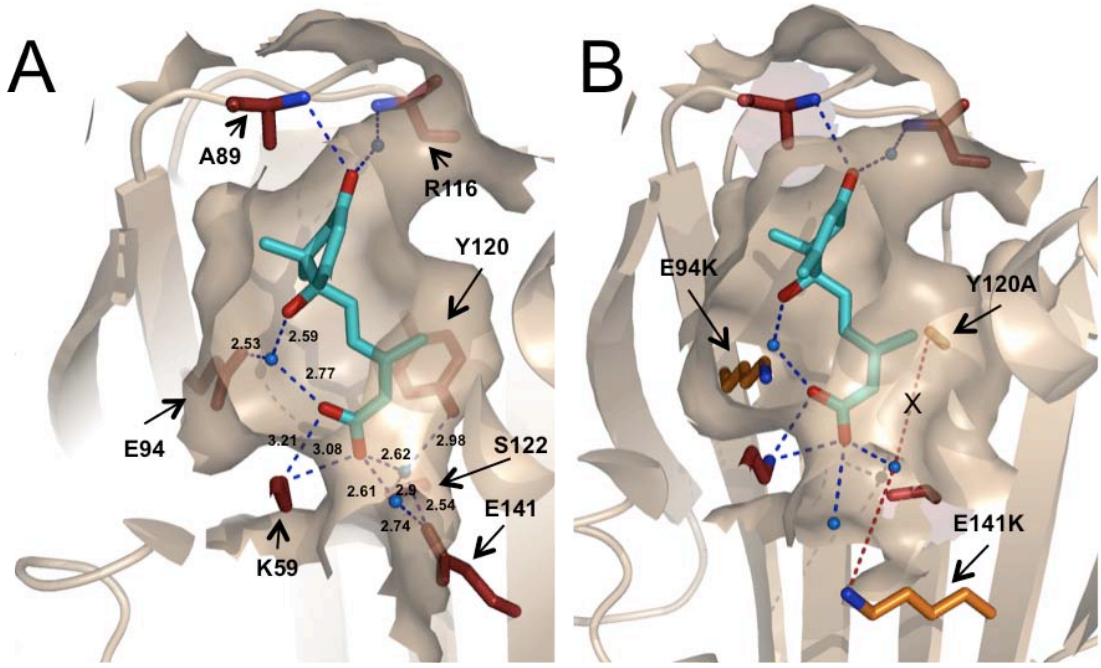


Figure 1. **Conserved residues in the PYR1 pocket which are critical for ABA binding. A,** Representation of ABA in PYR1 binding site showing direct polar contacts and water-mediated hydrogen bonds between the hydroxyl and carboxylic groups of the ABA molecule and well conserved residues in the inner part of the cavity E94, K59, Y120, S122 and E141.These interactions present to be critical in the coordination of the molecule in the pocket. They place the cyclohexene ring of ABA facing the gating loops of the receptor, attracting them and leading them to adopt a closed conformation. This closed conformation generates the PP2C-binding surface. In order to stabilize this conformation, the ketone group of ABA establishes a polar contact and a water-mediated hydrogen bond with the backbone amide of two residues located in the switching loops, A59 and R116, respectively. ABA is represented in cyan, residues in red sticks and water molecules in blue spheres. Blue dotted lines represent the contacts between ABA and the PYR1 pocket residues. Interaction distances are indicated in Å. **B,** Modelling of mutated residues E94K, E141K and Y120A and its effect in the binding of ABA. The change from a negatively charged to a positively charged residue, in the case of E94K and E141K, would significantly reduce the strength of the newly polar interactions established, severely affecting the ABA anchoring in the pocket. In the case of the Y120A mutation, the effect would be double. In the first place, the water mediated interaction through the tyrosin oxygen will be lost. Secondly, the loss of the tyrosin residue will disrupt the hydrophobic contacts between the tyrosin ring and the methyl group of the hormone, losing another important anchoring point of the ABA molecule in the pocket. ABA is represented in cyan, mutated residues are in orange sticks, non-mutated residues remain in red sticks and water molecules are shown in blue spheres. Red dotted lines represent the interactions affected by residue mutations. This structural information gives support to the biochemical results presented in Results Appendix Chapter 2.

Residue recognition of ABA by the receptor, leads to orientation of the ABA molecule in the pocket with its hydrocarbon chain buried in the inner part of the pocket and the cyclohexene ring facing the switching loops surrounding the entrance of the cavity. The key residues, Glu94, Lys59, Tyr120, Ser122 and Glu141 in PYR1 (and the corresponding in PYL1 and PYL2) recognize the hydroxyl and carboxyl groups of ABA

establishing direct polar contacts, like in the case of the Lys59, or water-mediated hydrogen bonds (Figure 1A), playing a critical role in anchoring ABA inside the cavity (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). The Lys120 plays a dual role, since besides establishing the water-mediated hydrogen bond between the OH from the ring and the oxygen from the carboxylic group of ABA, the ring from the Tyr also establishes hydrophobic interactions with the methyl group from C6 of ABA, contributing to the anchoring of the hormone in the pocket. This hormone orientation places the cyclohexene ring facing the gating loops, which acts as a magnet attracting them to adopt the closed conformation. In order to stabilize this closed conformation, a polar contact and a water-mediated hydrogen bond are also established between the ketone group of ABA and the backbone amide of Ala89 and Arg116, respectively.

The mutational analysis performed in Results Appendix Chapter 2, gives support to the relevance of ABA binding in PYR1 activation. Mutations of several of the above mentioned key residues located in the inner part of the cavity, severely impaired or completely abolished the interaction and inhibition of the phosphatase. In Figure 1B, a structural explanation of how binding of ABA would be affected by these mutations is presented by a modelling of the mutated residues Glu94Lys, Glu141Lys and Tyr120Ala inside the cavity.

This change in conformation, induced upon binding of the hormone, involves the closing up of the gating loops over the cavity, generating an interaction surface for the PP2C to dock (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009). In Figure 2A is presented the surface of ABA-bound PYR/PYL/RCAR receptors involved in the interaction with the PP2C. This surface comprehends the β3-β4 loop, the β5-β6 loop and the β7-α5 loop. Mutation of any of the key residues of the surface completely abolishes the interaction with the phosphatase, revealing to be the transmission vehicle of signal transduction (Figure 2B). The molecular mechanism, by which the information is transferred through the gating loops, has been provided by the structure resolution of the ternary complex PYR/PYL/RCAR-ABA-PP2C. PP2Cs are able to perceive the ABA signal through two key docking points with the ABA-bound receptors. One of them involves a Trp residue, highly conserved among clade A PP2Cs, which establishes water mediated interactions with the ketone group of ABA, and conserved Pro and Arg residues (for instance: Pro88 and Arg116 in PYR1), located at the β3-β4 and the β5-β6 loops of the receptor, respectively (Melcher et al., 2009; Miyazono et al., 2009; Dupeux et al., 2011). This interaction has revealed to be critical in the transfer of information since

biochemical analysis have shown that phosphatase mutants in that Trp are refractory to inhibition by ABA-bound PYR/PYL/RCAR receptors (Miyazono et al., 2009; Dupeux et al., 2011). This hormone sensing mechanism has also been proven to be critical *in planta*. Thus, engineering of a hab1^{W385A} dominant allele has led to strong ABA-insensitive phenotypes, which supports the relevance of this residue for the formation of ternary Receptor-ABA-Phosphatase complexes (Dupeux et al., 2011).

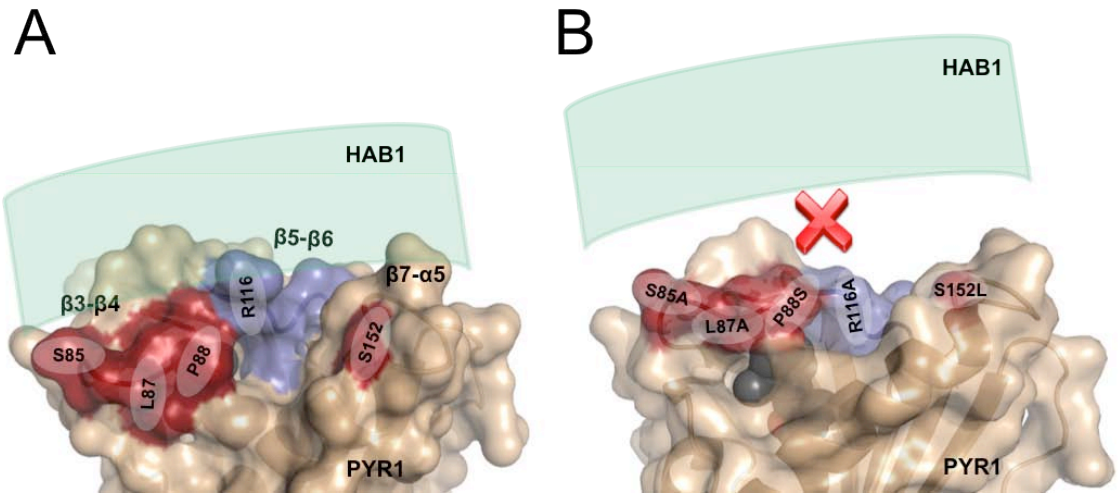


Figure 2. Surface of the ABA-bound PYR/PYL/RCAR receptors involved in the interaction with the PP2C. **A**, Interaction surface of ABA-bound PYR1. Critical residues of the gating loops are indicated, as well as the residue S152 from the loop β7-α5, that also suffers a change in conformation upon ABA binding, moving back to allow lid closure. **B**, Surface modelling of mutations in the key residues that completely disrupt the interaction with the phosphatase. ABA is represented in grey spheres. The SGLPA loop is represented in red and the HRL loop in blue surface.

The other key interaction for inhibition of PP2C activity, involves a conserved Ser located at the β3-β4 loop, which inserts into the PP2Cs' active site (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011). This interaction also seems to explain a competitive inhibitory mechanism of PP2Cs by ABA-bound PYR/PYL/RCAR proteins, by blocking the access of natural substrates to the active site (Melcher et al., 2009; Dupeux et al., 2011). The structure information reveals this conserved Ser (Ser85 of PYR1, Ser112 of PYL1 and Ser89 of PYL2) entering the active site and establishing contacts with two conserved residues of the PP2C catalytic site, a Gly and a Glu (Figure 3A). Disruption of these interactions, also results in PP2C mutants that are refractory to inhibition by PYR/PYL/RCAR receptors. The hab1^{G246D} biochemical data presented in Results Appendix Chapter 2, show that this mutation renders the phosphatase insensitive to inhibition by various PYR/PYL/RCAR proteins. This means that this phosphatase mutant escapes from the ABA-dependent PYR/PYL/RCAR inhibitory mechanism, leading to the subsequent constitutive inhibition

of OST1 activity. Structure modelling of Gly246Asp mutation in HAB1, illustrates that the bulky Asp residue would likely disrupt the interactions between the Ser and the active site residues, and cause steric hindrance (Figure 3B). All these data provide a framework to explain the ABA insensitivity effect of abi1^{G180D} and hab1^{G246D} mutations and their behaviour as hypermorphic mutations (Robert et al., 2006; Dupeux et al., 2011).

