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Identification of Targets and Auxiliary Proteins of PYR/PYL/RCAR ABA receptors: Protein Phosphatases Type 2C (PP2Cs) and C2-Domain ABA-related Proteins (CARs)

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By

Lesia Natacha Rodriguez Solovey

Supervisors

Pedro L. Rodriguez Egea

Miguel Gonzalez Guzman

El **Dr. Pedro Luís Rodríguez Egea**, Investigador científico del Consejo Superior de Investigaciones Científicas perteneciente al Instituto de Biología Molecular y Celular de Plantas (IBMCP, UPV-CSIC) de Valencia

El **Dr. Miguel González Guzmán**, contratado como Doctor por el Consejo Superior de Investigaciones Científicas en el Centro de Investigaciones Biológicas (CIB) de Madrid,

CERTIFICAN que Lesia Natacha Rodríguez Solovey ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas, el trabajo titulado **"Identification of Targets and Auxiliary Proteins of PYR/PYL/RCAR ABA receptors: Protein Phosphatases Type 2C (PP2Cs) and C2-Domain ABA-related Proteins (CARs)"** y que autorizan su presentación para optar al grado de Doctor en Biotecnología.

Fdo: Dr. Pedro Luís Rodríguez Egea

Fdo: Miguel González Guzmán

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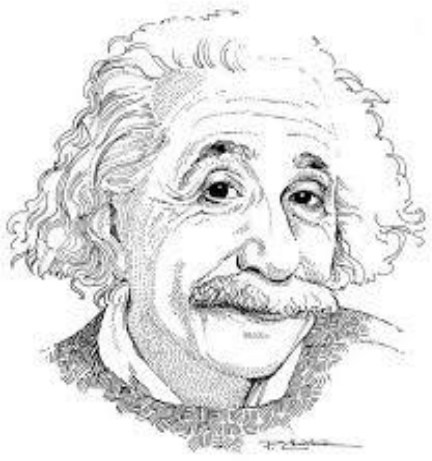

"If you want different results,
do not do always the same."

- Albert Einstein -

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... To my Mom, for all sacrifices

she made to get me where I am today.

ABSTRACT

Abscisic acid (ABA) signaling plays a critical role in regulating root growth and root system architecture. ABA-mediated growth promotion and root tropic response under water stress are key responses for plant survival under limiting water conditions. In this work, we have explored the role of *Arabidopsis* (*Arabidopsis thaliana*) PYR/PYL/RCAR receptors (PYRABACTIN RESISTANCE1 (PYR1)/PYR1 LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS) for root ABA signaling. As a result, we discovered that PYL8 plays a nonredundant role for the regulation of root ABA sensitivity. Unexpectedly, given the multigenic nature and partial functional redundancy observed in the PYR/PYL family, the single *pyl8* mutant showed reduced sensitivity to ABA-mediated root growth inhibition. This effect was due to the lack of PYL8-mediated inhibition of several clade A phosphatases type 2C (PP2Cs), since PYL8 interacted in vivo with at least five PP2Cs, namely HYPERSENSITIVE TO ABA1 (HAB1), HAB2, ABAINSENSITIVE1 (ABI1), ABI2, and PP2CA/ABA-HYPERSENSITIVE GERMINATION3 as revealed by tandem affinity purification and mass spectrometry proteomic approaches.

Membrane-delimited abscisic acid (ABA) signal transduction plays a critical role in early ABA signaling, but the molecular mechanisms linking core signaling components to the plasma membrane are unclear. We show that transient calciumdependent interactions of PYR/PYL/RCAR ABA receptors with membranes are mediated through a 10-member family of C2-domain ABA-related (CAR) proteins in Arabidopsis thaliana. Specifically, we found that PYL4 interacted in an ABA-independent manner with CAR1 in both the plasma membrane and nucleus of plant cells. CAR1 belongs to a plant-specific gene family encoding CAR1 to CAR10 proteins, and bimolecular fluorescence complementation and coimmunoprecipitation assays showed that PYL4-CAR1 as well as other PYR/PYL-CAR pairs interacted in plant cells. The crystal structure of CAR4 was solved, which revealed that, in addition to a classical calcium-dependent lipid binding C2 domain, a specific CAR signature is likely responsible for the interaction with PYR/PYL/RCAR receptors and their recruitment to phospholipid vesicles. This interaction is relevant for PYR/PYL/RCAR function and ABA signaling, since different *car* triple mutants affected in *CAR1, CAR4, CAR5, and CAR9* genes showed reduced sensitivity to ABA in seedling establishment and root growth assays. In summary, we identified PYR/PYL/RCAR-interacting partners that mediate a transient Ca^{2+}-dependent interaction with phospholipid vesicles, which affects PYR/PYL/RCAR subcellular localization and positively regulates ABA signaling.

RESUMEN

La señalización por la hormona vegetal ácido abscísico (ABA) desempeña un papel crítico en la regulación del crecimiento de la raíz y en la arquitectura del sistema radical. La promoción de crecimiento de la raíz en condiciones de estrés hídrico mediada por ABA es clave para la supervivencia de las plantas bajo condiciones limitantes de agua. En este trabajo, hemos explorado el papel de los receptores PYR/PYL/RCAR (PYRABACTIN RESISTANCE1 (PYR1)/PYR1 LIKE (PYL)/ REGULATORY COMPONENTS OF ABA RECEPTORS) de *Arabidopsis* (*Arabidopsis thaliana*) en la ruta de señalización de ABA en raíz. Así, hemos descubierto que el receptor de ABA PYL8 juega un papel no redundante en la regulación de la percepción de ABA en raíz. Inesperadamente, dada la naturaleza multigénica y la redundancia funcional parcial observada en la familia PYR/PYL/RCAR, el mutante *pyl8* fue el único mutante sencillo de pérdida de función de los receptores PYR/PYL/RCAR que mostraba una sensibilidad reducida a la inhibición del crecimiento mediada por ABA en raíz. Este efecto se debe a la falta de inhibición mediada por PYL8 de varias fosfatasas del grupo A tipo 2C (PP2Cs), ya que PYL8 es capaz de interactuar *in vivo* con al menos cinco PP2Cs, denominadas HYPERSENSITIVE TO ABA1 (HAB1), HAB2, ABAINSENSITIVE1 (ABI1), ABI2, and PP2CA/ABA-HYPERSENSITIVE GERMINATION3 según lo han revelado la purificación por afinidad en tándem (TAP por sus siglas en inglés) y estudios proteómicos de espectrometría de masas.

La transducción de la señal del ABA localizada en la membrana plasmática celular juega un papel crucial en los pasos iniciales de la señalización de la fitohormona, pero los mecanismos moleculares que unen los componentes básicos de la señalización y la membrana plasmática no están claros. Estudiando las interacciones de los receptores del ABA PYR/PYL/RCAR con la membrana plasmática hemos encontrado que éstos pueden interaccionar transitoriamente con ella de forma dependiente de calcio gracias a una familia de proteínas con dominios C2 relacionadas con la ruta de señalización de ABA (denominadas C2-domain ABA-related (CAR) proteins). Específicamente, se encontró que PYL4 interacciona de manera independiente de ABA con CAR1 tanto en la membrana plasmática como en el núcleo de las células vegetales. La proteína CAR1 pertenece a una familia multigénica constituida por 10 miembros en *Arabidopsis thaliana*, desde CAR1 hasta CAR10, y que solo se encuentra en plantas. Los ensayos de complementación bi-molecular de fluorescencia y de co-immunoprecipitación confirmaron la interacción en células vegetales tanto de PYL4-CAR1 como de otras parejas de PYR/PYL-CAR. La cristalización de la proteína CAR4 reveló que, además de un dominio C2 clásico de unión a lípidos dependiente de calcio, las proteínas de la familia CAR presentan un dominio específico que probablemente es responsable de la interacción con los receptores PYR/PYL/RCAR y de su posterior reclutamiento a las vesículas de fosfolípidos. Esta interacción es relevante para la función de los receptores PYR/PYL/RCAR

en la señalización del ABA, ya que diferentes mutantes triples *car* de pérdida de función, que tienen afectados los genes CAR1, CAR4, CAR5, y CAR9, demostraron una reducción de la sensibilidad al ABA en ensayos de establecimiento de plántula y crecimiento de la raíz. En resumen, hemos identificado nueva familia de proteínas que son capaces mediar las interacciones transitorias dependientes de Ca^{2+} con vesículas de fosfolípidos, lo que a su vez afecta localización de PYR/PYL/RCAR y regula positivamente la señalización de ABA.

RESUM

La senyalització per l'hormona vegetal àcid abcíssic (ABA) exerceix un paper crític en la regulació del creixement de l'arrel i també en l'arquitectura del sistema radical. La promoció del creixement de l'arrel en condicions d'estrés hídric, regulada per ABA és clau per la supervivència de les plantes sota condicions limitants d'aigua. Amb aquest treball, hem investigat el paper dels receptors PYR/PYL/RCAR (PYRABACTIN RESISTANCE1 (PYR1)/PYR1 LIKE (PYL)/ REGULATORY COMPONENTS OF ABA RECEPTORS) d'Arabidopsis (Arabidopsis thaliana) en el camí de senyalització d'ABA en arrel. Així, hem descobert que el receptor d'ABA PYL8 exerceix un paper no redundant en la regulació de la percepció d'ABA en arrel. Inesperadament, donada la naturalesa multigènica i la redundància funcional parcial que s'observa en la família PYR/PYL/RCAR, el mutant *pyl8* va ser l'únic mutant senzill de pèrdua de funció dels receptors PYR/PYL/RCAR que mostrava una sensibilitat reduïda a la inhibició del creixement mitjançada per l'ABA en l'arrel. Doncs aquest efecte es deu a la falta d'inhibició regulada per PYL8 de diverses fosfatases del grup A tipus 2C (PP2Cs), ja que PYL8 té la capacitat d'interactuar *in vivo* almenys amb cinc PP2Cs, anomenades HYPERSENSITIVE TO ABA1 (HAB1), HAB2, ABAINSENSITIVE1 (ABI1), ABI2, and PP2CA/ABAHYPERSENSITIVE GERMINATION3 segons ho han revelat per una banda la purificació per afinitat en tàndem (TAP són les seues sigles en anglés) i per altra banda, estudis proteòmics d'espectrometria de masses.

Pel que fa a la transducció del senyal del l'ABA, la qual es localitza en la membrana plasmàtica cel·lular, juga un paper molt important en els primers instants de la senyalització de la fitohormona, no obstant això els mecanismes moleculars que uneixen els components bàsics d'aquesta senyalització amb la membrana plasmàtica, no es troben del tot clars. Per tant, s'han estudiat les interaccions que tenen els receptors del ABA PYR/PYL/RCAR amb la membrana plasmàtica, i hem trobat que aquests tenen la capacitat d'interaccionar transitòriament amb la membrana de forma dependent al calci, gràcies a una família de proteïnes amb domini C2, les quals es troben relacionades amb la ruta de senyalització d'ABA(anomenades C2domain ABArelated (CAR) proteins).Específicament, es va trobar que PYL4 interacciona d'una manera independent al ABA amb CAR1, tant en la membrana plasmàtica, com en el nucli de les cèl·lules vegetals. La proteïna CAR1 pertany a la família multigènica constituïda per 10 components en Arabidopsis thaliana, des de CAR1 fins CAR10, que tan sols es troba en plantes. Els assajos de complementació bimolecular de fluorescència i de co-immunoprecipitació, van confirmar la interacció en cèl·lules vegetals, tant de PYL4CAR1 com d'altres parelles de PYR/PYL-CAR. La cristal·lització de la proteïna CAR4 va revelar que, a més d'un domini C2 clàssic de unió a lípids dependent del calci, les proteïnes de la família CAR presenten un domini PYR/PYL/RCAR, i del seu posterior reclutament a les vesícules fosfolipídiques. Doncs, aquesta interacció és rellevant en la funció dels receptors PYR/PYL/RCAR, ja que participa en la

senyalització del l'ABA. Aquesta interacció es clau per a la funció dels receptors, ja que diferents mutants triples *car* de pèrdua de funció, els quals posseïxen afectats els gens CAR1, CAR4, CAR5 i CAR9, van mostrar una reducció de la sensibilitat a l'ABA en assajos d'establiment de plàntula i creixement de l'arrel. En conclusió, hem identificat una nova família de proteïnes amb la capacitat d'organitzar les interaccions transitòries dependents del calci amb vesícules de fosfolípids, fet que al seu torn afecta la localització de PYR/PYL/RCAR i regula positivament la senyalització d'ABA.

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1. INTRODUCTION

1. INTRODUCTION

1.1 DISCOVERY, CHEMICAL FEATURES AND ABA PHYSIOLOGICAL ROLE IN THE PLANT

The plant phytohormone abscisic acid (ABA) was first discovered in the 1960s by two independent groups as an active endogenous plant regulator compound. Addicott´s group in the United States identified and isolated an abscission factor named "abscissin II" implicated in the senescence of the young cotton fruits (Ohkuma et al., 1963). At the same time, Cornforth's and Wareing's groups in the United Kingdom isolated the bud and seed dormancy hormone from sycamore leaves naming it "dormin"(Cornforth et al., 1965). Chemical studies and comparison of "abscisin II" and "dormin" showed they were the same compound and, subsequently, it was renamed abscisic acid (ABA) (Cornforth et al., 1966; Addicott et al., 1968). Later, it was discovered that ABA levels increase considerably when plants wilt, supporting the role of endogenous ABA in closing stomata as water-stress hormone (Wright and Hiron, 1969; Mittelheuse and Van Steveninck, 1969).

Although ABA has been identified and chemically characterized as an universal stress-hormone in higher plants, it has been found in organisms across all kingdoms from bacteria to animals, excepting *Archaea*. ABA biosynthetic genes and enzymes have been reported in vascular plants as well as in mosses, plant-associated bacteria, plant pathogenic fungi, certain cyanobacteria, algae, lichens, protozoa and sponges (Zocchi et al., 2001; Puce et al., 2004; Nambara and Marion-Poll, 2005). Surprisingly, ABA has also been identified as an endogenous hormone in mammals, regulating different cell functions including inflammatory process, stem cell expansion, insulin release and glucose, thus pointing to a potential anti-cancer compound use in humans (Page-Degivry et al., 1986; Bruzzone et al., 2007). The presence of ABA along different kingdom on the evolution scale confirms its ancient origin (Nambara and Marion-Poll, 2005; Wasilewska et al., 2008). In addition, comparison of the components of ABA signaling pathway, based on phylogenetic and transcriptome data, suggests the idea that appearance of the ABA signaling mechanism may have played a crucial role in land colonization by plants. Indeed, establishment of the ABA signaling core from bryophytes to angiosperms is a result of the evolutionary processes, caused by high selective pressure due to water limitation during land colonization 420 million years ago (Umezawa et al., 2010; Hauser et al., 2011).