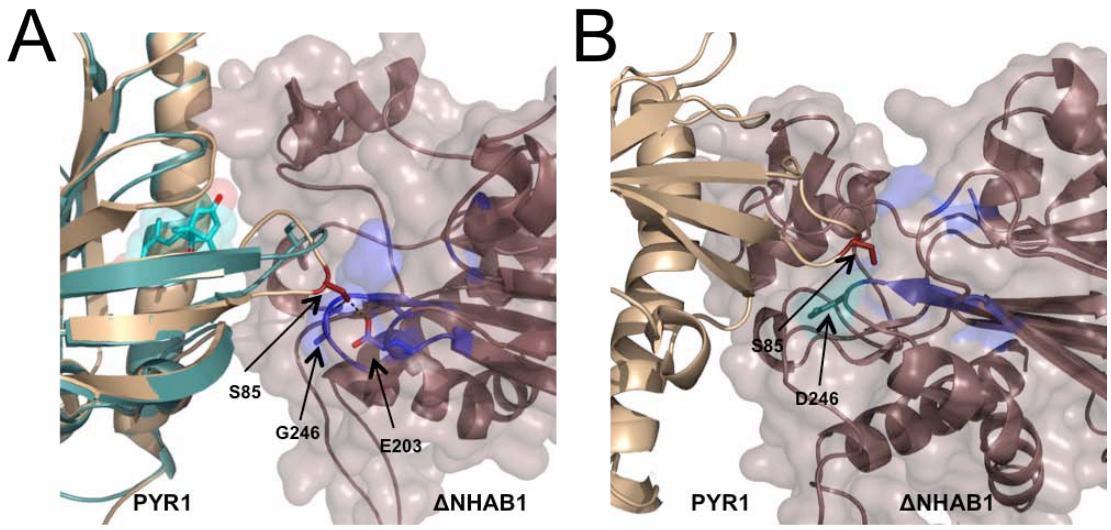


Figure 3. A, Detail of the interaction between the PYR1 loop containing the Ser85 residue and the phosphatase active site, particularly Gly246 and Glu203 residues. Hydrogen bonds are indicated by dotted lines. **B**, Modelling of the Gly246Asp mutation in the HAB1 active site. This amino acid substitution leads to disruption of the hydrogen bonds shown in A and steric hindrance for the interaction with the PYR1 loop.

The activation mechanism of PYR/PYL/RCAR proteins, to become PP2C inhibitors, depends on their oligomeric state. Biochemical and structural characteristics of PYR/PYL/RCAR proteins suggest that they are divided in two distinct sub-classes, monomeric and dimeric receptors in their apo form. Even though they present a different oligomeric state in the absence of ABA, PYR/PYL/RCAR receptors form 1:1 monomeric complexes with PP2Cs after ABA binding. This implies that dissociation of homodimeric receptors is necessary for their activation and ABA signal transduction. (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009B; Yin et al., 2009; Dupeux et al., 2011; Results Chapter 3 of this work). Figure 4A, illustrates a general view of PYR1 dimerization surface (similar in PYL1 and PYL2). This dimerization region largely overlaps with the PP2C interaction surface (Results Chapter 3 of this work). Detail in Figure 4B shows that dimerization involves the gating loops of juxtaposed monomeric subunits of the protein. Since both loops are critical in the interaction with the PP2C, this structural organization suggests that homodimerization and PP2C binding compete for the same interaction surface. This

way, dimer formation reveals as a mechanism to prevent constitutive interaction with the PP2C (Results Chapter 3 of this work).

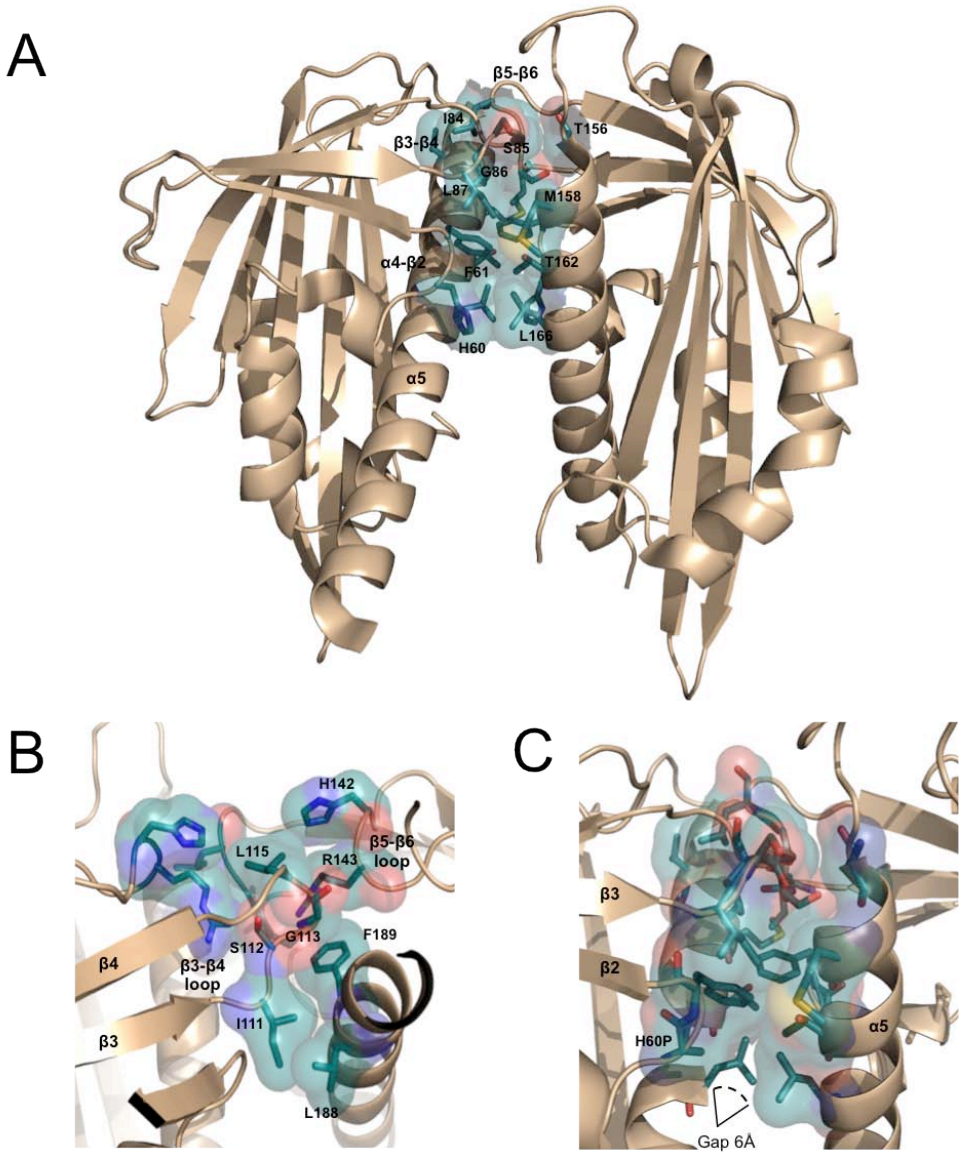


Figure 4. **PYR/PYL/RCAR dimerization region and modelling of the effect of PYR1 His60Pro mutation. A**, General view of PYR1 dimerization surface. Dimerization region involves α4-β2 loop, the β3-β4 loop that interacts with the β5-β6 loop of the juxtaposed molecule, and the N-terminal part of the α5 helix. **B**, Detail of the upper part of the dimerization surface of apo-PYL1, involving β3-β4 and β5-β6 loops of juxtaposed molecules. Since both gating loops are critical in the interaction with the PP2C, dimer formation seems to be a mechanism for preventing constitutive interaction with the phosphatase. **C**, Modelling of the PYR1 His60Pro mutation and its effect on the dimerization surface. The change to a proline creates a gap in the interaction surface with the residue Leu166 from the other chain. This Leu166 has been described to be critical in the dimerization interface since its single mutation to Arg generates a monomeric PYR1 (Peterson et al., 2010). Moreover, the more restricted backbone of the proline (Ho et al., 2005) might also affect the backbone conformation of neighbouring residues, such as Phe61, also affecting the dimer formation. Residues involved in dimerization are shown as sticks with surface.

The different oligomeric state of PYR/PYL/RCARs apo forms confers them distinct properties. Dimeric receptors, such as PYR1, PYL1 or PYL2, display lower apparent affinities for the hormone and they absolutely require binding to ABA in order to interact with the phosphatases, while monomeric receptors, such as PYL5, PYL6 or PYL8, present a higher intrinsic ABA affinity and are able to form low affinity complexes with PP2Cs in the absence of the hormone (Santiago et al., 2009A; Szostkiewicz et al., 2009; Results Chapter 3 of this work). The effect of the oligomeric form over the receptors' properties is well illustrated when comparing the PYR1 wild-type and the monomeric variant of $PYR1^{H60P}$. The $PYR1^{H60P}$ mutant protein is affected in its dimerization surface. Figure 4C illustrates the negative effect of the His60Pro mutation on PYR1 dimer formation. Both proteins share identical ABA binding pockets and PP2C interaction regions, but since $PYR1^{H60P}$ cannot form a stable dimer it shows higher ABA affinity and restores OST1 activity, by inhibiting PP2C at lower ABA concentrations (Results Chapter 3 of this work).

A His residue can be found in other dimer representatives such as PYL1 or PYL2 (Figure 5A), whereas a Pro at equivalent position can be found in monomeric members such as PYL8. This would suggest that some receptors whose oligomeric structure is not known such as PYL7, PYL9 or PYL10, would likely be monomeric (Figure 5B). However, additional features regulate the oligomeric structure of other receptors such as PYL5 and PYL6, since for instance monomeric PYL6 has a His residue in that position.

ABA plays a dual role in the activation of dimeric receptors. Abscisic acid has to promote dimer dissociation, to release the PP2C-interaction region, as well as to induce the closed conformation of the gating loops. The difference in ABA affinity between monomers and dimers can be explained by the thermodynamic penalty imposed by dimer dissociation in the activation process. In dimeric receptors' activation, two opposite thermodynamic processes happen at the same time. Hormone binding is an exothermic process that releases heat, whereas dimer dissociation is endothermic, and ends up reabsorbing the heat released by ABA binding. This turns out net positive enthalpies and less favourable ΔG for the whole activation process, resulting in lower apparent affinities for the hormone. In the case of the monomeric receptors, their activation process does not involve this unfavourable energetic process, which explains their higher intrinsic affinity for the hormone (Results Chapter 3 of this work).

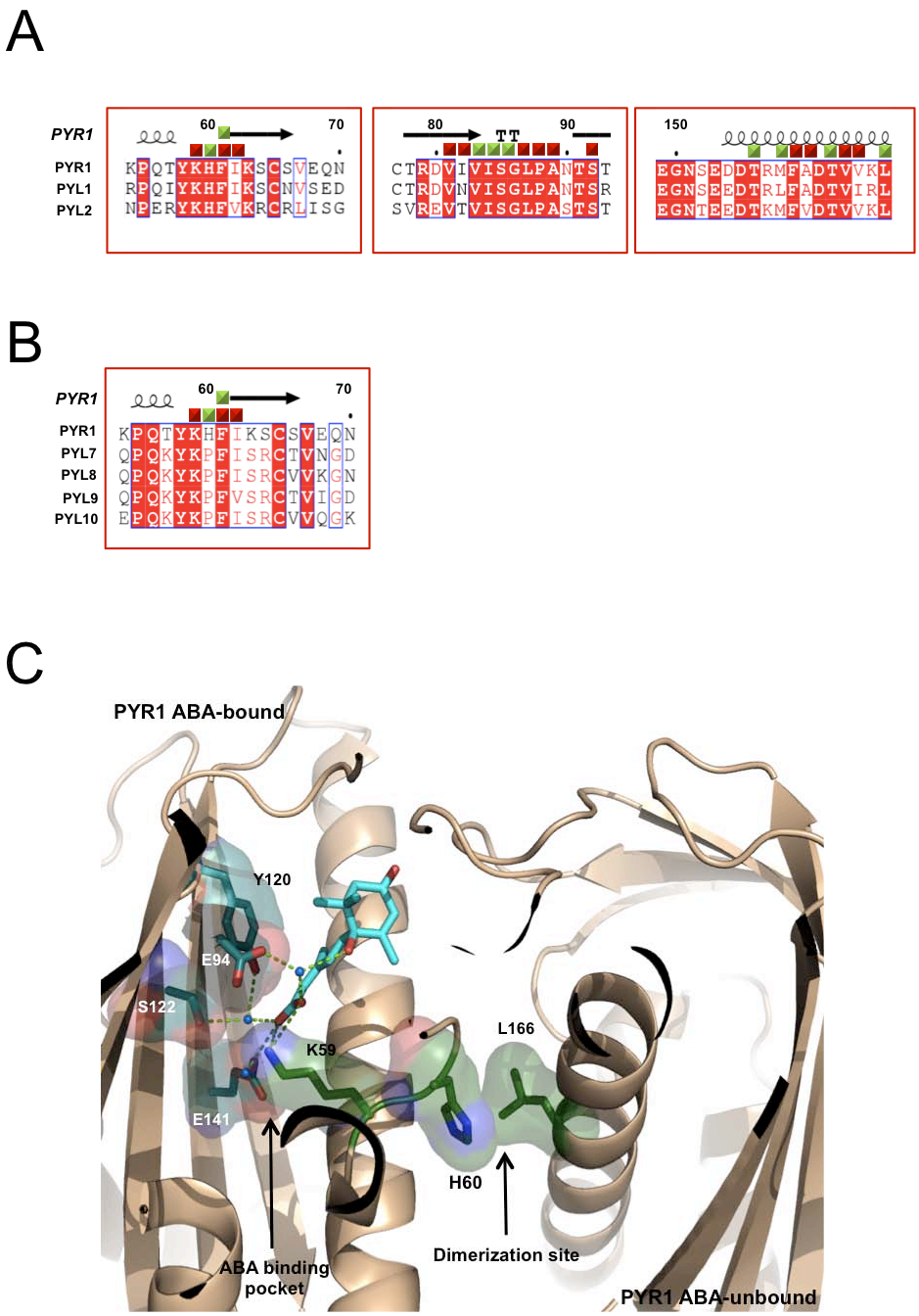


Figure 5. A model to explain transmission of ABA binding to the dimerization zone of PYR/PYL/RCAR dimers, eliciting dimer breakage. **A,** Multiple sequence and secondary structure alignment of selected regions, containing amino acids involved in (+)ABA binding (red squares) and dimerization surface (green squares) of dimeric PYR/PYL/RCAR proteins. Amino acids involved in dimerization are surrounded or closed to amino acids implicated in ABA binding. **B,** The substitution of the residue equivalent to H60 of PYR1 to proline, does naturally occur in some members of the PYR/PYL/RCAR family. $PYR1^{H60P}$ mutant has revealed to be a weaker dimer with higher dimer dissociation constant. Experimental evidences indicate that PYL8 (see Results Chapter 3) is a monomeric receptor. Color coding similar to (**A**). **C,** Structural representation of the key residue K59, crucial in ABA binding, connected to H60, involved in dimer formation. ABA is represented in cyan, residues involved in ABA binding are represented in steel blue sticks and K59 and dimerization residues, H60 and L166, are shown in green sticks.

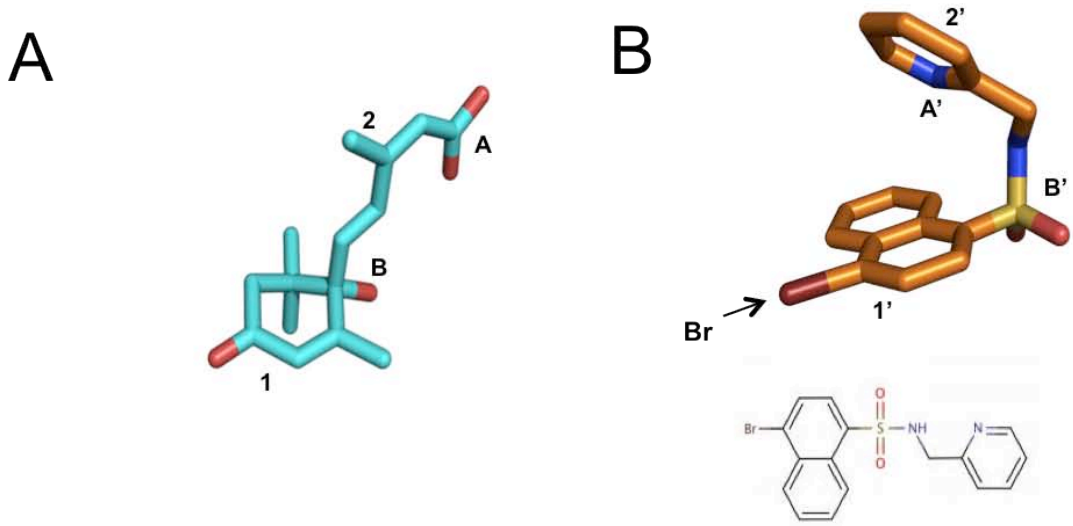
The ABA binding must elicit dimer breakage, which can be better understood when analyzing the ABA binding region and surrounding residues. In dimeric receptors like PYR1, PYL1 and PYL2, it can be seen that residues involved in ABA binding are surrounded or adjacent to amino acids implicated in dimerization (Figure 5A). A significant weakening of the dimer interface would be caused by the induction of the closed conformation. Attraction of the gating loops by ABA, as previously explained, would break both the polar and hydrophobic contacts with the neighbouring subunit, reducing this way the dimerization surface. Similarly, in Figure 5C we illustrate, how ABA binding signal could also be transmitted to critical residues involved in dimerization such as His60, affecting their connections with other residues from the neighbour subunit. This transmission model helps to understand how ABA binding could result in changes in the dimerization surface, reducing the number of contacts between the two subunits and inducing a change in orientation of both protomers, which would eventually lead to dimer breakage.

Selective agonist activation of pyrabactin *vs.* ABA

Pyrabactin was the synthetic ABA agonist that through a chemical genetics approach led to the discovery of PYR1, the representative member of the PYR/PYL/RCAR receptor family. Pyrabactin was shown to act as a selective ABA agonist among the members of the family (Park et al., 2009). The question is what these two molecules share in common to elicit agonistic responses among the PYR/PYL/RCAR proteins. Interestingly, when looking at both molecules (Figure 6A and 6B) there is no apparent similarity between their chemical structures. However, structure resolution of PYR1 and PYL1 in complex with pyrabactin has shed light on which are the key points to induce receptor's activation (Hao et al., 2010; Peterson et al., 2010). Despite their distinct chemical structures, ABA and pyrabactin present a correspondence in their polar and hydrophobic regions, which exert an analogous function by being recognized by similar key residues in the pocket. Both ligands are amphipathic and comprehend two analogous polar groups, buried deep inside the pocket, and two hydrophobic regions. The two polar portions of pyrabactin, are the pyridyl nitrogen (indicated as A' in Figure 6B) and the sulphonamide group (indicated as B' in Figure 6B), which correlate with the role played by the caboxylate (indicated as A in Figure 6A) and hydroxyl (indicated as B in Figure 4A) groups in ABA. In the case of the hydrophobic regions, the function of the cyclohexene ring of ABA (indicated as 1 in Figure 6A) is exerted by the bromo-naphthalene ring in pyrabactin (indicated as 1' in Figure 6B), and the ABA methyl group from C6 (indicated as 2 in Figure 6A) is replaced by the pyridine ring (indicated as 2' in Figure 6B). Pyrabactin's polar groups adopt the

same orientation and mimic some of the ABA interactions, being recognized and coordinated by almost identical residues in the receptor's cavity such as Glu121, Lys86 and Glu171 in PYL1 (corresponding to Glu94, Lys59 and Glu141 in PYR1). Figure 6C and 6D illustrate the correlation between the two molecules in the receptor's pocket. Indeed, a water molecule, which mediates the interaction between the pyridyl nitrogen and Lys86 and Glu171, is located at the same position as the carboxylate oxygen atom in ABA, which directly contacts Lys59 in PYR1.

Similarly, a water molecule that mediates the interaction between the hydroxyl group of ABA and Glu94 of PYR1, overlaps in position with the amine group of pyrabactin, which directly forms hydrogen bonds with Glu121 of PYL1. These two water molecules complete the polar regions, compensating for the lack of the corresponding function groups in either ligand. This pyrabactin arrangement in the receptor's pocket, positions the pyridine ring in a similar position as the methyl group from C6 of ABA, also establishing hydrophobic interactions with the hydrophobic residues surrounding it, helping to anchor the ligand in the cavity. Similarly, the bromo-naphthalene ring adopts a similar orientation than the cyclohexene ring of ABA, parallel to the gating loops, functioning as the major driving force to attract the loops to achieve the closed conformation (Figure 6D). This structural analysis highlights the essential role of the position of the pyridyl nitrogen for pyrabactin's function. If this nitrogen was located at any other site of the ring, it will be totally out of reach from the key pocket residues, even in the presence of water molecules, preventing from the molecule to adopt the right orientation. Additionally, the nitrogen in any other position might also interfere with the hydrophobic environment created by the rest of the ring within the receptor (Hao et al., 2010; Peterson et al., 2010). This also explains why apyrabactin, a pyrabactin structural analogue in which the pyridyl N is replaced by a C-H, results to be completely inactive (Park et al., 2009). However, despite pyrabactin being able to induce the activation of some PYR/PYL/RCAR receptors, its binding affinity seems to be much lower. PP2C activity assays have shown that inhibition by pyrabactin requires 10 times higher concentration of the agonist compound than of ABA, to reach a similar level of inhibiton (Hao et al., 2010; Peterson et al., 2010). This could be explained by pyrabactin not being able to establish absolutely the same contacts as ABA with residues of the receptor's cavity. For instance, pyrabactin loses the interaction with Tyr120, which has revealed to be critical in the receptors' activation by ABA (Results Chapter 3 of this work). This would affect anchorage of pyrabactin inside the pocket, also affecting the process of dimer breakage in the case of the dimeric receptors.