Chemically, ABA ($C_{15}H_{20}O_4$) is a weak acid characterized by pKa 4.7 and 15-C sesquiterpenoide structure with one asymmetric carbon at the position 1'of the ring. Rotation of

the ABA molecule around this chiral C1' carbon, with a swap of the relative positions of the 7' methyl and 8', 9'- dimethyl groups on the ring distinguishes between ABA enantiomers. The naturally active occurring form is the (+) –S –ABA [(+) ABA] with the side chain in 2-cis, -4-trans orientation and its mirror image is unnatural, synthetic (-) –R –ABA [(-) ABA] isomer (Figure 1.1). The two molecules are very similar in shape, differing only in the location of the axial 8'-methyl group. Several studies indicate that the exact ABA chemical structure is essential for its physiological activity and modification at the structure as, for example, at the carboxyl group, hydroxyl group or the stereochemistry at C1' in the ciclohexane ring greatly reduce ABA-like activities (reviewed in Cutler et al., 2010).

Since the discovery of ABA, the biological and biochemical activity of (+) –S –ABA and (-) –R –ABA have been extensively investigated. The S and R forms cannot be interconverted in plants. Comparisons between two enantiomers have showed that the unnatural enantiomer –R-ABA is active in different well known ABA response such as germination, root growth inhibition of seedlings and stomata closure (Walkersimmons et al., 1994; Nambara et al., 2002; Lin et al., 2005; Huang et al., 2007; Cutler et al., 2010). To explain the bioactivity of –R –ABA form, two mechanisms were hypothesized. (i) One of them was proposed by Milborrow over 40 years ago as a model of the spatial requirements for an ABA receptor, where in the active site, ABA adopts a conformation with the side chain equatorial, so that the C7' and C8' methyl groups have similar equatorial orientations. In order to occupy the same site like –S –ABA, the –R –ABA could flip 180º the ciclohexane plane accommodating C7' into the space normally occupied by the C8' of the –S –ABA, remaining the rest of the functional groups intact (Milborrow, 1974). Later, the first crystallographic studies of *PYR/PYL/RCAR* ABA

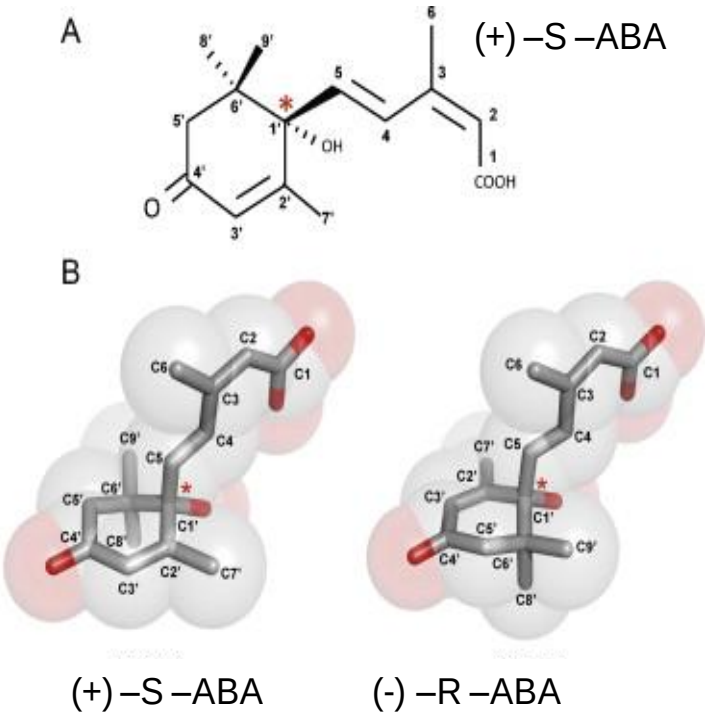

Figure 1.1 Chemical structure of the phytohormone ABA. (A) 2D structure of the natural S(+) form of ABA. The red asterisk points out the C1′ asymmetric carbon of the molecule. (B) 3D structure of the two ABA enantiomers. The (−) enantiomer has been rotated to illustrate the structural difference of the ring methyl groups with respect to the (+) enantiomer. From Santiago et al., 2012

receptors in complex with (+) ABA proved Milborrow's ''flip'' hypothesis (Nishimura et al., 2009). (ii) Another mechanism was the dual selectivity of ABA receptors. In such model, there are some members of the PYR/PYL family that differentiate between the two stereoisomers, accommodating them with different binding pocket orientation (Nambara et al., 2002). This hypothesis was validated for the probed with PYL5 complex with (-) ABA (Zhang et al., 2013)

The concentration of ABA in plant tissues is the result of combination of biosynthesis, transport, catabolic and conjugation processes. Moreover, the endogenous ABA levels are regulated in response to environmental or developmental signals such as drought, salinity, temperature, light, other hormones, intrinsic developmental programs and plant circadian rhythms. Regulation exerted by these processes changes endogenous ABA levels in the range from nanomolar to micromolar (Verslues and Zhu, 2005; McCourt and Creelman, 2008; Seung et al., 2012). The biosynthetic pathway of ABA in higher plants has been largely delineated. The early steps of ABA biosynthesis take place in plastids and occur through the carotenoid pathway via a series of oxidation and isomerization reactions followed by the cleavage of C40 carotenoid to yield the sesquiterpene xanthoxin. This cleavage is considered to be the rate-limiting step on the ABA biosynthesis pathway, and the enzyme responsible for this activity is 9-CIS-EPOXY CAROTENOID DIOXYGENASE (NCED). Xanthoxin is exported from the plastids to cytoplasm for the two final steps in which xanthoxin is converted to ABA aldehyde by AtABA2 enzyme (Gonzalez-Guzman et al., 2002; Cheng et al., 2002a), and subsequently oxidized to ABA by an ABSCISIC ALDEHYDE OXIDASE (AAO) (Seo et al., 2000; Gonzalez-Guzman et al., 2004). The expression pattern of ABA biosynthetic genes indicates that the synthesis of ABA takes place in the seeds, in the vasculature and in guard cells. Moreover, it has been shown that ABA primarily is synthesized in vascular tissues, and then is transported to target tissues of root and shoots via xylem and phloem. Taking into the account the nature of ABA as a weak acid, it can be transported by passive diffusion from a low to a high pH environment. However, in the last years, cell-to-cell ABA transport is seen also to be mediated by two plasma membrane bound ATP-binding cassette (ABC) transporters (AtBCG25 ABA exporter and AtBCG40 ABA importer) (Kang et al., 2010; Kuromori et al., 2010) and a family of low-affinity nitrate transporter AIT1/ NRT1.2 (Kanno et al., 2012).

In addition to ABA synthesis, catabolism and conjugation are major mechanism for regulation of ABA levels. There are two major pathways of ABA catabolism: (i) hydroxylation of ABA at the 8' position catalyzed by a P450 monooxygenase (CYP707A) to give an unstable intermediate (8'-hydroxy-ABA) that is isomerized to phaseic acid (Xu et al., 2002), and (ii) esterification of ABA to ABA–glucosyl ester (ABA-GE) produced by ABA glycosyltransferases. ABA-GE is an inactive conjugated storage and long-distance translocation form of ABA which is accumulated in the vacuoles, ER, and the apoplast (Cutler and Krochko, 1999; Dietz et al., 2000; Nambara and Marion-Poll, 2005). Mobilization of inactive ABA-GE

by the b-glucosidase homologs, AtBG1 and AtBG2, localized at the endoplasmic reticulum and vacuole, respectively, has been described as a fast mechanism which allows plants to quickly adjust the ABA levels in response to changing environmental conditions (Dietz et al., 2000; Lee et al., 2006; Xu et al., 2012).

1.1.1. The role of ABA in abiotic stress

ABA plays a key role in the regulation of numerous physiological processes in the plant. It is well recognized as the universal stress hormone, especially in response to abiotic stresses such as drought, salinity, extreme temperatures and excess of light (Christmann et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Qin et al., 2011). These stresses are similar in that they all produce water deficit altering the plant water status. The status of water in soils, plants and atmosphere is generally quantified in terms of water potential (Ψw). Differences in the Ψw define the direction of water flux, where water flows to the medium with more negative Ψw. Thereby, plants can withdraw water from the soil only when root Ψw is more negative than soil Ψw, and then facilitate translocation of water from the soil to the shoot plant tissues along the Ψw gradient. In water-deficit condition, when soil water content and Ψw is low or atmospheric vapour pressure deficit is high, the plant Ψw also decreases. Decreased plant Ψw makes it more difficult for the plant to take up water and alters plant water status (Verslues et al., 2006). Perception of these hydraulic changes in Ψw leads to increase of ABA levels as a result of an enhanced biosynthesis, inhibition of ABA degradation and release of ABA from ABA-GE. Accumulation of ABA in plant cells is the key factor involved in the downstream adaptive plant physiological responses to stress by controlling the internal water status in plants (Cutler et al., 2010; Raghavendra et al., 2010; Hauser et al., 2011; Joshi-Saha et al., 2011). These responses can be divided into two phases (i) low Ψw stress avoidance and (ii) low Ψw stress tolerance (Figure 1.2). The first response of plants to water deficit is to avoid low Ψw trough reducing water loss and/or increasing water uptake. In this way, one of the initial responses of plants is to reduce stomatal aperture to minimize water loss via transpiration. ABA is a major mediator of the stomatal closure and its signaling mechanisms are well characterized. ABA is known not only to be implicated in stomatal closure, but also in the inhibition of stomatal opening (reviewed in Schroeder et al., 2001; Kim et al., 2010; Joshi-Saha et al., 2011). During dehydrative stresses, ABA is transported from the biosynthesis sites to guard cells, where its accumulation induces a reduction of the guard cell turgor and volume, resulting in stomatal closure (MacRobbie, 1998; Hu et al., 2010). This effect of ABA on stomatal closure is explained by depolarization of the plasma membrane caused, especially by (1) the inhibition of the plasma membrane H^+-ATPase, (2) inhibition of the K^+ influx, and (3) activation of the slow (S-type) anion channels. Membrane depolarization activates K^+ efflux from the cell, causing

reduction of the cell ionic concentration that leads to water efflux and reduced guard cell volume, producing stomata closure (Kim et al., 2010).

In the long term response to low Ψw avoidance, during mild water stress, ABA stimulates elongation of the main root, arrests lateral root meristem growth and suppresses shoot growth. This adjustment leads to an increased root/shoot ratio and allows increment of the water uptake. In fact, ABA-biosynthesis deficient mutants as well as inhibition of endogenous ABA synthesis in wild type plants by fluridone markedly suppress root elongation under water deficit conditions compared with wild type plants (Saab et al., 1990; Sharp et al., 2004). This maintenance of root elongation at low water potentials highlights the putative role of ABA in the capacity of roots to perceive the moisture gradient. Additionally, ABA is key factor in the process named hydrotropism, which is defined as the root capacity to perceive moisture gradients. This process makes possible the search of water by the plant root under moderate stress conditions. Several reports have confirmed implication of the ABA "core signaling pathway" in the plant hydrotropic response (Moriwaki et al., 2012; Antoni et al., 2013). In contrast to mild water stress, severe stress inhibits germination, root and shoot growth (Vartanian et al., 1994; Duan et al., 2013). Recently, Planes et al. suggested that this growth inhibition can be caused by ABA accumulation and activation of ABA signaling pathway, leading to cytosolic acidification and changes in membrane potential and following altered ion homeostasis (Planes et al., 2015).

In spite of these strategies, low Ψw stress avoidance doesn't allow the long time plant maintenance and survival. For instance, improvement of the water-use efficiency responses by

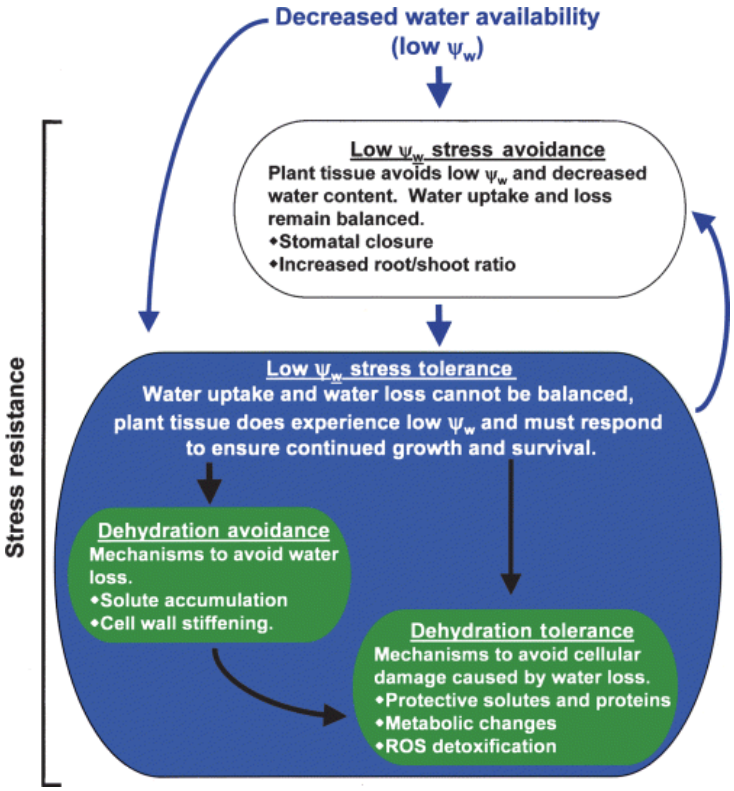

Figure 1.2 Conceptual diagram of the plant responses triggered by decreased water availability. In initial stages, the plants tend to avoid low water potentials by preventing water loss. Later, when the stress is prolonged in time, plants employ strategies directed to tolerate these conditions. Between these strategies it can be found the osmotic adjustment by solute accumulation or the synthesis of compounds directed to preserve the cellular components from the damage caused by low water availability. From Verslues et al., 2006

the stomata closure and inhibition of shoot growth causes reduction of CO_2 uptake and decreases the photosynthesis rate. On the other hand, inhibition of the lateral root growth decreases uptake of resources from the soil and the stress becomes more severe. Therefore, to maintain stress tolerance, plants activate responses to achieve low water potential while avoiding water loss. In plant cells, Ψw is the sum of osmotic potential (Ψs) and pressure (turgor) potential (Ψp). One of the mechanisms to diminish plant Ψw is decreasing Ψs (more negative) by accumulating compatible solutes inside the cell (it is named also as osmotic adjustment). For instance, ABA induces accumulation of some group of solutes that could function as osmolytes like proline, glycine betaine, sugars, small hydrophilic proteins, etc. The accumulation of these solutes does not interfere with metabolism or cellular function and may also have other properties such as maintenance of protein and membrane structure, cell growth or regulate red-ox balance contributing to cell homeostasis under water shortage (Voetberg and Sharp, 1991; Strizhov et al., 1997; Ishitani et al., 1997). Additionally, low water potential could be maintained by regulation of the mechanical forces exerted at the cell wall and at the cell wall-plasma membrane interface. In the absence of solute accumulation, cell wall hardening causes decrease of turgor and Ψw, allowing the cell to avoid further water loss. Nevertheless, this strategy prevents any further expansion of the cell, therefore, it is the most frequently strategy of the non-growing tissues. Moreover, solute accumulation is still required for this cell to lower its Ψw and take up water.