A
2
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1
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2'
A'
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Br
1'
Br
NH
N
C
I110
Y120
E94
I62
K59
E141
PYR1 ABA-bound
D
I89
3.4-4.6 Å
I137
E121
R106
K86
E171
PYL1 pyrabactin-bound
E
Non-productive
binding
11-13 Å
90 °
V114
V67
E98
Y124
K64
R83
E147
PYL2 pyrabactin-bound

Figure 6. Selective agonist effect of pyrabactin vs. ABA in different PYR/PYL/RCAR members. A and **B**, ABA ($C_{15}H_{20}O_4$) and pyrabactin ($C_{16}H_{13}BrN_2O_2S$) molecules are structurally different but a correspondence between analogous polar and hydrophobic regions can be observed. Polar regions are indicated by letters and hydrophobic regions by numbers. **C**, Detail of the ABA molecule inside the PYR1 pocket, illustrating the direct polar contacts and water-mediated hydrogen bonds between oxygen atoms of the hormone and key residues of the pocket. These interactions are crucial for the anchoring and orientation of the molecule in the cavity. The hydrophobic region composed by the methyl group from C6, establishes hydrophobic interactions with the Tyr120 ring, also helping to anchor the ligand in the pocket. Finally, the last hydrophobic ABA region, the cyclohexene, ring, is placed facing to the upper part of the cavity towards the gating loops. This module will be the major driving force to pull the gating loops over to achieve the closed conformation. **D**, Pyrabactin mimics ABA interactions and it is recognized by almost identical residues in the receptor's cavity. Detail of PYL1 bound to pyrabactin. The two polar portions of pyrabactin, the pyridyl nitrogen and the sulphonamide group, adopt the same orientation. These polar regions establish polar interactions and water-mediated hydrogen bonds with key binding residues K86, E121 and E171 of PYL1. Similarly, the hydrophobic region of pyrabactin, the bromo-naphthalene ring, adopts a similar orientation than the cyclohexene ring of ABA. The secondary hydrophobic area, that comprises the pyridine ring of pyrabactin, would also establish hydrophobic interactions with the hydrophobic residues surrounding it, helping to anchor the ligand; similarly as the methyl group from C6 of ABA. **E**, Molecular basis of pyrabactin's selectivity. Detail of pyrabactin bound to PYL2, which produces a non-productive binding. This non-productive binding is due to a change in orientation of pyrabactin molecule in PYL2 (E) relative to that in PYL1 (D). Responsible for this non-productive orientation are the residues Val67 and Val114 in PYL2, that happen to be isoleucines in PYL1 and PYR1, resulting both in productive pyrabactin orientation (C) and (D). The loss of side chain methyl groups, from Ile to Val, is enough to allow the molecule to shift orientation. Both gating loops, in the closed and open conformation, are represented in red. ABA molecule is shown in cyan and pyrabactin in orange. Key residues are represented in sticks.

As we have mentioned before, pyrabactin is a selective agonist; meaning that it is able to induce the activation of some of the PYR/PYL/RCAR members. For instance, pyrabactin is able to bind PYL2 but unable to induce its activation, leaving the gating loops in an open conformation (Peterson et al., 2010; Melcher et al., 2010; Yuan et al., 2010). This has been qualified as a non-productive binding (Peterson et al., 2010). Interestingly, coordination of pyrabactin in PYL2 pocket is still mediated by both polar and hydrophobic interactions with the key residues above described. However, when superimposing pyrabacting-bound PYR1 or PYL1 and PYL2 structures it can be appreciated that pyrabactin is rotated 90 degree in PYL2 relative to that in PYL1 or PYR1 (Figure 6D and 6E). As a consequence, the bromo-naphthalene ring of pyrabactin that attracts the gating loops is now in a perpendicular position, being unable to exert its attraction effect over the loops and stabilize the closed conformation. The pyrabactin selectivity in PYL2, is due to subtle changes in surrounding residues; in particular two Val (Val67 and Val114) that happen to be Ille in PYL1 and PYR1. Both these residues are pointing towards the inside of the cavity and the main difference between them lies on the extra methyl group of the side chain of the Ille with respect to

the Val. The loss of these side-chain methyl groups at positions 67 and 114 in PYL2, would impair the hydrophobic interactions between them and pyrabactin, giving to the molecule the enough degrees of freedom to rotate to a non-productive orientation (Figure 6E). This selective model seems to also work for the other pyrabactin-insensitive receptor, PYL4 (Park et al., 2009).

Pyrabactin has played a critical role in dissecting the mechanisms underlying ABA signalling. However, this ABA agonist presents some limitations, since it only controls ABA responses in seeds but not in vegetative tissues. A new important goal would be finding other agonist molecules that would control ABA responses in seedlings and could be used in agriculture. Since ABA has been the molecule selected by nature to activate these responses, we might expect that the binding affinity of these new compounds may also be lower. This situation could be overcome by increasing the dosage as shown with pyrabactin.

8.3 BIOTECHNOLOGICAL APPLICATION

Drought happens to be one of the major causes of poor plant performance and limited crop yield worldwide (Buchanan et al., 2000). This becomes particularly serious in developing countries, where crop growth and productivity mainly depend on rainfall patterns. Climate appears to be globally changing, causing a rise in temperatures and a change in rainfall patterns, which creates a general drying trend that has been proved to have a clear detrimental impact on biomass yields (Zhao and Running, 2010), seriously affecting agriculture.

The recent identification of this ABA intracellular receptors family and their establishment as the apex elements of the core signalling pathway, provides a potential biotechnological tool for controlling the drought stress response in plants.

Representatives of this protein family can be found in multiple cultivated species (Figure 7), suggesting that modulation of ABA responses through the PYR/PYL/RCAR receptors family seems to be a conserved regulatory mechanism among cultivated plants. Comparative sequence analyses show that these putative receptors exhibit high homology in sequence amino acid and secondary structure. They present a similar pattern of seven β-sheets sandwiched by two main α-helices. Important to highlight of these proteins is that sequences encoding for the ‘gating loops’, ‘SGLPA’ (between β3 and β4) and ‘HRL’ (between β5 and β6), that play a critical role in signal transduction conferring the receptors their inhibitory capacity upon ABA binding, are well conserved. This provides additional support to the idea of these putative proteins behaving as ABA

receptors and turning into PP2C inhibitors upon binding of the hormone. In the particular case of *Vitis vinifera*, it has been recently reported the identification of three VvPYR/PYL/RCAR members. These proteins have been shown to resemble the PYR/PYL/RCAR proteins in *Arabidopsis* and behave as ABA receptors, regulating the signalling pathway through the inhibition of *Vitis vinifera* PP2Cs (Li et al., 2011).

In our case, when looking for the PYR/PYL/RCAR orthologues members in *Oryza sativa* we found that its family was also comprehended by 14 members (Figure 1B from Results Chapter 4). A similar number of 16 members was also be found in *Solanum lycopersicum* (Figure 1C from Results Chapter 4).

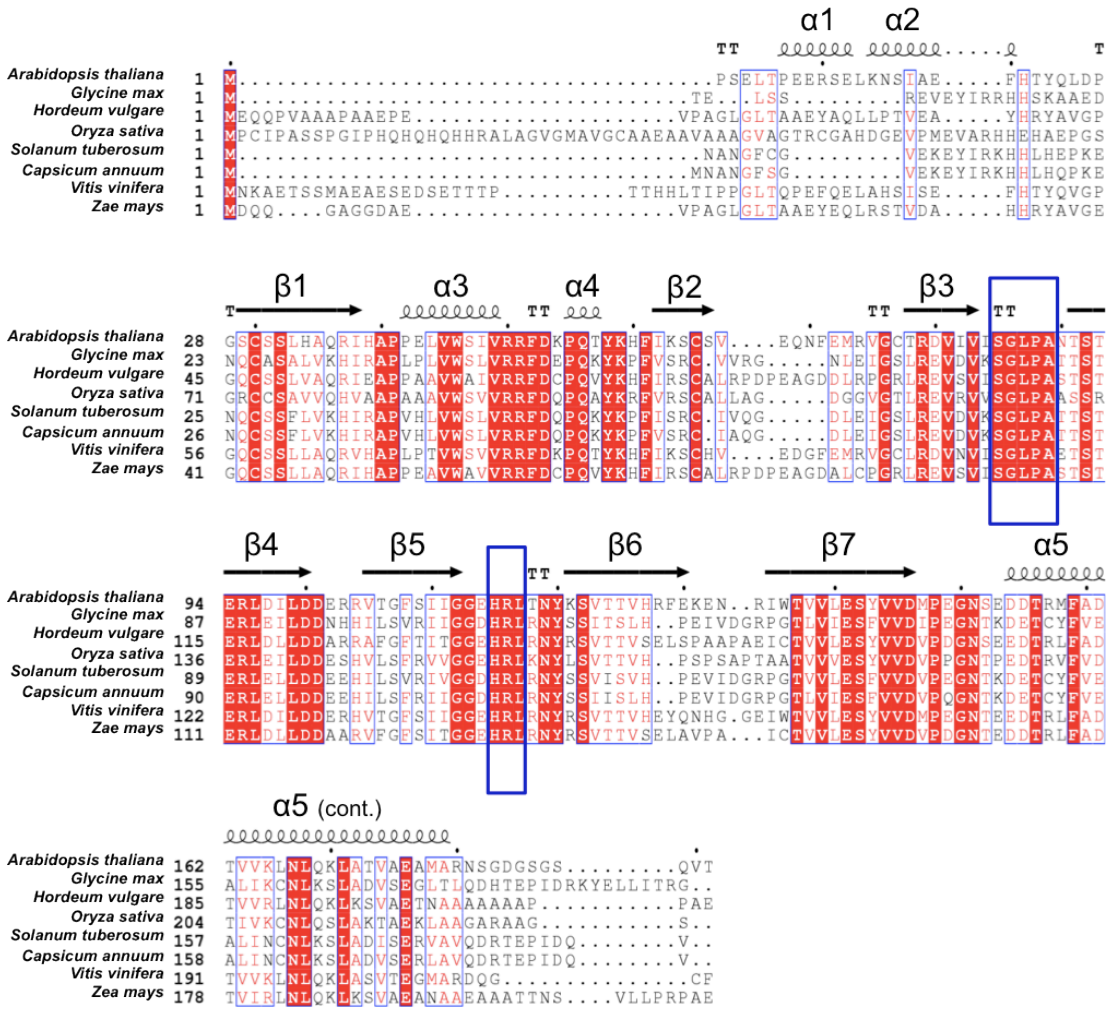

Figure 7. Modulation of ABA responses through the PYR/PYL/RCAR receptors is conserved in different cultivated plants. Multiple sequence and secondary structure alignment illustrating the presence of this receptors family in different crop plants. In blue squares is highlighted the conserved sequence corresponding to the "gating loops", suggesting a similar molecular regulatory mechanism. Figure generated with ESPript 2.2 (Gouet et al., 1999).

This receptor family offer different biotechnological approaches in order to improve water stress tolerance in plants, and therefore crop yields under drought conditions. A possible approach would be the generation of overexpressing lines of these positive regulators, similarly as done in *Arabidopsis* (Results Chapter 1 of this work), obtaining plants with a clear enhanced resistance to drought. Another option would be the modulation of their function by taking advantage of their role as hormone receptors. In that case understanding the mechanistic of PYR/PYL/RCAR receptors' regulation and activation, provides crucial information in order to design or screen for novel ABA selective agonist or antagonist compounds. These new molecules may have applicable potentials in agriculture and fewer restrictions than ABA in terms of stability and cost production. Alternatively, another avenue could be the screening or design of PYR/PYL/RCAR mutants that might be activated by chemicals already approved and in use in agriculture. Future understanding of whether these proteins are fully redundant or have specialized functions, will add to the equation the possibility to artificially regulate targeted PYR/PYL/RCAR functions.

9. CONCLUSIONS

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The PYR/PYL/RCAR proteins are positive regulators of the pathway and they act as inhibitors of the phosphatase activity of HAB1, ABI1 and ABI2 in an ABA-dependent manner.

One of the PYR/PYL/RCAR members, PYL5, specifically binds ABA with micromolar or nanomolar affinity in the absence or presence of HAB1, respectively. PYL5 presents particial stereoespecificity, being able to bind the (-) ABA form.

PYL5 is localized both in cytosol and nucleus

PYL5 is linked to the control of drought resistance in plants. Its overexpression leads to an enhanced response to ABA and drought resistance. This can represent a potential biotechnological application.

Thus, PYR/PYL/RCAR proteins are a new family of intracellular ABA receptors involved in the activation of the ABA signalling pathway through the inhibition of some members of the clade A PP2Cs.

PYR1 is an ABA receptor. Its structure consists of a central seven-stranded β-sheet flanked by two α-helixes. This bent β-sheet presents a curved disposition, and together with the long carboxy-terminal α-helix (α5), produces a central cavity where the hormone accommodates.

The structure resolution of the PYR1 receptor in complex with ABA has provided definitive evidence of their role as ABA receptors. The structure resolution reveals a specific recognition and anchoring of the molecule in the pocket that elicits the activation of the receptor by inducing a series of conformational changes that favour the interaction with the PP2C.

The PYR/PYL/RCAR family divides in two subclasses with different oligomeric state in their apo form: dimeric and monomeric. This oligomeric state correlates with their affinity for the hormone, conferring them a different activation mechanism. These differential properties might lead to a different contribution to regulation of the ABA responses in the plant.

The ABA-PYR/PYL/RCAR-PP2C signaling pathway is conserved in cultivated plants, being susceptible of regulation in order to improve crop yield under drought conditions.

10. REFERENCES

10. REFERENCES (Introduction and General discussion)

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Structure figures generated with PyMOL (http://www.pymol.org).

11. APPENDIX 1

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Review

Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs

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ABSTRACT

Abscisic acid (ABA) plays an essential function in plant physiology since it is required for biotic and abiotic stress responses as well as control of plant growth and development. A new family of soluble ABA receptors, named PYR/PYL/RCAR, has emerged as ABA sensors able to inhibit the activity of specific protein phosphatases type-2C (PP2Cs) in an ABA-dependent manner. The structural and functional mechanism by which ABA is perceived by these receptors and consequently leads to inhibition of the PP2Cs has been recently elucidated. The module PYR/PYL/RCAR-ABA-PP2C offers an elegant and unprecedented mechanism to control phosphorylation signaling cascades in a ligand-dependent manner. The knowledge of their three-dimensional structures paves the way to the design of ABA agonists able to modulate the plant stress response.

Contents

1. Introduction

ABA plays a pivotal role to coordinate plant response under water stress situations as well as to regulate plant growth and development [1]. Chemically, ABA ($C_{15}H_{20}O_4$) is a sesquiterpenoid derived from isopentenyl pyrophosphate synthesized in plastids through the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway [2]. ABA contains one asymmetric carbon atom at C1′, the natural form is S(+)ABA and the side chain of the molecule is present in the 2-cis,4-trans isomeric state (Fig. 1). A plane of symmetry can be defined in the ABA molecule through the optical center, which defines two sides that differ only in that C6′ carries two methyl group whereas C2′ carries one and a double bond. Consequently, the non-natural (−)enantiomer only differs structurally in these positions from the (+)enantiomer (Fig. 1B) [3].

Plant hormone research has greatly benefited from genetic screenings aimed at the identification of key components of the hormone signaling pathways. Arabidopsis mutants showing reduced or enhanced sensitivity to a hormone usually pointed out to critical loci that, once cloned, revealed crucial components of the hormone signaling pathway [4]. However, such screenings, although highly successful for different hormone signaling pathways, failed to identify ABA receptors. Functional redundancy or pleiotropic effects including embryo or gamete lethality looked as sound arguments to justify this failure. Indeed, the first approaches to identify ABA receptors, marked by retracted data in one case

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[5], used biochemical techniques to identify ABA-binding proteins (CHLH/ABAR/GUN5) [6] or followed pharmacological evidence suggesting the involvement of G-protein coupled signaling in the ABA pathway (GTG1/GTG2) [7]. Finally, a chemical genetic approach using a synthetic selective ABA agonist, pyrabactin, made it possible to identify a family of soluble ABA receptors, named PYR/PYL for pyrabactin resistance1/PYR1-like [8]. In a convergent approach, yeast-two hybrid screenings [9,10] and *in planta* interaction studies [11] identified these receptors through their capacity to bind to clade A phosphatases type-2C (PP2Cs), and Ma et al., (2009) named them RCAR for regulatory components of ABA receptors [9]. The clade A PP2Cs are key negative regulatory components of the ABA pathway involved in the dephosphorylation of certain sucrose non-fermenting 1-related subfamily 2 (SnRK2) kinases [12–14]. Since structural studies have adopted the PYR/PYL nomenclature, we will use it along this review. In order to guide the reader, amino acid sequence alignment of PYR/PYLs can be found in different articles [8–10].

The PYR/PYL family of ABA receptors has witnessed a rapid explosion of structural data, since five independent groups have reported structural and functional mechanisms on ABA signaling [15,16,17,18,19], and even more recently, using the seed ABA-agonist pyrabactin (Fig. 1C), the structural basis for selective activation of certain receptors and the mechanism of antagonism in other members [20,21,22]. Such wealth of structural information is not available for other ABA receptors, such as the Mg-chelatase H subunit and the GTG1/GTG2 proteins, therefore we will focus this review on the recent structural data reported for the PYR/PYL ABA receptors and their inhibitory interaction with PP2Cs. Additionally, different studies have provided a clear connection of PYR/PYL receptors with downstream signaling components, such as PP2Cs, SnRK2s and ABA-responsive elements binding factors (ABFs/AREBs) or the Slow Anion Channel 1 (SLAC1), whose key role in ABA signaling had been previously revealed by biochemical and genetic studies. These findings have been recently reviewed [1,23].

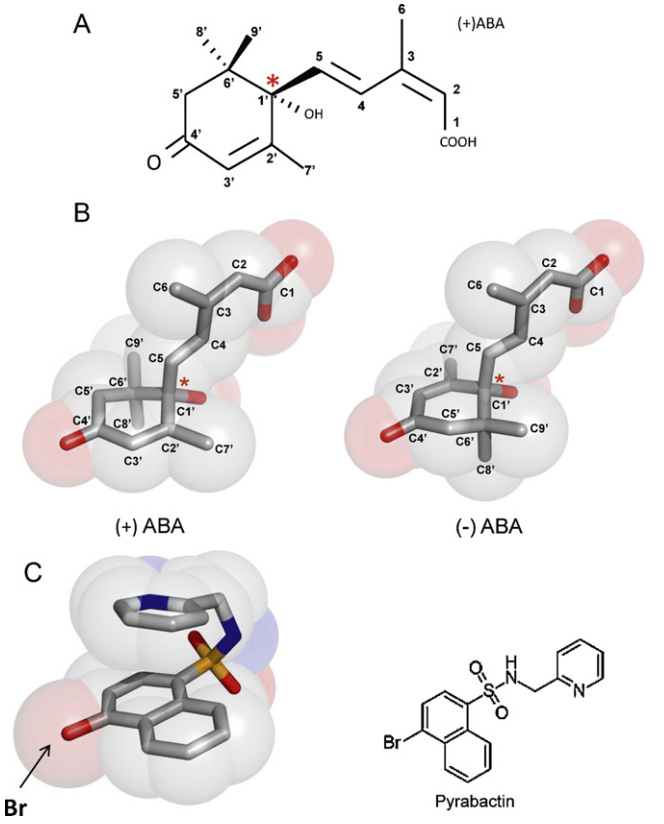

Fig. 1. Chemical structure of the phytohormone ABA and the seed ABA-agonist pyrabactin. (A) 2D structure of the natural S(+) form of ABA. The red asterisk points out the C1′ asymmetric carbon of the molecule. (B) 3D structure of the two ABA enantiomers. The (−)enantiomer has been rotated to illustrate the structural difference of the ring methyl groups with respect to the (+)enantiomer. (C) 2D and 3D structures of pyrabactin.

2. The structure of PYR/PYL receptors

The crystal structure of three ABA receptors, PYR1, PYL1 and PYL2, has been reported to date (see Protein Data Bank codes in Table 1). As predicted, these data confirm that the PYR/PYL family belongs to a branch of the Bet v superfamily, which is structurally characterized by the presence of the Bet v fold or START domain [24]. This structure comprises a seven-stranded β-sheet flanked by two α-helices, which is designated as helix-grip fold. Additionally, the PYR/PYL family contains a α-helical segment at the N-terminus, which is not present in the Bet v fold and therefore, it can be considered as a particular feature of this family of ABA receptors.

Crystal structures were obtained both in the apo form (ABA-free) and in the presence of ABA. For instance, apoPYL1 [15], apoPYL2 [15,19], ABA-bound PYL1 [16] and ABA-bound PYL2 [15,19] structures have been resolved. In the case of PYR1, in the crystallographic asymmetric unit, there is one ABA-bound and one ABA-free subunit [17,18]. A high degree of structural similarity is evident from the superposition of the three structures (Fig. 2), however, recent works using the pyrabactin molecule reveal subtle differences among the receptor binding pockets with important functional consequences, since pyrabactin is an agonist of PYR1 and PYL1, whereas it is an antagonist of PYL2 [20,21,22]. Different experimental data indicate that these receptors exist as dimers in solution, however, the receptor-PP2C

Table 1
PDB codes of structural data on ABA-receptors of the PYR/PYL/RCAR family.