Many aspects of these adaptive processes to prolonged stresses are started by profound changes in gene expression, being a key point for stress tolerance maintenance. As many abiotic stresses are characterized by both the water deficit and osmotic imbalance, there is an overlap in gene expression patterns between different stresses, suggesting the existence of a significant cross-talk between stresses to maintain cellular homeostasis. It has been seen that some of stress-inducible genes respond to ABA, whereas others do not, indicating that there are not only ABA-dependent pathways but also ABA-independent pathways involved in the abiotic stress response (reviewed in Yamaguchi-Shinozaki and Shinozaki, 2006; Cutler et al., 2010; Huang et al., 2012). Global gene expression analysis has shown that, in *Arabidopsis* and in several other plants, between 1-10% of the protein-coding genes are ABA-regulated (Seki et al., 2002; Hoth et al., 2002; Nemhauser et al., 2006). Analysis based on gene ontology (GO) showed that ABA-repressed genes generally encode proteins involved in growth and development, such as ribosomal, chloroplast, cell wall, and plasma membrane proteins. In contrast, ABA-induced genes include those that encode (1) proteins associated with stress response and tolerance, including LEA proteins; (2) a number of regulatory proteins, such as transcription factors, protein kinases, and phosphatases; (3) a variety of transporters (ion transporters, protein channels and aquaporins); and (4) enzymes involved in the biosynthesis of osmolytes, detoxification and general metabolism, phospholipid signaling, etc. (Choudhury and Lahiri,

2011; Fujita et al., 2011). Promoter analysis of ABA-inducible genes showed that most of them contain a conserved, cis-acting sequence, named as ABA-responsive element (ABRE; PyACGTGG/TC) (reviewed in Fujita et al., 2011; 2013; Nakashima and Yamaguchi-Shinozaki, 2013). Proteins that bind to ABRE are called ABRE-binding proteins (AREB) or ABRE-binding factors (ABFs). They are basic leucine zipper (bZIP) transcription factors that regulate ABA-dependent gene expression, acting as major transcription factors under abiotic stress conditions (reviewed in Fujita et al., 2011; 2013). The AREB/ABF transcription factors are induced by abiotic stress and their transcriptional activities are ABA-dependent. Over-expression of these factors in transgenic plants results in ABA hypersensitivity in germination and seedling growth, and also in enhanced drought tolerance (Kang et al., 2002; Kim et al., 2004a; Fujita et al., 2005; Furihata et al., 2006; Abdeen et al., 2010). There are other families of transcription factors like AP2/ERF, R2R3-MYB, NACS, HD-ZIP, BHLH, C2H2, AFLB3 and WRKYs which are also involved in ABA-regulated gene expression under abiotic stress conditions.

Changes in gene expression allow to re-establish cell homeostasis (both ionic and osmotic) and to activate detoxification and repairing stress damages pathway. For instance, homeostatic mechanisms appear to play major role in response to salt stress, in which the ion homeostasis is restored by restriction of salt uptake and water/osmosis homeostasis is adjusted trough synthesis of compatible solutes. In fact, toxic levels of sodium are regulated by Na^+/H^+ antiporter SOS1 (Shi et al., 2000), the Na^+ influx transporter HKT1 (Rus et al., 2001; 2004) and the tonoplast Na^+/H^+ antiporter AtNHX1(Apse and Blumwald, 2002). These channels are known to be important to avoid toxic ion compartmentalization and also are determinants in ion homeostasis. On the other hand, ABA-induced synthesis and accumulation of osmotic protectants (such as compatible solutes and protein channels involved in movement of water through membranes, and enzymes involved in osmoprotectant synthesis) or proteins that may protect macromolecules and membranes (late embryogenesis abundant proteins (LEA), osmotin, antifreeze protein, chaperons, and mRNA binding proteins) are important response for re-establish water/osmotic homeostasis. Furthermore, the accumulation and activation of these genes have been reported to adapt the plants to stress conditions and their over expression in transgenic plants produce stress-tolerant phenotypes (Gilmour et al., 2000; Ito et al., 2006). For instance, LEA proteins are high molecular weight proteins that not only are abundant during late embryogenesis but also accumulate during seed desiccation and in response to water stress (Galau et al., 1987). Constitutive over expression of group3 LEA proteins has resulted in improved growth characteristics and drought tolerance. Over expression of HVA1 (group3 LEA) gene from barley (Xu et al., 1996) and a PMA1959 (group1 LEA) from wheat (Cheng et al., 2002b), among others, have been reported to confer drought and salt stress tolerance in terms of cell integrity. Also it has been suggested the role of LEA proteins in anti aggregation of

enzymes under desiccation and freezing stresses (Goyal et al., 2005). In addition, aquaporins may also have a role in acclimating to water stress. ABA regulates expression and activity of several aquaporins, although there are many conflicting reports on the role of ABA in this process (Jang et al., 2004; Alexandersson et al., 2005; Kline et al., 2010; Shatil-Cohen et al., 2011).

Another negative aspect of the abiotic stress tolerance is the increase of oxidative stress caused by generation of reactive oxygen species (ROS) (such as H_2O_2, superoxide (O_2^-) and singlet oxygen ($^1O_2$)). High levels of ROS lead to disruption of the cellular homeostasis by reacting with lipids, proteins, pigments, and nucleic acids resulting in lipid peroxidation, membrane damage, and the inactivation of enzymes, affecting cell viability (Bartels and Sunkar, 2005). Reduction of oxidative damage could provide enhanced plant resistance to abiotic stress. In order to control high levels of ROS and prevent its uncontrolled accumulation, plants have developed complex antioxidant system which includes various oxidative protection enzymes, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases (Roxas et al., 1997) and non-enzymatic metabolites that may also play a significant role in ROS signaling in plants (Vranova et al., 2002). Surprisingly, increased levels of ROS associated with ABA accumulation have been proposed as a key component "of cross-talk tolerance" to multiple types of stress. Despite the damage effect at high ROS concentrations, there is now evidence that ROS act as second messengers in the acclimation response to stress stimuli, mediated by ABA (Wang and Song, 2008). Moreover, genetic evidences show that the *Arabidopsis* double mutant *rbohD/F* (impaired in NADPH oxidases) lakes ABA-induced stomatal closure, ABA promotion of ROS production, and ABA-activated plasma membrane Ca^{2+}-permeable channels, while the exogenous application of H_2O_2 rescued these responses (Kwak et al., 2003). As a result, ABA-induced ROS lead to high concentration of cytoplasmatic Ca^{2+} ($[Ca^{2+}]$ cyt) and further stomatal closure (Kim et al., 2010).

1.1.2 The role of ABA in biotic stress

In their natural environments, plants are continuously challenged by biotic stresses imposed by other organisms and include pathogenic interactions with bacteria, viruses, fungi, oomycetes, insects, as well as other plants. Plant response to biotic stresses is mediated by phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), which create a complex network to modulate quickly and efficiently plant defense response. In addition to these well characterized pathways, there are other plant hormones that may act as co-regulators of the biotic stress responses (Bari and Jones, 2009). One of them is ABA, which is not only crucial for mediating abiotic stress responses, but also plays a multifaceted and pivotal role in disease susceptibility, resistance to pathogen infection, and interaction with other biotic stress

hormones (Ton et al., 2009; Cao et al., 2011) (Figure 1.3). ABA is reported to play an ambivalent role in pathogen defense, it can act as positive or negative regulator of plant defense depending on the plant species, on the pathogens and their mode of infection (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008; Ton et al., 2009; Cao et al., 2011). For example, mutants affected in ABA biosynthesis or ABA signaling in *Arabidopsis* (*abi1-1*, *abi2-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *pyr1pyl1pyl2pyl4*) and tomato (*sitiens*) were shown to have enhanced resistance to different biotrophs pathogens such as *Botrytis cinerea*, *Pseudomonas syringae*, *Plectosphaerella cucumerina* and *Hyaloperonospora parasitica* (Audenaert et al., 2002; Mohr and Cahill, 2003; Torres-Zabala et al., 2007; Sanchez-Vallet et al., 2012). However, a positive role of ABA was seen in response to some necrotrophs pathogens, such as *Alternaria brassicicola*, *Ralstonia solanacearum*, and *Pythium irregulare*, as ABA-deficient and ABA-insensitive mutants (*abi1-1*, *abi2-1*, *abi4-1*, *aba1-6*, *aba2-12*, *aao3-2*) were found to be more susceptible than wild-type plants to these pathogens (Adie et al., 2007; Hernandez-Blanco et al., 2007; Flors et al., 2008; Garcia-Andrade et al., 2011).

Different mechanisms of ABA action in the modulation of pathogen resistance had been proposed. There are some similarities between the biotic and abiotic responses. For example, the stomatal closure not only leads to prevent water loss during drought stress but also, in early stages of pathogen invasion, serves as defense barriers to halt pathogens and, at the same time, prevents unnecessary activation of costly SA- and JA-dependent defenses (Melotto et al., 2006). For instance, ABA-deficient and ABA-insensitive mutants (*aba3-1* and *ost1-2* respectively) with suppressed stomatal closure were found to be more susceptible than wild type plants to the bacteria *P.syringae* in *Arabidopsis* (Melotto et al., 2006). A second layer of defence in response to infection is rapid callose deposition in the apoplast and generation of reactive oxygen species (ROS). The function of ABA in this post-invasive penetration resistance seems to be conditioned by the plant-pathogen interaction. For instance, ABA suppresses early ROS production and causes increased susceptibility, as observed upon infection of tomato with *B. cinerea* (Asselbergh et al., 2007). In plant pathogen-induced callose deposition the role of ABA is more controversial. In infection by fungi, ABA production can enhance resistance by stimulation of callose deposition to block pathogen entry (Flors et al., 2008; Asselbergh et al., 2008). In contrast, a negative effect of ABA in callose deposition has been reported from different studies in defense against bacteria as described in *Arabidopsis* cotyledons upon treatment with bacterial PAMP flagellin (Clay et al., 2009). A feasible explanation for this antagonistic ABA effect might be the activation of different pathways to trigger callose in bacteria and fungi (Ton et al., 2009). In later stage of pathogen response, the effect of ABA against pathogen attack is modulated by interaction with other hormones. As a result, ABA suppresses SA- and ET/JA-dependent response, and modulates synergistic cross-talk with JA signaling (Anderson et al., 2004; Mauch-Mani and Mauch, 2005; Adie et al., 2007) (Figure 1.3).

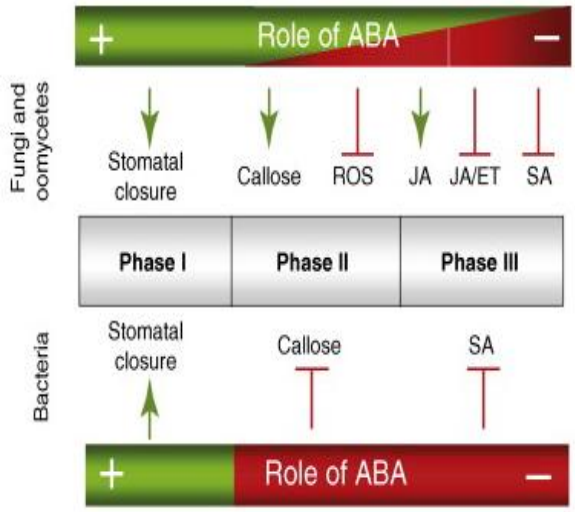

Figure 1.3 Contribution of ABA to disease resistance or susceptibility. ABA has a multifaceted role throughout different phases of plant defense. During Phase I, ABA stimulates resistance against fungi and oomycetes by mediating stomatal closure. Throughout Phase II, it promotes callose deposition in response to infection by fungi and oomycetes. However, in certain cases, ABA production can suppress early ROS production and cause increased susceptibility. By contrast, components in the ABA response pathway can suppress bacteria-induced callose deposition and, therefore, contribute negatively to resistance. During Phase III, ABA interacts with SA-, JA- and ET-dependent defense pathways. From Ton et al., 2009

Interestingly, as a strategy to manipulate the plant hormone machinery, some pathogens have evolved abilities to produce ABA or ABA-mimetic in order to interfere with the host defence response. For example, several fungal species produce ABA, including *B. cinerea*, *Rhizoctonia solani*, *Ceratocystis fimbriata*, and *Rhizopus nigricans* and use ABA as the virulence factor to suppress hormone balance in plant resistance (Inomata et al., 2004).

1.1.3 The role of ABA in plant growth and development

Plants continuously need to adapt to a changing environment while they must maintain growth, development, and reproduction. Plant hormones integrate plant development and growth processes with the environmental stress signals in spatiotemporal manner. Hence, in addition to the role as stress hormone, ABA also has multiple roles in plant growth and development, being part of complex regulatory networks with other hormones as auxin, ethylene, cytokinin, gibberellins (GA), brassinosteroids and jasmonate, among others. For instance, ABA regulates key growth and developmental processes, such as embryo and seed development, promotion of seed desiccation tolerance and dormancy, seed germination, seedling establishment, vegetative development as well as general growth and reproduction.

Despite the fact that ABA hormone is involved in a wide range of these developmental processes, the ABA effect on seed development is the most studied process. Developing embryos enter maturation phase when they undergo a transition from growth by cell division to growth by cell enlargement and begin to accumulate storage reserves. In this growth phase transition, there are two peaks of ABA accumulation. The early peak takes place 9 DAP (Days After Polinization), just before the maturation stage. This peak comes from maternal ABA and in combination with the leafy-cotyledon *FUS3* and *LEC* transcription factor genes, is important for preventing premature germination at the end of the cell division phase of embryogenesis

(Raz et al., 2001). The later ABA peak occurs at 15-16 DAP, it is mostly from embryonic tissue and is essential for induction of dormancy and desiccation tolerance (Karssen et al., 1983). During seed maturation, accumulation of storage and LEA proteins, fatty acid reserves and sugars, are required for germination of mature embryos. Reserve accumulation and expression of LEA proteins is ABA-dependent. This seed specific and ABA-inducible gene expression is controlled by a subset of embryo and seed specific ABA response transcription factors, such as members of B3-domain (ABI3), AP2 domain (ABI4) and bZIP domain (ABI5) families (reviewed in Finkelstein et al., 2002), which promote embryo maturation, seed dormancy maintenance and transition to the next phase of germination. But ABA is not the only hormone involved in these processes. Pioneer genetic studies indicated that the balance between relative levels of ABA and GA mediates environmental regulation of germination in antagonistic manner (Koornneef et al., 1982). This antagonism is dependent on environmental conditions, such as after-ripening, stratification or darkness, which lead to decreased ABA levels in seeds, or cold and light, which lead to accumulation of GA. Therefore, release from dormancy is characterized by increased ABA catabolism and increased GA synthesis (reviewed in Seo et al., 2009). For example, ABA-deficient mutants present over-expression of GA biosynthetic genes, whereas in ABA over-producer mutants these genes are under-expressed (Seo et al., 2009). In addition, ABA effects on germination are also antagonized by other hormones such as ethylene (Beaudoin et al., 2000; Cheng et al., 2009; Linkies et al., 2009), brassinosteroids (Li et al., 2001; Steber and McCourt, 2001), cytokinins (Wang et al., 2011) and strigolactones (Toh et al., 2012). In contrast, auxins enhance the inhibitory effect of ABA (Liu et al., 2007a). On the other hand, nutrient availability can also regulate germination through effects on ABA levels. In fact, exogenous application of nitrate during germination leads to decreased ABA levels and favours germination (Matakiadis et al., 2009). In contrast, low sugar concentrations (5-300 mM) delay germination, while high sugar concentrations (>300mM) can inhibit seedling growth in an ABA-dependent manner that cannot be explained simply by the osmotic effects of sugar (Gibson, 2005).

For many years, ABA has commonly been considered to be a growth inhibitor modulator. This view was based on: (i) under water deficit plants accumulate ABA high levels that correlate with growth inhibition, and (ii) ABA application in seedlings arrests their development, provoking the browning of cotyledons and inhibition of root growth, resulting in impaired general growth (Quarrie and Jones, 1977; Trewavas and Jones, 1991). However, in the last years, several reports have suggested the promoter role of ABA in vegetative growth. In fact, under high humidity conditions, ABA-deficient mutants like *aba1* and *aba2* are often smaller and have smaller leaves than wild types, while exogenous ABA application can substantially restore the wild type phenotype (Barrero et al., 2005). Furthermore, ABA-insensitive mutants of the various members of the core ABA signaling pathway, like multiple

PYR/PYL/RCAR ABA receptors mutants or multiple bZIP transcriptional factor mutants *abf2/abf3/abf4*, present strongly impaired growth and fertility (Yoshida et al., 2010; Gonzalez-Guzman et al., 2012). Gonzalez-Guzman et al. showed that under impaired root growth of pentuple and sextuple *PYR/PYL/RCAR* ABA receptor mutants *in vitro* conditions of high humidity can be restored by ABA supplementation (Gonzalez-Guzman et al., 2012). These results confirm the reported role of ABA to maintain primary root growth during water deficit (Sharp et al., 2004) and of low ABA concentrations (<1 µM) to stimulate root growth under non-stress conditions (Zeevaart and Creelman, 1988). On the other hand, these findings confirm conclusions that endogenous ABA is required to maintain both shoot and root development under stress and non-stress conditions, indicating the positive role of ABA in plant growth (Barrero et al., 2005).

1.2 THE CORE ABA SIGNALING PATHWAY

Since the discovery of ABA and its structure in 1960s, broad advances have been made in understanding ABA metabolism, synthesis and hormone responsive genes, whereas ABA perception and signal transduction have remained unclear until last decade due to the complex network of proteins involved (Hirayama and Shinozaki, 2007). In 2009, it was proposed the existence of a "core" ABA signaling pathway composed by (1) PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE 1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR) ABA receptors, (2) group A of PROTEIN PHOSPHATASEs 2C (PP2Cs), (3) ABA-activated subfamily 2 of SNF1-RELATED PROTEIN KINASEs (SnRK2), i.e. SnRK2.2, SnRK2.3, SnRK2.6 and (4) their downstream targets (Umezawa et al., 2009; Fujii and Zhu, 2009; Vlad et al., 2009). These components are assembled as a double negative regulatory system and form a signalling complex known as 'ABA signalosome' (Figure 1.4).

The use of multidisciplinary approaches was required to identify key components of ABA signaling: genetics, biochemistry, pharmacology/cell biology and bioinformatics. Clade A PP2Cs were originally identified in the mid-90s upon map-based cloning of the ABA-insensitive *Arabidopsis* mutants, *abi1-1* and *abi2-1* (Leung et al., 1994; Meyer et al., 1994; Rodriguez et al., 1998) and later, clade A PP2Cs were characterized as negative regulators of the ABA signaling pathway (Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004). Discovery of these phosphatases indicated the importance of protein phosphorylation events in ABA signaling, which was confirmed by the identification of OST1/SnRK2.6, as a key positive regulator of ABA (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002). Biochemical analysis showed that PP2Cs were able to interact with ABA activated SnRK2s (Yoshida et al.,

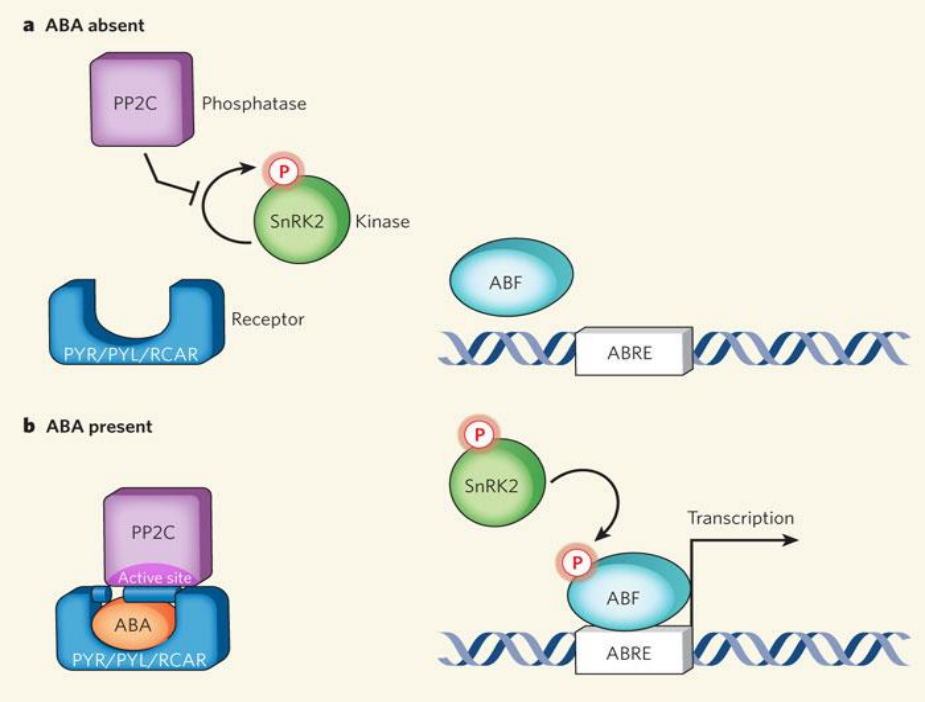

Figure 1.4 Minimal abscisic acid (ABA) signaling pathway. (a) In the absence of the plant hormone ABA, the phosphatase PP2C is free to inhibit autophosphorylation of a family of SnRK kinases. (b) ABA enables the PYR/PYL/RCAR family of proteins to bind to and sequester PP2C. This relieves inhibition on the kinase, which becomes auto-activated and can subsequently phosphorylate and activate downstream transcription factors (ABF) to initiate transcription at ABA-responsive promoter elements (ABREs). From (Sheard and Zheng, 2009)

2006) and inactivate them efficiently through dephosphorylation at specific amino acid residues (Umezawa et al., 2009; Vlad et al., 2009). These kinases/phosphatases are the main effector proteins that drive cellular responses in a highly reversible manner. Since SnRK2s are activated in an ABA-dependent manner, not only traditional genetic strategies but also biochemical/cell/proteomic approaches and bioinformatic screenings were used to identify the complex network of downstream phosphorylated targets in the nucleus such as b-ZIP AREBs/ABFs transcriptional factors that initiate ABA-regulated gene expression (Johnson et al., 2002; Furihata et al., 2006; Fujii et al., 2007; Umezawa et al., 2009; Fujita et al., 2009). As well as other targets in the plasma membrane such as ion channels SLAC1, KAT1 and NADPH oxidases, responsible for turgor-mediated stomatal closure (Kwak et al., 2003; Geiger et al., 2009; Sato et al., 2009; Sirichandra et al., 2009; Lee et al., 2009). Unfortunately, the use of classical genetic screenings for identification of ABA receptors was unsuccessful for more than 30 years. However, in 2009, chemical genetics and protein-protein interaction approaches made possible the discovery of a new type of soluble ABA receptors called PYR/PYL/RCAR proteins (Park et al., 2009; Ma et al., 2009; Nishimura et al., 2009; Santiago et al., 2009b). This finding and the fast elucidation of PYR/PYL/RCAR structures (Nishimura et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Santiago et al., 2009a) provided a major breakthrough in our understanding of the ABA signaling pathway. In fact, the current model can be described as follows: (i) in the absence of ABA, PP2Cs interact and inactivate ABA-

activated SnRK2s via dephosphorylation of multiple Ser/Thr residues in the activation loop (Hirayama and Umezawa, 2010); (ii) in the presence of ABA, interaction of PYR/PYL/RCAR and PP2C is promoted, resulting in PP2Cs inhibition and release of SnRK2s inhibition. Next, SnRK2s are auto-phosphorylated and activate downstream targets, including transcription factors, NADPH oxidases, and ion channels (Raghavendra et al., 2010; Klingler et al., 2010). In this model the regulation of the activity of SnRK2s by PP2Cs and the regulation of the activity of PP2Cs by ABA-bound PYR/PYL/RCARs have emerged as a central mechanism of PYR/PYL/RCARs-mediated ABA signaling. Description of these core components and their molecular mechanism of action will be explained in the followings points.

1.2.1 PYR/PYL/RCAR proteins: soluble ABA receptors

1.2.1.1 Discovery and function of the PYR/PYL/RCAR receptors

During the past decade most of the key receptor proteins for the classical plant hormones (auxin, gibberellins, cytokinin, and ethylene) were discovered using the traditional forward genetics approach (Chang et al., 1993; Ueguchi-Tanaka et al., 2005; Dharmasiri et al., 2005; Kepinski and Leyser, 2005) while, in the case of ABA, it was unsuccessful. It suggested that ABA receptors may belong to a gene family with redundant functions or that mutations in the ABA receptors confer lethality (McCourt, 1999). Earlier studies based either on induction of ABA response following delivery of ABA to the cytoplasm by microinjection or treatment with impermeable ABA analogs suggested that ABA may have both intracellular and extracellular perception (Schwartz et al., 1994; Allan et al., 1994). This led biologists to use direct biochemical screenings searching for proteins able to directly bind ABA with high affinity and to trigger ABA signaling events. However, such putative ABA receptors remain controversial. Thus the role as ABA receptors of (1) the plasma membrane localized G-Protein Coupled Receptor 2 (GCR2) (Liu et al., 2007b), (2) G-protein coupled receptor (GPCR)-type G proteins (GTG1 and GTG2) (Pandey et al., 2009), (3) plastid-localized ABA binding protein (ABAR)/Mg-chelatase H subunit (CHLH)/Genomes uncoupled 5 (GUN5) (Zhang et al., 2002; Shen et al., 2006) and (4) nuclear flowering time control protein A (FCA) (Razem et al., 2006) has been questioned by different works (Johnston et al., 2007; Gao et al., 2007; Illingworth et al., 2008; Risk et al., 2008; Razem et al., 2008; Risk et al., 2009; Muller and Hansson, 2009; Cutler et al., 2010; Chen et al., 2010; Antoni et al., 2011; Tsuzuki et al., 2011; Jaffe et al., 2012) and structural evidence showing ABA binding to these putative receptors is lacking in all cases.

The situation dramatically changed in 2009, when four different groups (Cutler, Grill, Rodriguez and Schroeder) independently isolated as ABA receptors a new family of soluble proteins known as PYR (pyrabactin resistant), PYLs (PYR-like) or RCARs (regulatory

component of ABA receptor) able to bind ABA and interact with group A of PP2Cs (Park et al., 2009; Ma et al., 2009; Santiago et al., 2009b; Nishimura et al., 2010). This breakthrough marked the beginning of the "core ABA signalling pathway" (reviewed in Cutler et al., 2010; Miyakawa et al., 2013). The new class of ABA signaling proteins was identified in *Arabidopsis thaliana* using different methods: (i) chemical genetics by Cutler's group (ii) yeast two-hybrid screening by Grill's and Rodriguez's group and (iii) proteomic/mass-spectrometry analysis by Schroeder's group. In the chemical genetics approach, Sean Cutler's group used a synthetic small molecule, named pyrabactin, which inhibits seed germination, to screen for mutants able to germinate in the presence of pyrabactin (*pyr1* alleles). Through map-based cloning of the *pyr1-1* mutant, it was identified the Pyrabactin Resistance 1 (PYR1) gene and several PYR1-like homologues in *Arabidopsis* (PYLs) as interacting partners of PP2Cs, thus connecting them with ABA signaling pathway (Park et al., 2009). In an independent study, Grill's group, using PP2C ABI2 (ABSCISIC ACID INSENSITIVE 2) as a bait in yeast two-hybrid screening, isolated two members of the PYR/PYL family, PYL8 and PYL9, and named them Regulatory Component of ABA Receptor 1 and 3 (RCAR1/RCAR3) (Ma et al., 2009). In an analogous yeast two-hybrid screening followed by Rodriguez's group, using as a bait HAB1 (HYPERSENSITIVE TO ABA 1), were isolated as HAB1-interacting partners several members of the PYR/PYL family, i.e. PYL5, PYL6 and PYL8 proteins (Santiago et al., 2009b). In an alternative approach, using *Arabidopsis* YFP-tagged ABI1 (ABSCISIC ACID INSENSITIVE 1) lines, Schroeder's group performed *in planta* identification of ABI1-interacting proteins by proteomic/mass-spectrometry analysis. This method identified nine members of the PYR/PYL/RCAR family (Nishimura et al., 2010). The simultaneous reports of PYR/PYL and RCAR proteins have created two sets of nomenclature for this family (Figure 1.5).

The Arabidopsis genome analysis indicates the presence of 14 members of the

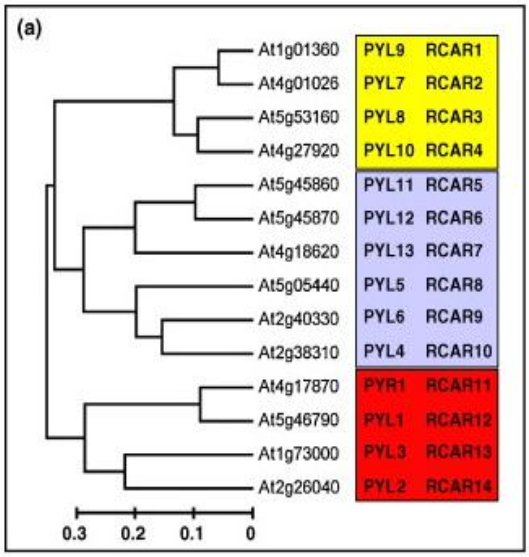

Figure 1.5 Phylogenetic tree of RCAR/PYR1/PYL ABA binding proteins from *Arabidopsis*. The proteins can be grouped into three subfamilies I, II and III highlighted in yellow, blue and red, respectively. The RCAR and PYR1/PYL numbering is given as well as the gene numbers. From Raghavendra et al., 2010

PYR/PYL/RCAR gene family with high homology at the amino acid sequence level. All members encode small proteins, between 159 and 211 amino acid residues and belong to the START domain/Bet v domain superfamily, which has a conserved hydrophobic ligand-binding pocket. PYR/PYL/RCAR proteins are able to bind ABA in their ligand-binding pocket with different affinities in a ratio of one ABA molecule per PYR/PYL/RCAR receptor, which was confirmed by binding assays (Park et al., 2009; Ma et al., 2009; Nishimura et al., 2010; Hubbard et al., 2010). It has been shown that PYR/PYL/RCAR proteins not only can directly bind ABA but also interact with PP2Cs in the presence of ABA, thereby inhibiting phosphatase activity *in vitro* (Nishimura et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Santiago et al., 2009b). Moreover, Bimolecular Fluorescence complementation (BiFC) experiments have reported that these interactions occur either in the cytosol and nucleus defining PYR/PYL/RCAR proteins as intracellular ABA receptors (Park et al., 2009; Santiago et al., 2009b).