Authors	Apo-structures	ABA-bound PYR/PYL	PYL-ABA-PP2C structures
[15] Melcher et al. (2009)	apoPYL1 3KAY apoPYL2 3KAZ	ABA-bound PYL2 3KB0	PYL2-ABA-HAB1 3KB3
[16] Miyazono et al. (2009)		ABA-bound PYL1 3JRS	PYL1-ABA-ABI1 3JRQ
[19] Yin et al. (2009)	apoPYL2 3KDH	ABA-bound PYL2 3KDI	PYL1-ABA-ABI1 3KDJ
[17] Nishimura et al. (2009)	PYR1 (subunit B) 3K3K	PYR1 (subunit A) 3K3K	
[18] Santiago et al. (2009)	PYR1 (subunit B) 3K90	PYR1 (subunit A) 3K90	

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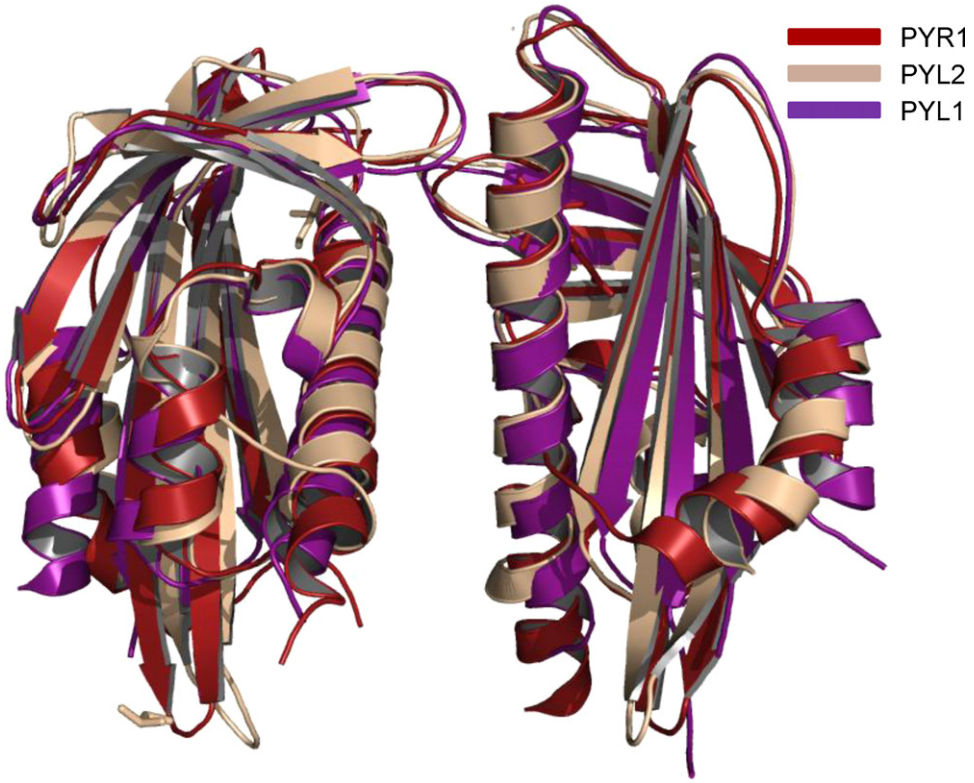

Fig. 2. Superposition of PYR1, PYL1 and PYL2 dimers. Figure generated with PyMOL (http://www.pymol.org) using the Protein Data Bank (PDB) codes 3KAZ (PYL2), 3KAY (PYL1) and 3K90 (PYR1).

complexes have a 1:1 stoichiometry, implying that the receptor dimers have to dissociate before interacting with the PP2C [17,18,19].

2.1. ABA-binding pocket

The structure of the ABA-bound forms of PYR1, PYL1 and PYL2 reveals the nature of the interactions stabilizing the hormone into the receptor binding pocket [15–19]. The ABA sits in a deep cavity almost completely buried from the external medium (see Fig. 3). The walls of this cavity match perfectly the chemical character of the different functional groups of the hormone. The cyclohexene ring and the isoprene moiety establish hydrophobic interactions with apolar PYR1 side chains, while the carboxylic, hydroxyl and ketone groups of the ABA molecule are stabilized through interactions with polar side chains. Strikingly, many of these polar interactions involve hydrogen bonds with water molecules (which are sandwiched between the hormone and the walls of the receptor

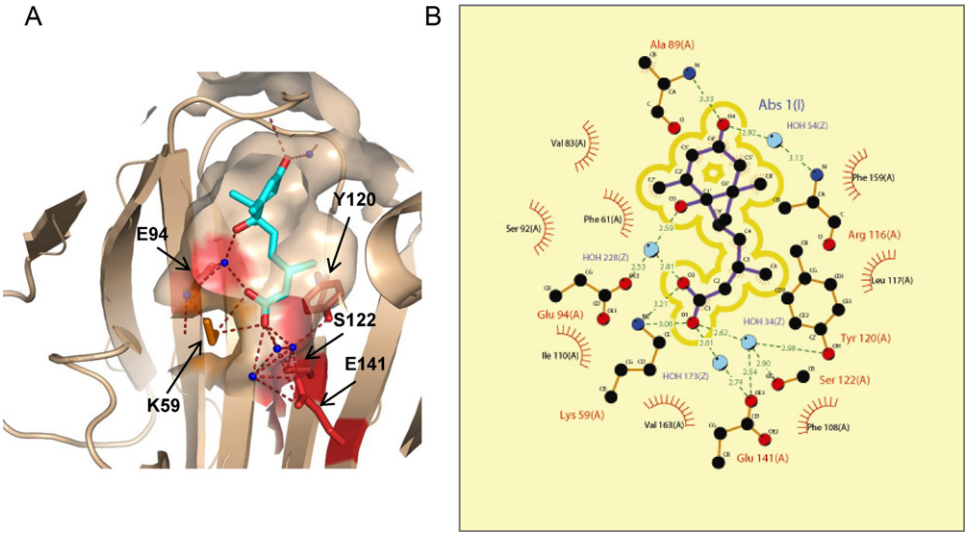

Fig. 3. ABA binding in the PYR1 cavity. (A) ABA (in light blue) buried in the PYR1 cavity. Lys59 (orange sticks), which is located at the bottom of the cavity, establishes a direct polar contact between its amine group and the carboxylate group of ABA. Interactions of Glu94, Glu141, Ser122 and Tyr120 (red sticks) with the carboxylate group are established through hydrogen bonds mediated by water molecules. (B) 2D map of interactions between ABA and PYR1 [18]. Hemispheres represent hydrophobic interactions whereas polar interactions are represented by lines. HOH indicates water molecules. The yellow ribbon around the ABA molecule indicates low solvent accessibility.

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cavity) rather than direct side chain hormone contacts. For instance, the carboxylate group of ABA establishes interactions with the side chains of Glu94, Glu141, Ser122 and Tyr120 through hydrogen bonds mediated by three internal water molecules (Fig. 3). Additionally, a direct polar contact is found between the amine group of Lys59 and the carboxylate group, which is buried into the ABA binding pocket, away from the loops flanking the entry. These data are in agreement with the requirement of the carboxylate group for ABA bioactivity [3], and indeed, different mutations in these residues, e.g. Glu94Lys, Glu141Lys, Lys59Gln, abolish or reduce PYR1 function. In this context, it is interesting to mention that coupling of ABA through its carboxylic group to the amino group of a 10-atom spacer arm of a Sepharose resin has been used as a tool to identify ABA binding proteins, which resulted in the identification of the Mg-chelatase H subunit as an ABA receptor [6,25]. Such approach is likely to interfere with the binding of the carboxylate group to the receptor, so the mechanism of ABA binding by Mg-chelatase has still to be explained. The addition of bulky groups to any part of the hormone molecule is likely to interfere with binding to PYR/PYL receptors, since this kind of receptor wraps very tightly around the hormone.

The architecture of the ABA-binding pocket and the structure of ABA enantiomers suggest that both molecules can be accommodated into the pocket of at least some PYL receptors, although with different affinity. Stereospecificity would be contributed by steric constraints imposed by the mono-methyl group at position C2′ and the dimethyl group at C6′. Indeed, binding of (−)ABA to the PYL5 receptor has been measured using isothermal titration calorimetry [10], although the Kd for the natural (+)enantiomer was 20-fold lower (1.1 versus 19.1 μM), which indicates a higher affinity for ABA binding of the natural form. Additionally, (−)ABA promoted interaction of PYL2, PYL3 and PYL4 with HAB1 [8]. Structural studies on PYL2 also show that the (−)enantiomer can be accommodated into the ABA-binding pocket [19], although the dimethyl group flipped in the (−)enantiomer would cause some steric hindrance with the narrow pocket that accommodates the monomethyl group [15].

2.2. ABA-induced conformational changes

Since the PYR1 dimer crystal structure contains both unbound and ABA-bound subunits, comparison of both structures made it possible to reveal the ABA-induced subunit conformational changes (Fig. 4A and B) [17,18]. Thus, superposition of the ABA-bound and ABA-free subunits of the PYR1 dimer revealed notable differences in two loop regions (loops β3–β4 and β5–β6) and the N-terminal part of the C-terminal α-helix. Specifically, the loop β3–β4 (S_{85}GLPA$_{89}$) and the loop β5–β6 (H_{115}RLT$_{118}$), upon ABA-binding, fold over ABA to complete ABA enclosure, in contrast, in the ABA-free subunit, these loops adopt an open conformation that allows entry of ABA into the cavity and hence they have been called the gating loops. The region comprising the loop between β7 and the N-terminal part of the last α-helix (M_{147}PEGNSEDDTRM$_{158}$) is also involved in the stabilization of the closed conformation of the gating loops, as well as in interaction with the PP2C. Indeed, in addition to trapping the ABA molecule into the receptor cavity, these conformational changes are crucial to generate a favourable interaction surface for the binding of the PP2C (Fig. 4C and D). For instance, the mutations P88S and S152L severely reduce the capacity of PYR1 to interact with HAB1. Another key example is the flipping movement of S85 (equivalent to S89 in PYL2 and S112 in PYL1), which is exposed in the surface of PYR1 upon binding of ABA. This residue is crucial for the interaction with the active site of the PP2C (discussed below).

Similar conclusions have been obtained by comparison of the ABA-free and ABA-bound forms of dimeric PYL2 [19]. In addition, these authors have analysed the changes generated in the dimer interface of PYL2 upon ABA binding. ABA binding induces a slight change in the relative orientation of one PYL2 protomer with respect to the other. As a result, a significant rearrangement of the interface residues is generated, leading to a diminished number of van der Waals contacts and hydrogen bonds and consequently a weakening of the dimer interface. SAXS studies performed with PYR1 also reveal significant changes in the dimer assembly upon ABA-binding, which leads to a flatter, more compact form of PYR1, which also indicates an orientation change between both subunits [17].

3. Architecture of ternary complexes PYL-ABA-PP2C

The crystal structure of two ternary complexes has been described, i.e. PYL1-ABA-ABI1 and PYL2-ABA-HAB1 [15,16,19]. It is important to note that only the catalytic core of the ABI1 (residues 125–429) and HAB1 (residues 172–511) PP2Cs has been used for these studies. Therefore, it has not yet been elucidated the structure of the N-terminal region of the PP2Cs, which is expected to play an important role either for biochemical regulation of PP2C activity or for regulation of the interaction with other partners.

First of all, these works provide the first structures of plant PP2Cs, and comparison with the pre-existing structure of human PP2C reveals that the active-site residues are highly conserved (Figs. 5 and 6). Thus, critical active-site residues of ABI1 are Arg138, Glu142, Asp143, Asp177, Gly178, His179, Asp347 and Asp413, which correspond in HAB1 to Arg199, Glu203, Asp204, Asp243, Gly244, His245, Asp432 and Asp492, respectively. Both PP2Cs adopt a similar folding pattern as human PP2C, with two central five-stranded β-sheets sandwiched by two pairs of α-helices. The catalytic site is located at the edge of the two central β-sheets and contains 3 atoms of either Mn^{++} or Mg^{++} ions.