On the other hand, mutants impaired in pyr/pyl/rcar loci have provided key genetic evidence on the role of PYR/PYL/RCAR proteins in ABA signaling. The *pyr1* single mutant did not show altered response to ABA due to functional redundancy with other 13 family members whereas progressive inactivation of them in the triple *pyr1/pyl1/pyl4* or quadruple *pyr1/pyl1/pyl2/pyl4* and more recently in the sextuple *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* mutants showed dramatic ABA insensitivity in germination, root growth and stomatal closure, besides reduced or null activation of ABA-activated SnRK2s (Park et al., 2009; Nishimura et al., 2010; Gonzalez-Guzman et al., 2012). In contrast, over-expression of PYL4, PYL5, PYL8 or PYL9 produced hypersensitivity to ABA including inhibition of seed germination, inhibition of root elongation and stomatal closure, which led to enhanced drought resistance in *Arabidopsis* (Ma et al., 2009; Santiago et al., 2009b; Saavedra et al., 2010; Pizzio et al., 2013). In addition, the ABA pathway was reconstructed *in vivo* using transformation of *Arabidopsis* protoplasts, which confirmed that all PYR/PYL/RCAR proteins (except PYL13) have a role as ABA receptors, able to transduce the ABA signal to activate gene expression, thus confirming that PYR/PYL/RCAR proteins together with PP2Cs and ABA-activated SnRK2s constitute the core of the ABA signaling pathway (Fujii and Zhu, 2009).

Additionally, the structure of PYR/PYL/RCAR in ABA-free and ABA-bound forms together with the structure of the ternary complex PYR/PYL/RCAR-ABA-PP2C have provided insights on the molecular details and mechanism of action of ABA receptors (Nishimura et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Santiago et al., 2009a; Dupeux et al., 2011a; 2011b).

Analysis of transcriptome databases such as the Electronic Fluorescent Pictograph (eFP) Browser (Winter et al., 2007) and Genevestigator (Zimmermann et al., 2005) have shown variation in gene expression profiles among the members of PYR/PYL/RCAR family (Klingler et al., 2010). For example, PYL3, PYL10, PYL11, PYL12 and PYL13 have low expression

levels in all tissue/conditions analyzed. GUS reporter gene analysis of PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8 revealed overlapping expression patterns, as well as some differences in their expression pattern (Gonzalez-Guzman et al., 2012)

These expression data together with genetic evidence and biochemical properties of PYR/PYL/RCAR proteins suggest that the function of each member of the family is only partially redundant and could play a distinctive role in ABA signaling *in planta*. In fact, ABA sensitivity in analysis of the expression in root for several PYR/PYL/RCAR proteins as well as comparative analysis of *pyr/pyl/rcar* mutants served to identify a singular role of PYL8 in root ABA signaling (Antoni et al., 2013).

Moreover, under ABA treatment the expression of PYR/PYL/RCAR proteins generally is repressed, in contrast to the expression of the clade A PP2C genes which are strongly induced by ABA treatment (Santiago et al., 2009b; Gonzalez-Guzman et al., 2012). Thus, the initial response to ABA achieved by ABA-dependent PYR/PYL/RCAR inactivation of PP2Cs is attenuated later on by ABA-mediated up-regulation of PP2Cs and down-regulation of PYR/PYL/RCAR proteins. As a result this dynamic mechanism allows the re-setting of the ABA signaling pathway and modulation of ABA response (Santiago et al., 2009b).

Recently, several reports have described orthologous PYR/PYL/RCAR genes in crops, such as tomato (Sun et al., 2011; Gonzalez-Guzman et al., 2014), strawberry (Chai et al., 2011; Jia et al., 2011), rice (Kim et al., 2012; 2014), grape (Boneh et al., 2012; Li et al., 2012), citrus (Romero et al., 2012) and soybean (Bai et al., 2013). These recent studies in a wide array of species, given the physiological and practical implications of the ABA signaling pathway, show PYR/PYL/RCAR ABA receptors as a useful tool to improve crop performance under stress and non-stress environmental conditions (Santiago et al., 2009b; Ben Ari, 2012; Gonzalez-Guzman et al., 2014).

1.2.1.2 Structural insights into PYR/PYL/RCAR proteins as ABA specific receptors

In late 2009, the elucidation of the atomic structure of PYR1, PYL1 and PYL2 by five groups revealed a gate-latch-lock mechanism of ABA perception and signal transduction (Nishimura et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Santiago et al., 2009a) PYR/PYL/RCAR ABA receptors are members of the large superfamily of START/Bet v I proteins characterized by conserved “helix grip” fold that forms a central open hydrophobic ligand-binding pocket (Radauer et al., 2008; Melcher et al., 2009). In addition to PYR/PYL/RCAR ABA receptors, the START/Bet v 1 superfamily also contains different hormone-binding proteins as PR10 (brassinosteroids (Markovic-Housley et al., 2003)) and the cytokinin-specific binding protein CBP (Markovic-Housley et al., 2003). However,

PYR/PYL/RCAR proteins are the only START proteins specifically able to bind ABA, interact with PP2Cs and by inhibiting their activity to transduce the ABA signal.

Interestingly, the PYR/PYL/RCAR members are classified into three subfamilies based on amino acid sequence identity: subfamily-I (PYL7-10/RCAR1-4), subfamily-II (PYL4-6/RCAR8-10 and PYL11-13/RCAR5-7) and subfamily-III (PYR1/RCAR11 and PYL1-3/RCAR12-14) (Park et al., 2009) (Figure 1.5). Up to date, eight PYR/PYL/RCAR ABA receptors have been crystallized, PYR1 (Santiago et al., 2009a; Nishimura et al., 2009), PYL1 (Mizayono et al., 2009), PYL2 (Melcher et al., 2009; Yin et al., 2009), PYL10 (Hao et al., 2011; Sun et al., 2012), PYL3 (Zhang et al., 2012; 2013), PYL5 (Zhang et al., 2013), PYL9 (Zhang et al., 2013; Nakagawa et al., 2014) and PYL13 (Li et al., 2013a). These crystal structures have been obtained both in ABA-bound form and in apo-form (without ABA) and reveal the structural changes induced by the binding of ABA. Thus, the walls of the ligand-binding pocket are constituted by large C-terminal α-helix (α3) enfolded by a seven-strand anti-parallel β-sheet and two small helices close the bottom side of the cavity. The entry to the ligand binding cavity is flanked by two β-loops that were named as *gate* (between β3-β4 sheets, SGLPA loop) and *latch* (between β5-β6 sheets, xHRLxNYxS loop), and by β7-α3 loop (Figure 1.6). These three loops are characterized by highly conserved sequences and show allosteric changes upon transition from the ABA-free to ABA-bound states. In the absence of ABA, these loops show an open conformation of the ligand-binding pocket. Once the intracellular hormone levels increase, ABA molecule enters inside the receptor-binding pocket and anchors its carboxyl group in the bottom part of the cavity. In this position, the binding of ABA to PYR/PYL/RCAR proteins is mediated by a combination of (1) direct ionic interaction of the carboxyl group of ABA with a conserved lysine (K64/K86/K59 in PYL2/PYL1/PYR1, respectively) (what makes the ABA's carboxyl group critical for its biological activity) and (2) water-mediated interactions between hydroxyl and carboxyl groups of ABA with side chains of several conserved receptor residues (Figure 1.7A). The recognition of ABA leads to orientation of the hormone cyclohexene ring at the upper part of the cavity and induces conformational changes that lead to the closing up of

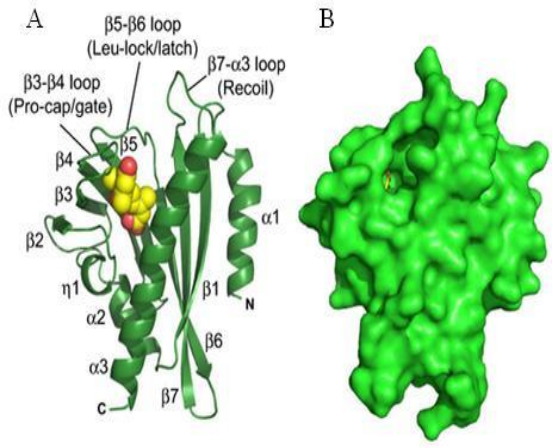

Figure 1.6 Overall structure of the PYL9-ABA complex. PYL9 displays a core domain of the Bet v 1-fold consisting of seven β-strands (β1–β7) forming a bent antiparallel β-sheet that produces a central cavity. (A) Three loops, β3–β4 (Pro-cap/gate), β5–β6 (Leu-lock/latch) and β7–α3 (Recoil), which possess highly conserved sequences, cover the ABA-binding pocket. (B) Molecular surface representation with the same view as in A with the bound ABA (stick model) located at the mouth of the deep ABA-binding pocket. From Nakagawa et al., 2014

the gate and latch loop over the cavity by direct interaction with the cyclohexene ring of ABA. In addition to these two loops, β7-α3 loop induces a conformational change to interact with gate loop thus stabilizing closed conformation. This shift has two important consequences: (1) shielding ABA inside of the receptors from solvent exposure and (2) generation of the favorable interaction gate-latch interface for the binding of the PP2Cs. For instance, structural and mutational data show that single amino acid mutations in any of the gating loops as well as residues that bind the ABA's carboxylate group impair the capacity of the receptor to interact with ABA and PP2Cs, and therefore, impair or abolish PP2C inhibition by PYR/PYL/RCARs. Thus, a gate-latch-lock mechanism is essential for ABA signal transduction (Miyazono et al., 2009; Nishimura et al., 2009; Yin et al., 2009; Melcher et al., 2009) (Figure 1.7B).

Analysis of the ternary complex PYR/PYL/RCAR-ABA-PP2C (Miyazono et al., 2009; Yin et al., 2009; Melcher et al., 2009; Dupeux et al., 2011a; Hao et al., 2011) has revealed that interaction of the ABA-bound receptor with the PP2C occurs both through the gating loops in their closed conformation and N-terminal part of the α3 helix. PP2Cs are able to perceive the ABA molecule, accommodated into the PYR/PYL ABA binding pocket, through a flap subdomain, which contains a conserved tryptophan residue (W385 in HAB1 and W300 in ABI1). This residue is highly conserved among clade A PP2Cs and is critical for the interaction with ABA-bound PYR/PYLs, formation of the ternary complex and inhibition of PP2C phosphatase activity. This tryptophan inserts between the gate and latch loops establishing water-mediated hydrogen bond with ABA's ketone group and two conserved residues of the gating loops: a proline and an arginine (P92/P115/P88 and R120/R146/R116 in PYL2/PYL1/PYR1, respectivly). These interactions help both to stabilize the receptor closed conformation and receptor-phosphatase complex increasing more than 20-fold the ABA-binding affinity for PYR/PYL-PP2C ternary complexes (up to 20-40 nM) as compared to single PYR/PYL/RCAR receptors (> 1 μM) (Miyazono et al., 2009; Yin et al., 2009; Melcher et al.,

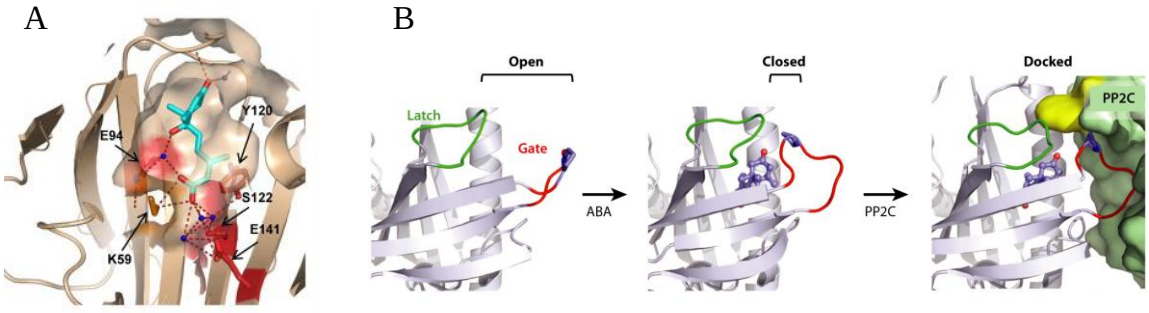

Figure 1.7 ABA binding in the PYR/PYLs cavity. (A) ABA (in light blue) buried in the PYR1 cavity. Lys59 (orange sticks) located at the bottom of the cavity, establishes a direct polar contact between its amine group and the carboxyl group of ABA. Interactions of Glu94, Glu141, Ser122 and Tyr120 (red sticks) with the carboxyl group are established through hydrogen bonds mediated by water molecules, from Santiago et al., 2012 (B) PYR/PYL proteins possess an open conformation of the gate and latch loops (*red* and *green*, respectively) that flank the ABA-binding pocket. Binding of ABA induces closure of the gate and latch, which in turn creates the interaction surface that recruits docking of type 2 C protein phosphatases (PP2C) onto the ABA-bound receptors, from Cutler et al., 2010.

2009; Dupeux et al., 2011b). Mutation of the tryptophan lock residue abolishes ABA dependent PYR/PYL-mediated PP2C inhibition (Miyazono et al., 2009; Dupeux et al., 2011a). Thus, the *Arabidopsis hab1*^{W385A} dominant allele has strong ABA-insensitive phenotypes, which reveals the relevance of this residue to form ternary receptor-ABA-phosphotase complexes (Dupeux et al., 2011a).