The structural studies of different groups have made it possible to define the molecular mechanism of the ABA-dependent inhibition of PP2C activity through PYR/PYL ABA receptors. Although a clear picture emerges from these works (see below), important questions are still open, for instance, a clear discrepancy is observed with respect to the role of the dimeric PYR/PYL proteins in the mechanism of action. Indeed, both the work of Melcher et al. [15] and Miyazono et al. [16] omit any consideration on the dimeric nature of the ABA-receptor and the role, if any, of the dimer in the regulation of PYR/PYL function. Taking into account that experimental evidence indicates that both PYR1 and PYL2 are dimers in solution, the contribution of Yin et al. [19] is particularly relevant in this context. Thus, this group postulates that PYR/PYLs exist as inactive homodimers in cells, unable to bind or inhibit PP2Cs. However, yeast two hybrid and biochemical experiments indicate that different PYLs are able to interact with PP2Cs (in a non-inhibitory manner) in the absence of exogenous ABA [8,9,10]. On the other side, experimental evidence indicates that ABA-binding induces a conformational rearrangement of the receptor, which weakens the dimerization interface of the PYLs and, in turn, generates an interaction platform to contact the PP2C [19]

3.1. Mechanism of ABA-dependent inhibition of PP2Cs by PYR/PYL receptors

In the structure of PYL1-ABA-ABI1 and PYL2-ABA-HAB1, the ABA receptor (PYL1 or PYL2) contacts the PP2C using the gating loops that cover the ABA-binding pocket, i.e. the β3–β4, β5–β6 as well as the β7–α5 loop (Fig. 4C and D). On the other hand, the PP2C contacts the ABA receptor through its active site and a small protruding region, which has been called the flap sub-domain [26] and

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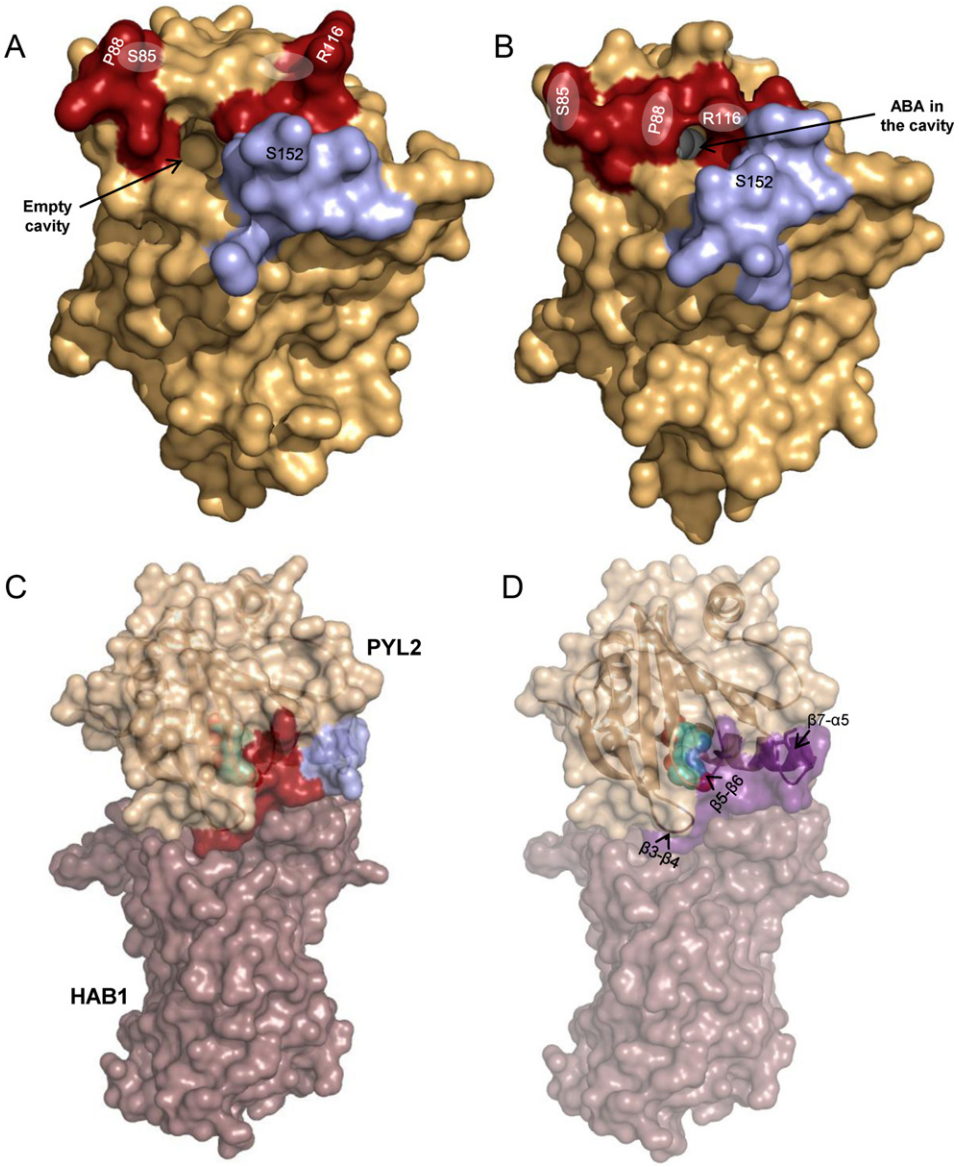

Fig. 4. Induction of conformational changes in the gating loops surrounding the PYR1 cavity upon ABA binding. Interaction of PYL2 and the catalytic core of HAB1. (A) Open conformation of the loops β3–β4, S_{85}GLPA$_{89}$, and β5–β6, H_{115}RLT$_{118}$, (in red), which maintains an open passage for ABA entry into the cavity. (B) ABA binding induces the closed conformation of the gating loops, which fold over the hormone and bury it into the cavity. In light blue, region comprising the loop between β7 and N-terminal part of the α5 (M_{147}PEGNSEDDTRM$_{158}$), which is also involved in stabilizing ABA into the cavity. (C) Surface representation of the complex PYL2-ABA-ΔNHAB1. In red, the PYL2 surface generated by the closed conformation of the gating loops. In light blue, the region above indicated. (D) Detail of the loops and α-helix of PYL2 involved in the contact with the PP2C (purple).

contains an important tryptophan residue (Fig. 6A). In the ternary complexes, access to the active-site cleft of the PP2C is blocked by the β3–β4 loop of PYL proteins, where the Ser112 of PYL1 or Ser89 of PYL2 establish hydrogen bonds with Gly180 of ABI1 and Gly246 of HAB1 and the metal-stabilizing residue Glu142 of ABI1 and Glu203 of HAB1, respectively (Fig. 6B) explaining the inhibitory action of receptor-hormone complexes on the activity of these phosphatases. Indeed, experiments by Melcher et al. [15] using a SnRK2.6 peptide that acts as physiological substrate of HAB1 indicate a competitive inhibitor mechanism between ABA-bound PYL2 and HAB1 in agreement with the structural data. These results however, contrast with those reported by Ma et al. [9], which using a non-peptidic PP2C substrate (methyl-umbelliferyl-phosphate) concluded that the mode of inhibition of ABI2 by RCAR1/PYL9 occurs through non-competitive inactivation of the enzyme.

In addition to the contact with the active-site cleft of the PP2C, amino acid residues Trp300 and Trp385 of ABI1 or HAB1, respectively, located at the flap sub-domain of the PP2C, are critical for the interaction with ABA-bound PYR/PYLs (Fig. 6A). This residue points into the ABA-binding pocket (through the loops that cover ABA) and establishes a water-mediated hydrogen-bond to the ketone group of ABA. This link has been interpreted for some authors as a direct proof that the PP2C serves as a co-receptor of ABA [15,16]. An alternative point of view is that most of the ABA molecule is previously buried within the ABA-binding pocket of the ABA-receptor and only one water-mediated hydrogen bond is established between ABA and the PP2C upon interaction of the ABA-bound PYL protein with the PP2C [19]. However, the work of Melcher et al. [15] shows that upon insertion of Trp385 of HAB1 into the PYL2 ABA-binding pocket, the β3–β4 (SGPLA residues) and β5–β6 (HRL) loops undergo a conformational change, generating additional contacts with the ABA molecule. Therefore, Trp385 would act as a locking mechanism to keep the β3–β4 and β5–β6 loops in the closed conformation. Similar conclusions were reached by Miya-

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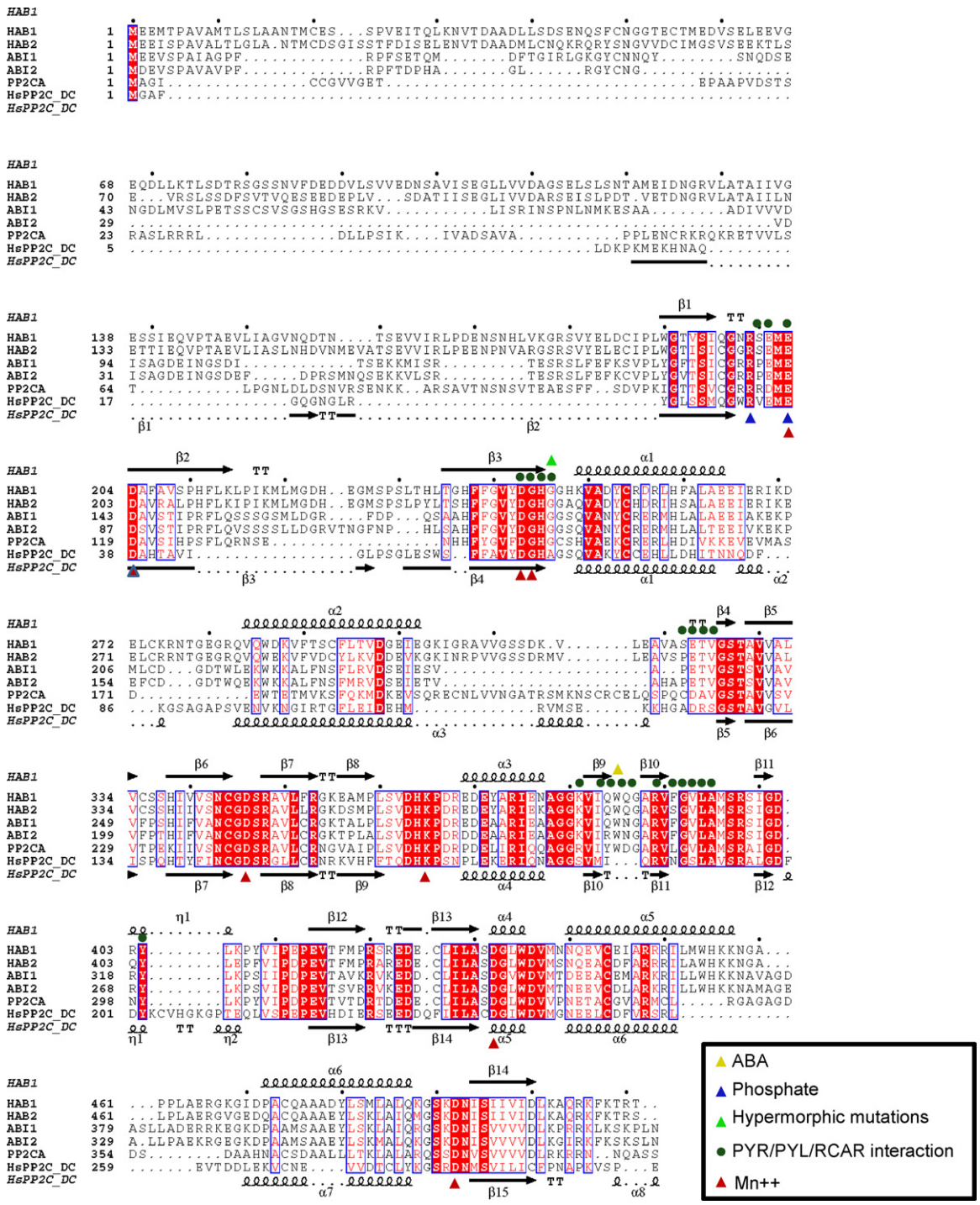