Key residues of the PP2C that establish contact with the receptor gating loops are: G246 in HAB1/G180 in ABI1 and the Mg^{2+} coordinating E203 in HAB1/E142 in ABI1. They establish hydrogen bonds with the conserved serine residue (S89/S112/S85 in PYL2/PYL1/PYR1, respectively) of the gate loop. This interaction inhibits PP2C activity by blocking substrate entrance to the catalytic site (Miyazono et al., 2009; Yin et al., 2009; Melcher et al., 2009; Dupeux et al., 2011a). Phosphatase mutants G246D/G180D/G168D in HAB1, ABI1, ABI2, respectively, escape from ABA-dependent receptor-mediated inhibition leading to subsequent inhibition of downstream regulated SnRK2s, even in the presence of ABA. Therefore, *abi1*^{G180D}, *abi2*^{G168D} and *hab1*^{G246D} are considered hypermorphic mutations in the presence of ABA that cause strong insensitivity to ABA *in planta* (Robert et al., 2006; Santiago et al., 2012). Thus, *abi1-1D (abi1*^{G180D}*)* and *ab2-1D (abi2*^{G168D}*)* are strong ABA-insensitive mutants that show diminished response to ABA in seeds and vegetative tissues, in contrast to loss-of-function alleles, which show ABA-hypersensitivity phenotype (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998; Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004; 2006)

The crystal structure of the SnRK2.6-HAB1 complex shows that the PP2C-ABA-PYR/PYL complex mimics the interaction between PP2C and SnRK2, making these interactions mutually exclusive. Thus, the conserved serine residue in the gate loop of

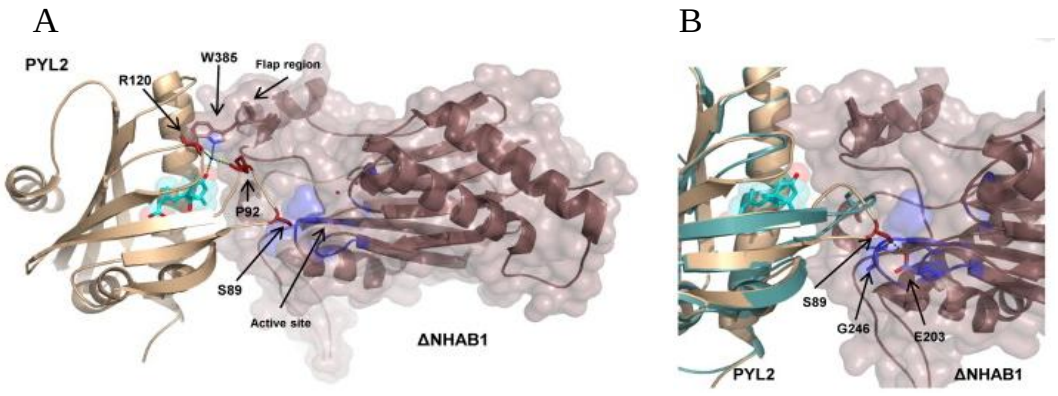

Figure 1.8 Structural details of the PYL2-ABA-HAB1 complex. (A) Overview of contact points between PYL2 and the catalytic core of HAB1. The PP2C contacts the receptor through its active site and the flap region containing the Trp385 residue. Detail of the interactions involving this residue, the PYL2 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. From Santiago et al., 2010

PYR/PYL mimics the phosphorylated Ser175 of SnRK2.6, which is the normal substrate of the phosphatase. Therefore, ABA-PYR/PYL acts as a competitive inhibitor at the substrate-binding site of the phosphatase (Ng et al., 2001; Dupeux et al., 2011a; Soon et al., 2012) (Figure 1.8).

Crystal structure of subfamily I of ABA receptors (Melcher et al., 2009; Nishimura et al., 2009; Santiago et al., 2009a; Yin et al., 2009) (PYR1, PYL1 and PYL2) showed they form dimers in solution in the absence of ABA yet they bind to PP2Cs as monomers with 1:1:1 (receptor:ABA:PP2C) stochiometry. Biochemical and structural data indicate that receptor family members present differences in their *oligomeric state*, which classifies them (in absence of ABA) in two subclasses, monomeric and dimeric receptors. Thus, receptors such as PYR1, PYL1, PYL2 and PYL3 form homodimers and PYL4, PYL5, PYL6, PYL8 and PYL10 are monomeric (Dupeux et al., 2011b; Hao et al., 2011). This observation suggests a model in which ABA binding dissociates dimeric PYR/PYLs and leads to the interaction of the monomeric form with PP2C proteins (Yin et al., 2009). The receptor dimerization region overlaps with the PP2C interaction surface, which suggests that dimer formation is a mechanism to prevent constitutive interaction with PP2Cs and ABA-induced dissociation is required for interaction with PP2C (Dupeux et al., 2011b). This monomer/dimer equilibrium found between PYR1/PYL family members can be explained by differences in their ABA-affinities. Dimeric receptors display 50-100 fold lower intrinsic affinities for ABA than the monomeric forms. In fact, the Kd of the monomeric receptors is approximately 1 µM (Santiago et al., 2009b; Szostkiewicz et al., 2010; Dupeux et al., 2011a; 2011b) while Kd of the dimeric receptors is > 50 µM (Santiago et al., 2009b; Miyazono et al., 2009; Dupeux et al., 2011b) (Figure 1.9A). In addition, one of the dimeric receptors, PYL3, has the unusual property that after ABA binding, it changes from the common cis-homodimer to a trans-dimer before complete dissociation (Zhang et al., 2012).

Since the discovery of ABA receptors, one of the most studied questions was the inhibitory effects of PYR/PYL/RCARs on clade A phosphatase activity. In absence of ABA, preliminary *in vitro* characterization suggested that dimerization in receptors like PYR1, PYL1, and PYL2 prevents basal interactions with the PP2Cs, whereas some monomeric receptors may bind to PP2Cs in the absence of ABA (Ma et al., 2009; Fujii and Zhu, 2009; Santiago et al., 2009b; Nishimura et al., 2010; Hao et al., 2011; Sun et al., 2012). However, in absence of ABA, these complexes between monomeric PYR/PYL/RCAR receptors and PP2Cs have a low affinity, whereas it increases to the nanomolar range in ABA-receptor-PP2C ternary complexes. Both dimeric and monomeric receptors in presence of ABA form high-affinity complexes with PP2Cs (Kd between 30-60 nM). In the absence of PP2Cs, monomeric receptors bind ABA with Kd of circa 1 µM, whereas dimeric receptors show Kd > 50 µM (Ma et al., 2009; Santiago et al., 2009b) (Figure 1.9A). As a result, only in presence of ABA PYR/PYLs can inhibit efficiently PP2Cs. Finally, in two recent studies, it has been reported that one of the members of

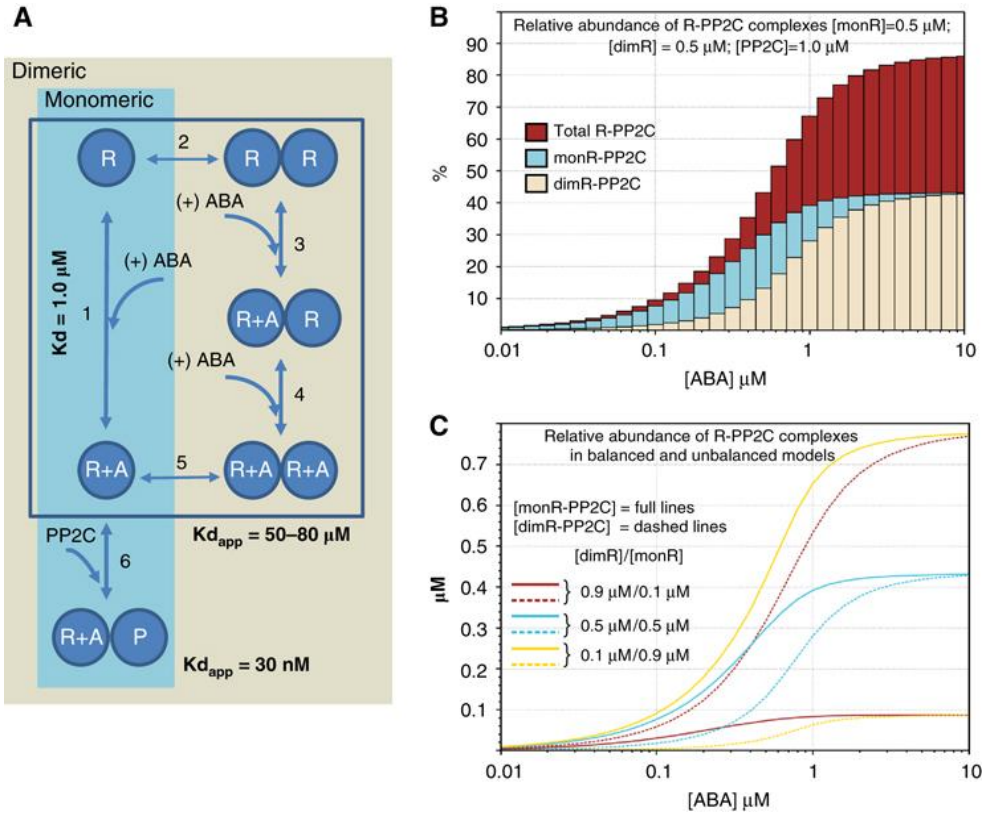

Figure 1.9 Model of the abscisic acid receptor activation process. (A) The reaction equilibria leading to the formation of receptor–ABA–PP2C ternary complexes for monomeric and dimeric PYR/PYL/RCAR proteins considered in the modelling study are indicated. The apparent Kds for the reactions measured experimentally (formation of receptor–ABA and receptor–ABA–phosphatase complexes) are indicated, and were used to parameterize the model. This model was used to explore the response to ABA when both monomeric and dimeric PYR/PYL/RCAR proteins compete for the same pool of ABA and PP2C molecules. (B) Results corresponding to a balanced model with dimer dissociation constant of 0.05 µM. The amount of total receptor–ABA–PP2C ternary complexes (red) as well as the proportion of ternary complexes formed with monomeric (monR-PP2C, cyan) and dimeric receptors (dimR-PP2C, brown) are indicated. (C) Activation of dimeric and monomeric receptors in balanced and unbalanced models. The concentration of ternary complexes formed by dimeric (dimR-PP2C dashed lines) and monomeric (monR-PP2C full lines) receptors at different ABA concentrations is indicated for situations involving different proportions of monomeric and dimeric receptors (as indicated). The dimer dissociation constant and the total receptor and PP2C concentrations were as in (B). As can be appreciated, these results indicate that overexpression of dimeric receptors can compensate for their thermodynamic disadvantage, and that the relative abundance of monomeric receptors has a strong influence on the concentration of ABA at which activation of dimeric receptors From Dupeux et al., 2011b

PYR/PYL/RCAR family, PYL13, because of changes in conserved amino acid residues involved in ABA binding does not bind ABA, but it is able to interact with and inhibit some members of clade A PP2Cs (PP2CA in particular) in an ABA-independent manner (Li et al., 2013; Zhao et al., 2013). In contrast, Fuchs et al. found no evidence for this claim and provided evidences that PYL13, as *bona fide* ABA receptor inhibits PP2CA, ABI2, and ABI1 only in the presence of ABA (Fuchs et al., 2014). Likewise, the recent work of Li et al. (2015) shows that the putative ABA-independent effect of PYL10 to inhibit PP2Cs was an artifact due to the presence of BSA in the reaction mixture (Li et al., 2015).

The existence of two distinct subclasses of PYR/PYLs ABA receptors, monomeric and dimeric receptors, with distinct properties suggests that they might have specialized function and contribute differentially to ABA signaling responses when they are considered in the context of the plant. Taking into account that both monomeric and dimeric receptors are expressed in the same cells, they may compete for the same pool of ABA and PP2Cs and form the ternary complexes. Thus, it has been proposed, through mathematical modeling of the receptor activation process that high-affinity monomeric receptors compete better than ones dimeric for low ABA concentrations and, therefore, might inhibit preferentially at low ABA concentrations (< 1µM). Thus, they might play a role for regulation of developmental processes related to plant survival and growth under non stress situations. The low-affinity dimeric receptors need higher ABA concentrations to dissociate, what suggests that they might play a crucial role under stress situations, where ABA levels increase between 10-40 fold (Dupeux et al., 2011b) (Figure 1.9B).

1.2.1.3 Combinatorial interaction of the ABA signalosome.

As described above, the current core model of ABA signaling is formed by three components: PYR/PYL/RCAR ABA receptors, group A PP2Cs and ABA-activated SnRK2s. The characterization of these core components has revealed a simple mechanism of action, which is based on a phosphorylation-dependent cascade. However, the existence of gene families encoding *Arabidopsis* 14 PYR/PYL/RCARs, 9 PP2Cs and 3 essential ABA-activated SnRK2 suggests a sophisticated fine-tuning modulation of ABA response (Ma et al., 2009, Park et al., 2009, Umezawa et al., 2009), which allow a combinatorial control of ABA signaling. Taken together, there are up to 126 (14x9) possible combinations for the assembly of potential PYR/PYL-ABA-PP2Cs complexes. However, *in vivo* experimental evidence for the existence of multiple PP2Cs interacting with a single ABA receptor was lacking at the beginning of this work. Additionally, there are up to 378 (126x3) regulatory combinations that might control ABA-activated SnRK2s, which in turn regulate numerous downstream targets such as plasma membrane proteins or transcriptional factors (e.g. AREB/ABFs). *In vivo*, this combinatorial variation in the ABA signalosome might be regulated by multiple determinants. First, differences between PYR/PYL proteins such as ABA sensitivity, oligomeric state, and basal activation levels as well as different interaction selectivity depending of PP2C partner might affect the formation of signaling PYR/PYL-ABA-PP2C complexes and their sensitivity to varying cytosolic phytohormone concentrations (Szostkiewicz et al., 2010). Second, transcriptional profiling showed that different members of the PYR/PYL/RCAR, PP2C and SnRK2 gene families were differentially expressed in different tissues and at different developmental stages (Rubio et al., 2009; Gonzalez-Guzman et al., 2012). This fact determines

spatio-temporal limitations for different combinations. Third, ABA-related stress conditions alter the transcript levels of distinct core elements resulting in altered sensitivity and plasticity of the response. As described above, under ABA treatment the expression of PYR/PYL/RCAR genes generally is repressed and PP2C genes are strongly induced (Santiago et al., 2009b; Gonzalez-Guzman et al., 2012), what suggests PYR/PYL/RCAR and PP2C are important not only in stress response but also in the recovery when ABA levels decay (Santiago et al., 2009b; Gonzalez-Guzman et al., 2012). Fourth, different PYR/PYLs, PP2Cs and SnRK2s are known to have selective protein–protein interactions (Umezawa et al., 2009; Antoni et al., 2013), which may modulate ABA responses, for instance, by targeting ABA signaling at different subcellular locations.

In summary, the effect of this combinatorial regulation on fine-tuning of ABA transduction, spatio-temporal determinants, stress-responsive gene expression patterns, subcellular localization as well as preferences in protein–protein interactions might be important issues in future studies. Eventually, it might lead to biotechnological applications through regulated activation/inactivation of key elements of the ABA pathway.

1.2.2 Clade A type 2C protein phosphatases (PP2C)

1.2.1.1 Clade A PP2Cs type as negative regulators of ABA signaling

Protein phosphatases type 2C (PP2Cs) are the best characterized group of protein serine/threonine (Ser/Thr) phosphatases involved in ABA signaling. Owing to the fact that the reversible protein phosphorylation/dephosphorylation is a key step of signal transduction and regulation of ABA signaling pathway, PP2Cs are considered with their protein kinase partners as a key element of the ABA response (Hirayama and Shinozaki, 2007).

The PP2Cs are monomeric enzymes that require Mg^{+2} or Mn^{+2} for their activity (Cohen, 1989; Rodriguez et al., 1998). In *Arabidopsis*, there exist 76 PP2C genes clustered in 10 groups (A-J), but only members of the group A have been implicated in the ABA signaling pathway (Schweighofer et al., 2004). This group contains nine members: ABI1, ABI2, HAB1, HAB2, ABA-HYPERSENSITIVE GERMINATION 3 (AHG3/ AtPP2CA), AHG1, and the recently characterized HIGHLY ABA-INDUCED 2 (HAI2)/ AKT1 INTERACTING PROTEIN 1(AIP1), HAI3/ AIP1 HOMOLOGS 1(AIPH1) and HAI1/AIPH2. All of them show a highly conserved catalytic core with different N-terminal extensions, and most of them have been shown to interact with PYR/RCAR receptors in presence of ABA.

A genetic screen in *Arabidopsis* for ABA insensitive mutants identified *abi1-1D* and *abi2-1D* alleles (Koornneef et al., 1984). Later, following a map-based positional cloning approach, ABI1 and ABI2 were characterized as highly homologous members of the PP2C

family of Ser/Thr protein phosphatases (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). The *abi1-1D* and *abi2-1D* alleles, encode dominant mutations of ABI1 and ABI2 described previously as $abi1^{G180D}$, $abi2^{G168D}$, with analogous amino acid substitution in their catalytic domains (conserved Gly residue to Asp), which causes a diminished phosphatase activity in the absence of ABA as compared to wt (Santiago et al., 2012). These hypermorphic enzymes repress ABA response and lead to strong insensitivity to ABA in seed germination, root growth, stomatal regulation and gene expression responses (Leung et al., 1994; 1997; Meyer et al., 1994; Rodriguez et al., 1998; Gosti et al., 1999). The identification and physiological characterization of loss-of-function alleles *abi1-1R1–abi1-1R7* of the ABI1 (Gosti et al., 1999) and *abi2-1R1* of the ABI2 (Merlot et al., 2001) provided the first evidence that these proteins act as negative regulators in the ABA signaling pathway. This finding was confirmed through the analysis of T-DNA mutants of HAB1, ABI1 and ABI2 genes (Saez et al., 2004; 2006; Rubio et al., 2009). Later on, additional members of this clade of PP2Cs have been identified: HAB2 (Saez et al., 2004; 2006), AHG1 (Nishimura et al., 2007), and AHG3/AtPP2CA (Cherel et al., 2002; Kuhn et al., 2006; Yoshida et al., 2006). Loss-of-function mutants of these PP2Cs exhibited ABA hypersensitive phenotypes with different strengths in germination, growth, stomatal closure and gene expression, supporting a role as major negative regulators of the ABA signaling pathway (Hirayama and Shinozaki 2007). In contrast, over-expression of PP2Cs induces ABA insensitivity in seed germination and growth as well as increased transpiration rates mainly due to reduced stomatal closure. (Saez et al., 2004: Khun et al., 2006). Concerning HAI1, HAI2, HAI3, based on microarray analysis and genetic characterization, it was suggested that they are redundant positive regulators of ABA response at germination, but negative regulators of post-germination drought response (Fujita et al., 2009; Bhaskara et al., 2012). However, more recent report demonstrated that one of them, HAI2, also is a negative regulator during seed germination (Kim et al., 2013). On the other hand, the high gene expression induction under osmotic stress of these three PP2Cs, and increased Pro and osmoregulatory solute accumulation at low Ψw suggest a predominant role of HAI1, HAI2 and HAI3 on osmotic adjustment under water stress (Fujita et al., 2009; Bhaskara et al., 2012).

It has been observed that some of the single loss-of-function mutants have weak ABA-hypersensitive phenotype (*hab1-1, abi1-2, abi2-2*), but double or triple PP2C loss-of-function mutants show an elevated hypersensitivity to ABA, which reflects partial overlapping function between them (Gosti et al., 1999; Khun et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007; Rubio et al., 2009). It suggests that integrated action of these genes acts as a fine-tuning modulation in ABA response as well as in development and plant growth processes (Saez et al., 2006; Nishimura et al., 2007, Rubio et al., 2009). However, functional hierarchy between PP2Cs can be established according to gene expression levels, tissue-specific expression

patterns and different preferences for interacting partners such as upstream PYR/PYL receptors as well as different downstream targets (Yoshida et al., 2006; Nishimura et al., 2007; Umezawa et al., 2009; Rubio et al., 2009).

1.2.2.1 Targets of clade A PP2Cs

Given the central role of PP2Cs in ABA signaling, identification of their protein interactors and substrates is crucial to better understand their mechanism of action. Among the targets of PP2Cs, the protein kinases (PKs) are well known substrates, such as calcium-independent kinases: SUCROSE NON-FERMENTING 1 (SNF1)-RELATED PROTEIN KINASEs from subfamily 1 and 2 (SnRK1s and SnRK2s) as well as calcium-regulated: CALCIUM-DEPENDENT PROTEIN KINASEs (CDPKs/CPKs) and SnRK3s/ CALCINEURIN B-LIKE PROTEIN (CBL) INTERACTING PROTEIN KINASES (CIPKs). In presence of ABA, PP2Cs are inhibited by PYR/PYL ABA receptors, which allows the phosphorylation and activation of PKs and downstream target proteins such as transcriptional factors, chromatin remodeling components and plasma membrane targets such as ion channels (i.e. QUICK ANION CHANNEL1 (QUAC1), SLOW ANION CHANNEL-ASSOCIATED1(SLAC1), K^+ influx CHANNEL *Arabidopsis thaliana* 1 (KAT1), K^+ UPTAKE PERMEASE 6, 8 proteins (KUP 6, 8)), PLASMA MEMBRANE PROTON ATPASES (PM H^+-ATPase) and NADPH oxidases, which are responsible for stomatal closure under water stress. Thus, it has been demonstrated that PP2Cs regulate protein kinase substrates indirectly or directly (Lee et al., 2007; Geiger et al., 2009; 2010; Antoni et al., 2012; Lynch et al., 2012; Demir et al., 2013).

- *Protein kinases*

Various stress-inducible PKs such as SnRKs and CDPK/CPKs families are implicated in ABA signaling as direct substrates of PP2Cs. The SnRKs form three subgroups based on sequence similarity and domain structure, SnRK1, SnRK2 and SnRK3 (Hrabak et al., 2003). The SnRK1 and SnRK2 subfamilies form the group of calcium-independent kinases. The SnRK3 protein subfamily together with CDPK/CPK family are calcium regulated PKs through interaction with calcium sensors (CBLs) or through the calmodulin domain, respectively.

- *Calcium-independent protein kinases: SnRK1s and SnRK2s*

The group of SnRK2s in *Arabidopsis* is formed by 10 members that are classified into three subclasses: subclass 1, activated by osmotic stress but not ABA, subclass 2, activated by osmotic stress and weakly by ABA and the subclass 3 that are activated by both ABA and

osmotic stress. Subclass 3 SnRK2s is represented by SnRK2.2, SnRK2.3, and SnRK2.6/OST1, all of them strongly activated by ABA (Boudsocq et al., 2004). The first reports of SnRK2s involved in ABA signalling were the wheat PKABA1 and the AAPK of *Vicia faba* (Gomez-Cadenas et al., 1999; Li et al., 2000). SRK2E/Open STOMATA1 (OST1)/SnRK2.6 was the first putative orthologous of AAPK identified in *Arabidopsis* by genetic screening for mutants defective in stomatal closure based on thermal imagining of drought stressed plants (Merlot et al., 2002). This *ost1*/*snrk2.6* mutant showed reduced stomatal responses to ABA and to mild drought conditions, but not to light and CO_2 suggesting that OST1/SnRK2.6 could be specifically involved in ABA signaling as a positive regulator (Mustilli et al., 2002; Yoshida et al., 2002). Later, genetic evidence showed that SnRK2.6 is expressed to high levels in guard cells but to low levels in other tissues, which explains why *ost1/ snrk2.6* single mutant was impaired only in ABA-mediated stomatal closure and not other ABA responses. SnRK2.2 and SnRK2.3 show an overlapping expression pattern and high functional redundancy between them, which explains why *snrk2.2* and *snrk2.3* single mutants do not have clear ABA response phenotypes, while *snrk2.2*/*2.3* double mutant is insensitive to ABA in seed dormancy, seed germination and seedling/root growth assay but shows weak water loss phenotype (Fujii et al., 2007). Interestingly, the triple knockout mutant, *snrk2.2/2.3/2.6*, completely blocks all ABA responses resulting in severe ABA-insensitive phenotypes in stomatal closure, water loss, germination, root and shoot growth or gene expression. Additionally, it is also impaired in growth and seed production. Thus, the phenotype of the *snrk2.2*/*2.3*/*2.6* triple mutant indicates the ABA signaling pathway converges at these 3 protein kinases which act as redundant positive regulators in ABA signaling (Yoshida et al., 2002; 2006; Fujita et al., 2009; Nakashima et al., 2009; Fujii and Zhu, 2009).

Structurally, SnRK2s contain N-terminal highly conserved kinase domain, and a C-terminal variable regulatory domain. This regulatory domain can be divided in two subdomains: domain I, which is conserved among all SnRK2s and domain II, conserved among ABA-responsive SnRK2s, which is responsible for their differential activation by ABA and interaction with clade A PP2Cs (Yoshida et al., 2006; 2010). Yeast two hybrid and structural analysis indicate that subclass 3 of SnRK2s interact with PP2Cs not only at the C-terminal domain II, but also at the catalytic kinase domain (Yoshida et al., 2006; Vlad et al., 2009; Soon et al., 2012). In the absence of ABA, PP2Cs interact with SnRK2.2, SnRK2.3, and SnRK2.6 (Yoshida et al., 2006; Umezawa et al., 2009; Fujii and Zhu, 2009), leading to kinase inactivation by dephosphorylation of a serine residue highly conserved in the kinase activation loop (Ser175 in SnRK2.6/OST1) (Yoshida et al., 2006; Park et al., 2009; Ma et al., 2009; Umezawa et al., 2009; Yin et al., 2009; Vlad et al., 2009; Soon et al., 2012). In the presence of ABA, PYR/PYLs bind to and inhibit PP2Cs. This leads to SnRK2 activation by the phosphorylation of several Ser/Thr residues in the activation loop including the Ser175 and the subsequent de-repression

of the signaling pathway by direct phosphorylation of downstream targets (Park et al., 2003; Ma et al., 2009; Umezawa et al., 2009; Lee et al., 2009; Fujii and Zhu, 2009). In fact, the triple mutant *abi1-2/hab1-1/pp2ca-1* exhibits partial constitutive activation of SnRK2.2, 2.3 and 2.6 (Fujii and Zhu, 2009) and the sextuple mutant, *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8*, presents lack of ABA-mediated activation of SnRK2s (Gonzalez-Guzman et al., 2012). Thus, the ABA-dependent activation of SnRK2.2, 2.3 and 2.6 kinases depends on the inhibition of PP2Cs by PYR/PYL ABA receptors. In addition, activation of SnRK2s involves autophosphorylation and phosphorylation by upstream kinases, such as the recently described GLYCOGEN SYNTHASE KINASE 3 (GSK3)-like kinases (Boudsocq et al., 2007; Cai et al., 2014).

SnRK2.2, 2.3 and 2.6 kinases are central elements of the ABA signalling pathway. Therefore, identification of their substrates has been a key research issue. Three recent phosphoproteomic studies have identified potential SnRK2 substrates *in vivo*, which sheds light on the role of the SnRK2 protein kinases and downstream effectors of ABA action (Kline et al., 2010; Umezawa et al., 2013; Wang et al., 2013). These results confirm that AREB/ABFs are the main substrate transcription factors involved in stress-responsive gene expression downstream of SnRK2.2/2.3/2.6. Other target proteins are implicated in the direct stomatal control (Kline et al., 2010; Umezawa et al., 2013; Wang et al., 2013) (see later section *Membrane targets*).

In addition to SnRK2s, SNF1-related kinases 1 (SnRK1) have been recently identified as interacting partners and substrates of clade A PP2Cs (Rodrigues et al., 2013). SnRK1s (SnRK1.1 (KIN10)/1.2(KIN11)/1.3 in *Arabidopsis*) and the plant target of rapamycin (TOR) kinase are central regulators that link growth and development to carbon nutrient and energy status (Smeekens et al., 2010; Robaglia et al., 2012). While plant TOR promotes growth in response to high sugar levels (Deprost et al., 2007), SnRK1 activity rises dramatically during sugar deficit. SnRK1 is a metabolic sensor that can decode energy deficiency signals and induce an extensive metabolic reprogramming being essential in development and for plant survival under stress conditions (Baena-Gonzalez, 2010). Recently, Baena-González's group have provided molecular, genetic, and physiological evidence for the role of two clade A PP2Cs, ABI1 and PP2CA (AHG3), as negative regulators of SnRK1 signaling in Arabidopsis through their direct interaction with the SnRK1 α-catalytic subunit, its dephosphorylation and subsequent inactivation (Rodrigues et al., 2013). Accordingly, SnRK1 repression is abrogated in double and quadruple *pp2c* knockout mutants, provoking, similarly to SnRK1 overexpression, sugar hypersensitivity during early seedling development. The activation of SnRK1 leads to phosphorylation of transcription factors and downstream targets implicated in transcriptional stress response. In fact, reporter gene assays and SnRK1 target gene expression analyses further demonstrate that PP2C inhibition by ABA results in SnRK1 activation, promoting SnRK1 signaling during stress and once the energy deficit subsides. Consistent with this, SnRK1 and ABA induce largely overlapping transcriptional responses. Hence, the PP2C hub allows the

coordinated activation of ABA and energy signaling, strengthening the stress response through the cooperation of two key and complementary pathways (Rodrigues et al., 2013).

- *Calcium-regulated protein kinases*

Ca^{2+} serves as an intracellular messenger in many signaling pathways, including ABA responses (Leung and Giraudat, 1998; Allen et al., 2000; 2001). Decoding of Ca^{2+} signaling by a large number of Ca^{2+} sensor proteins is essential in early steps of ABA signal transduction. In this context, two major groups of Ca^{2+} sensor proteins exist in *Arabidopsis:* Ca^{2+}-DEPENDENT PROTEIN KINASES (CDPKs/CPKs) and CALCINEURIN B-LIKE PROTEIN (CBL) INTERACTING PROTEIN KINASES (CIPKs/ SnRK3s).