Fig. 5. Amino acid sequence and secondary structure alignment of plant PP2Cs with the catalytic core of human PP2C (residues 1–300). Figure generated with ESPript 2.2 [39]. Colour codes indicate the amino acid residues involved in the interaction with ABA receptors and contact points with phosphate, metal, ABA and hypermorphic mutations.

zono et al. [16], which concluded that the docking of Trp300 of ABI1 into the PYL1 ABA-binding pocket is necessary for the β3–β4 loop to be properly located into the active site of ABI1. Modeling of a W385A mutation in the PYL2-ABA-ΔNHAB1 complex reveals the critical loss of the docking point provided by the interaction of the Trp residues with the ABA molecule (Fig. 6D). Overall, these PP2C-induced conformational changes of PYR/PYL and ABA interaction would explain the higher ABA binding affinity measured for PYR/PYLs in the presence of the catalytic core of the PP2Cs [9,10].

3.2. *abi1*^{G180D}, *abi2*^{G168D} *and hab1*^{G246D} *hypermorphic enzymes*

The ternary complex PYL1-ABA-ABI1 [16,19] provides an explanation to the dominant gain-of-function phenotype of the *abi1-1D* mutation (equivalent to *abi2-1D*), which is a missense mutation that replaces Gly180 by Asp (Gly168Asp in abi2-1). These mutations were isolated 25-years ago by genetic screenings in Arabidopsis, aimed to the identification of ABA-insensitive mutants [27] and the cloning of the mutant loci provided pioneering insights into ABA signaling [28,29,30,31]. Both *abi1-1D* and *abi2-1D* are strong

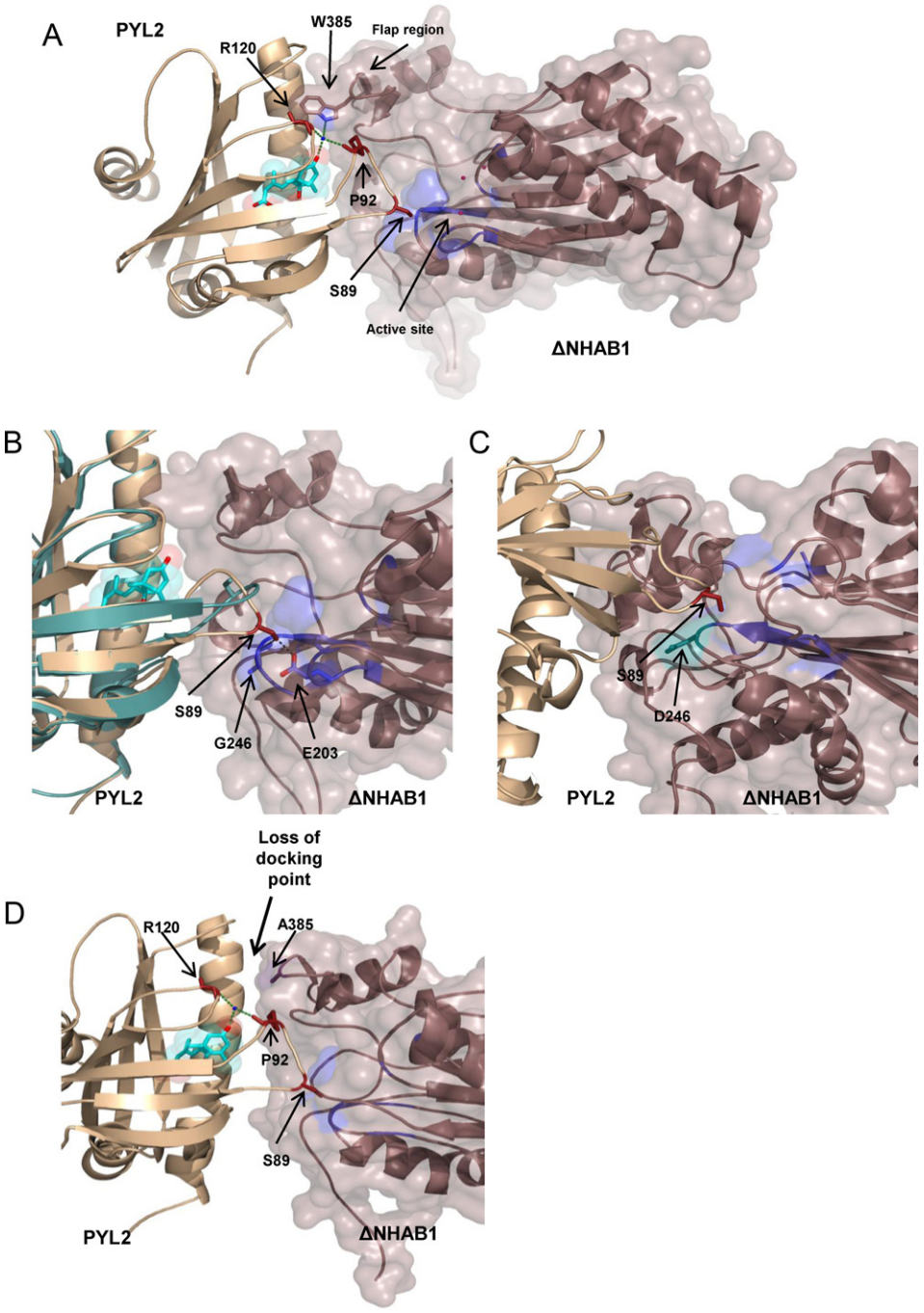

Fig. 6. Structural details of the PYL2-ABA-HAB1 complex. (A) Overview of contact points between PYL2 and the catalytic core of HAB1. The PP2C contacts the receptor through its active site and the flap region containing the Trp385 residue. Detail of the interactions involving this residue, the PYL2 gating loops, containing Pro92 and Arg120 residues, and the ketone group of ABA. The contacts are coordinated through a water molecule (in blue) located at the narrow channel between the loops. Mn^{++} ions are marked as pink dots. (B) Detail of the interaction between the PYL2 loop containing the Ser89 residue and the phosphatase active site, emphasizing Gly246 and Glu203 residues. Hydrogen bonds are indicated by dotted lines. (C). Modeling of the Gly246Asp mutation in the HAB1 active site. This amino acid substitution leads to disruption of the hydrogen bonds shown in B and steric hindrance for the interaction with the PYL2 loop. (D) Modeling of a Trp385Ala mutation in HAB1. This mutation leads to loss of the docking point provided by the interaction of the Trp residue with the ABA molecule.

ABA-insensitive mutants that show diminished response to ABA in seeds and vegetative tissues, however, analysis of loss-of-function alleles indicates that ABI1 and ABI2 gene products, as well as other clade A PP2Cs, are negative regulators of ABA signaling [32,33,34,35]. Since the phenotype of the *abi1-1D* and *abi2-1D* dominant alleles is just the opposite of loss-of-function alleles, they can't represent dominant negative alleles, as it was accurately noted by Robert et al. [36]. A molecular explanation is now provided by structural studies of the PYR/PYL receptors in complex with ABA and the PP2Cs. For instance, Gly180 of ABI1 establishes a hydrogen bond with Ser112 of PYL1, which is a key residue for the blockage of the active-site cleft of the PP2C (see above). The replacement of the glycine residue by a bulkier aspartate will likely introduce steric hindrance for the interaction of the β3–β4 loop with the PP2C. Indeed, it has been demonstrated that abi1-1 protein loses the interaction and is refractory to inhibition by PYR/PYL proteins [8,13], whereas this mutation does not block interaction with downstream targets of PP2Cs, such as SnRK2s [13,14,37].

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Similar considerations apply to the equivalent Gly246Asp mutation of HAB1 [14,36] and the ternary complex PYL2-ABA-HAB1 also provides a molecular explanation to the dominant nature of this mutation [15]. Thus, structural modeling of the mutated Asp246 residue in HAB1 suggests that its larger side chain compared to Gly246 will collide with the β3–β4 loop of the ABA-bound PYR1/PYL2 [15] (Fig. 6C). Moreover, biochemical studies performed with hab1^{G246D} reveal that this mutant PP2C, in contrast to HAB1 wt, escapes from ABA-dependent PYR1-mediated inhibition, and it is able to dephosphorylate OST1 in the presence of ABA and PYR1 (P.L. Rodriguez, unpublished results). Therefore, it qualifies as a hypermorphic mutation in the presence of ABA and a PYR/PYL receptor, although paradoxically, hab1^{G246D} as well as abi1^{G180D} and abi2^{G168D} proteins, have a lower specific activity as compared to wt in the absence of ABA.

4. Mechanism of action of pyrabactin

ABA-agonists harbour the potential to improve the yield of crop plants under drought stress or any other properties modulated by the ABA pathway in crop or ornamental plants. The identification of ABA antagonists would have also an important role in basic research, in order to manipulate ABA response in different tissues or developmental stages, which would represent a very useful tool to study ABA physiology. Recent studies with the first ABA agonist identified, pyrabactin, have provided a structural basis for understanding the selective activation of certain ABA receptors [20,21,22,38]. Additionally, these studies reveal that whereas pyrabactin is an ABA-agonist for some PYR/PYL receptors, e.g. PYR1 and PYL1, it is an antagonist for other members of this family, e.g. PYL2. Comparison of the crystal structures of PYL1-pyrabactin-ABI1 and PYL1/PYL2-pyrabactin complexes has provided the mechanism to distinguish between productive and non-productive pyrabactin binding. In the case of PYR1 and PYL1, pyrabactin binds inside the receptor cavity and establishes interactions that stabilize the closed conformation of the gating loops, as ABA does. In the case of PYL2 however, pyrabactin also binds inside the receptor cavity but adopts a different conformation which does not promote closure of the gating loops. These findings also establish the concept of ABA receptor antagonism as well as a framework for modifying agonist selectivity.

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