The *Arabidopsis* genome encodes 34 CDPK/CPK isoforms. Structurally, CPK are composed by a Ser/Thr protein kinase domain, an autoinhibitory domain, and calmodulin-like domain with EF-hand motifs (Hrabak et al., 2003). At basal Ca^{2+} levels, kinase domain is physically blocked by autoinhibitory domain, but upon increased intracellular Ca^{2+} levels, Ca^{2+} binding to the calmodulin-like domain allows displacement of the autoinhibitory domain and following activation of the kinase, resulting in activation of specific target proteins (Ludwig et al., 2004). Several CPKs have been implicated in ABA response with differential physiological functions according to their cellular localization and downstream targets (Boudsocq and Sheen, 2013). Some of them, such as CPK3, CPK6, CPK21 and CPK23, are plasma membrane targeted proteins mainly expressed in guard cells. They are implicated in regulation of ABA-mediated stomatal opening/closure through S-type anion channels (SLAC1, SLAH3) and Ca^{2+}-permeable channels in response to ABA (Mori et al., 2006; Geiger et al., 2010; 2011; Brandt et al., 2012; Demir et al., 2013). In fact, *cpk3/ cpk6* single and double mutants present decreased ABA-induced stomatal closure (Mori et al., 2006) whereas, surprisingly, *cpk21* and *cpk23* mutants do not exhibit any stomata phenotype (Geiger et al., 2011) probably due to the functional redundancy with other kinases such as SnRK2s (Boudsocq and Sheen, 2013). On the other hand, other CPKs, such as CPK4, CPK10, CPK11, CPK30 and CPK32, are nuclear localized protein implicated in ABA-induced transcriptional reprogramming through phosphorylation of ABFs, which have been identified as their substrates (Choi et al., 2005; Zhu et al., 2007). ABA insensitivity in seed germination, seedling growth, stomatal closure and increased drought sensitivity were reported in the *cpk4* and *cpk11* single and double mutants, which provided genetic evidence on their role in ABA response (Zhu et al., 2007). Moreover, different genetic and biochemical studies have allowed establish a connection between clade A PP2Cs and several CDPKs. It has been reported that some PP2Cs, such as ABI1 and ABI2, interact and inhibit CDPK activities, CPK10, CPK30, CPK21 and CPK23 in regulation of anion channels (Sheen, 1996; 1998; Geiger et al., 2010; 2011). On the other hand, addition of ABA and PYR/PYLs proteins results in inactivation of PP2Cs and of CPKs (Geiger et al., 2011), which

suggests a direct ABA-dependent regulation of CPKs activity by the PYR/PYL–PP2C ABA complex.

The second group, SnRK3/CIPK, represents a subfamily of SnRK family. SnRK3/CIPK kinases become activated by interaction with Ca^{2+}-sensor CBL proteins (Luan et al., 2009; Kudla et al., 2010) The *Arabidopsis* genome encodes 26 putative CIPK and 10 CBL proteins which form multiple and specific subsets of CBL-CIPK complexes involved in responses to several stresess, such as salt, cold, drought stress (Pandey et al., 2004; D'Angelo et al., 2006; Pandey et al., 2007; Batistic and Kudla, 2009; Luan, 2009; Li et al., 2009; Hashimoto and Kudla, 2011). To date, the well known targets of CBL–CIPK complexes are ion channels and transporters related to the influx or efflux of various ions, as Na^+, K^+, Ca^{2+}, NO^{3-} and H^+ (Xu et al., 2006; Ho et al., 2009; Held et al., 2011; Batistic and Kudla, 2012; Drerup et al., 2013). For instance, one of the first targets identified was the plasma membrane Na^+/H^+ antiporter SOS1, which is phosphorylated and activated by CBL4–CIPK24 (originally named as SOS3–SOS2), thereby being the CBL4-CIPK24 complex a key determinant element for salt tolerance in *Arabidopsis* (Shi et al., 2000; Quintero et al., 2002; D'Angelo et al., 2006).

Structurally, all CBL proteins share a conserved core region consisting of four EF-hand Ca^{2+} binding sites (Nagae et al., 2003; Kolukisaoglu et al., 2004). All CIPKs are composed by both conserved N-terminal kinase domain and C-terminal regulatory domain that includes a highly conserved NAF (Asn-Ala-Phe)/FISL (Phe-Ile-Ser-Leu) motif and a conserved protein phosphatase interaction (PPI) motif. Upon elevation of cellular Ca^{2+}concentration, CBL-Ca^{2+} proteins interact with NAF motif of CIPKs, which release kinase activity leading to the kinase activation and phosphorylation of downstream targets (Guo et al., 2001; Gong et al., 2002; Akaboshi et al., 2008). It has been reported that CBL proteins, in addition to the activation of CIPK activity, are also important to determine the localization of the CBL/CIPK complexes modulating their targets and functions (D'Angelo et al., 2006; Cheong et al., 2007; Batistic et al., 2010). On the other hand, the PPI domain mediates CIPK interaction with clade A PP2Cs such as ABI1, ABI2 and PP2CA/AHG3 (Ohta et al., 2003; Lee et al., 2007; Lan et al., 2011; Lyzenga et al., 2013). Crystallization studies of CBL4-CIPK24 complex suggest that PP2C interaction with the PPI domain of CIPKs would lead to displacement of the CBL protein from NAF domain, thus preventing the formation of trimeric complexes (Sanchez-Barrena et al., 2007). Competitive formation of either CBL/CIPK or CIPK/PP2C complexes would allow rapid control of the phosphorylation status of downstream target proteins (Kudla et al., 2010). Moreover, it has been reported that some knockout CIPK mutants exhibit ABA-hypersensitive phenotype in ABA seedling establishment assays, which confirms some CIPKs function as negative regulators of ABA signaling. For example, the CBL9–CIPK3 complex negatively regulates the ABA response during seed germination and silencing of CIPK15 gene by RNA interference confers plants hypersensitivity to ABA in seed germination, seedling growth,

stomatal closing and gene expression (Guo et al., 2002; Pandey et al., 2008). However, in the case of CIPK26 which interacts with the bZIP transcriptional factor ABI5, it plays a positive role in ABA signaling during the seed germination (Lyzenga et al., 2013). These results suggest that the effect of CBL-CIPK complexes depends on their downstream targets on ABA signaling.

- *Transcriptional regulators*

ABA signaling leads to large changes in gene expression in which transcriptional regulatory networks play an essential role (reviewed in Cutler et al., 2010). ABA-responsive gene expression is directly regulated by transcription factors (TFs) which, in turn, are controlled by the activity of ABA receptors, secondary messengers, protein kinase/phosphatase cascades and chromatin-remodeling factors.

Among TF targets of clade A PP2Cs identified up to date, the basic leucine zipper transcription factors (bZIP TFs), known as ABA-responsive element (ABRE) binding factors (AREB/ABFs) family, have been shown to play a central role in regulation of ABA-mediated gene expression in stress response (reviewed in Fujita et al., 2011, 2013). The first member identified and characterized was ABA-INSENSITIVE 5 (ABI5) and analysis of *abi5* loss-of-function mutant has revealed a key role as positive regulator of ABA signaling during seed maturation, germination and early seedling growth (Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Finkelstein et al., 2002; Lopez-Molina et al., 2002; Finkelstein et al., 2005). Other ABFs with partly redundant functions, such as ABF1, AREBB1/ABF2, ABF3, AREB2/ABF4, were characterized as positive regulators of ABA signaling in vegetative tissues (Fujita et al., 2005; Yoshida et al., 2010; 2015). Transcriptional activity of AREB/ABF TFs requires ABA-dependent phosphorylation, which highlights the essential role of kinases in their regulation (Fujita et al., 2005; Yoshida et al., 2010). Among these upstream kinases, the main regulators are SnRK2s (SnRK2.2/2.3/2.6) (Fujii and Zhu, 2009; Umezawa et al., 2010) as well as calcium-regulated CPKs (Zhu et al., 2007) and CIPKs (Hong et al., 2011; Lyzenga et al., 2013). On the other hand, AREB/ABFs are direct targets of clade A PP2Cs. The first evidence that AREB/ABFs are PP2Cs direct substrates was provided by Antoni et al. (2012) through *in vitro* kinase assays where two PP2Cs, AHG1 and AHG3, efficiently dephosphorylated ABF2, whereas coincubation with PYL8 in the presence of ABA abolished their activity (Antoni et al., 2012). Later on, Lynch et al., trough yeast two hybrid and BiFC assays, confirmed interaction between several AREB/ABFs (ABF1, ABF3,ABI5) and PP2Cs (AHG1 and AHG3) *in vivo* (Lynch et al., 2012).

Downstream regulated genes of the AREB/ABFs overlap with those regulated by SnRK2.2/2.3/2.6. They represent one-third of ABA-responsive genes downregulated in the *snrk2.2/2.3/2.6* triple mutant, which suggests that AREB/ABF TFs are key but not the only TFs

that regulate gene expression downstream of SnRK2.2/2.3/2.6 in ABA signaling (Fujita et al., 2009). For example, some members of the APETALA2-(AP2) domain family, such as ABA-INSENSITIVE 4 (ABI4), which are related to the DROUGTH RESPONSE ELEMENT BINDING (DREB) and ETHYLENE RESPONSE FACTOR (ERF) subfamilies and some members of B3-domain family, such as ABA-INSENSITIVE 3 (ABI3), are also involved in regulatory complex with some bZIP TFs. Thus, these different TFs present substantial overlap and some cross-regulation (Finkelstein and Somerville, 1990; Parcy and Giraudat, 1997; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002; Carles et al., 2002; Fujita et al., 2011; Moenke et al., 2012).

Additionally to AREB/ABFs, the class I homeodomain-leucine zipper (HD-ZIP) TFs has been characterized as direct targets of clade A PP2Cs. Two HD-ZIPs, ARABIDOPSIS THALIANA HOMEOBOX6 (ATHB6) and ATHB12 have been reported as direct interacting partners of ABI1 and ABI2 PP2Cs (Himmelbach et al., 2002; Valdes et al., 2012). Thus, constitutive over-expression of ATHB6/12 in transgenic *Arabidopsis* plants decreases their sensitivity to ABA during germination and stomatal closure, which suggests they act as negative regulators downstream of PP2Cs in the ABA signaling pathway (Soderman et al., 1996; Olsson et al., 2004). Moreover, ATHB6, ATHB7 and ATHB12 have been shown to act as positive transcriptional regulators of PP2C genes and negative regulators of genes encoding the ABA receptors PYL5 and PYL8 in response to ABA stimulus. Therefore they have been implicated in ABA signaling as part of negative feedback loop with the PP2Cs and PYR/PYL ABA receptors (Himmelbach et al., 2002; Elhiti and Stasolla, 2009; Valdes et al., 2012).

Finally, several chromatin-remodeling components have been identified as key regulators of ABA- or stress-inducible gene expression (reviewed in Chinnusamy et al., 2008). Among them, it has been shown that clade A PP2Cs, such as HAB1, PP2CA, ABI1 and ABI2, can directly interact *in vivo* with SWI3B, a subunit of the ATP-dependent chromatin remodeling complex (Saez et al., 2008). Analysis of ABA resistant *swi3b* mutants suggested that SWI3B act as a positive regulator of ABA signalling in seeds and vegetative tissues and is important for plant growth and development (Saez et al., 2008). Later on, characterization of BRAHMA (BRM) SWI/SNF ATPase mutants in different ABA responses allowed determine the role of the BRM/SWI/SNF chromatin-remodeling complex as a negative regulator of ABA signaling, acting as a repressor of ABA-responsive gene expression through the regulation of nucleosome stability in the ABI5 promoter (Han et al., 2012). However, the molecular mechanism whereby PP2Cs regulate SWI/SNF complexes remains unclear yet.

- *Membrane targets*

ABA signaling involves regulation of nuclear targets that modulate stress-responsive gene expression and membrane targets implicated in rapid physiological changes. These non-transcriptional changes are important for regulation of ion homeostasis and the control of stomatal movements (Kim et al., 2010). Several of the membrane targets were identified as direct kinase/phosphotase substrates. Among them, plasma membrane S- and R-type anion channels are key players implicated in the anion efflux required for membrane depolarization and ABA-mediated stomatal closure (Negi et al., 2008; Vahisalu et al., 2008; Kollist et al., 2011; Barbier-Brygoo et al., 2011; Roelfsema et al., 2012). For instance, regulation of slow S-type anion channels activity, mediated by SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) and SLAC1 HOMOLOGUE3 (SLAH3) is required for the efflux of chloride and nitrate from guard cells (Negi et al., 2008; Vahisalu et al., 2008). Thus, SLAC1 is phosphorylated and activated by SnRK2.6 and CPK23 in a calcium-independent manner and by CPK3/6/21 in a calcium-dependent manner, while SLAH3 is phosphorilated and activated by CPK3/6/ 21 and 23 but not by SnRK2.6 (Geiger et al., 2009; 2010; 2011; Brandt et al., 2012; Scherzer et al., 2012). On the other hand, channel activities can be abolished by clade A PP2Cs, such as ABI1, ABI2 and AHG3, via direct interactions and dephosphorylation of anion channels as well as by inhibition of the activating kinases (Geiger et al., 2009; Lee et al., 2009; Geiger et al., 2010; Demir et al., 2013). Recently, in addition to S-type anion channels, it has been shown that SnRK2s phosphorylate and activate rapid R-type anion channels, such as QUICK ANION CHANNEL 1 (QUAC1), which also can release organic anions (Meyer et al., 2010; Imes et al., 2013). Membrane depolarization caused by anion channels decreases activity of inward K^+ channels, such as K^+ CHANNEL *Arabidopsis thaliana* 1 (KAT1), KAT2, and activates outward K^+ channels, such as the GUARD CELL OUTWARD RECTIFYING K^+ channel1 (GORK1) and K^+ UPTAKE PERMEASE 6, 8 proteins (KUP6,8) (Ache et al., 2000; Hosy et al., 2003; Osakabe et al., 2013). ABA regulates through phosphorylation/dephosphorylation events the activity of these channels. For example, the inward K^+ channels KAT1, KAT2 are inactivated by SnRK2.6 and CPK10/13 phosphorylation (Sato et al., 2009; Zou et al., 2010; Ronzier et al., 2014), whereas outward K^+ channels, GORK1 and KUP6, are activated by SnRK2.6 phosphorylation leading to K^+ release and water efflux, which results in loss of turgor and final stomata closure (Osakabe et al., 2013).

ABA-dependent kinase/PP2C cascade is also essential in the regulation of other membrane targets implicated in different processes such as plant nutrition and maintenance of cell homeostasis. Among them, some inward K^+ channels, such as *Arabidopsis* K^+ TRANSPORTER2 (AKT1), the major root channel for uptake of K^+ from the soil (Gierth and Maeser, 2007) and AKT2/3, which regulates phloem loading and long-distance transport via modification of phloem potential (Deeken et al., 2002). For example, AKT1 is phosphorylated

and activated by the CBL1/9/10-CIPK23 complexes in a calcium-dependent manner under limiting K^+ conditions and inactivated by HAI2 dephosphorylation (Xu et al., 2006; Lee et al., 2007; Wang and Wu, 2013). On the other hand, CBL4-CIPK6 complex mediates translocation and activation of AKT2/3 at the plasma membrane, whereas interaction with AHG3 blocks its activity (Cherel et al., 2002; Held et al., 2011). Other well known targets of ABA signaling are PLASMA MEMBRANE PROTON ATPASES (PM H^+-ATPases) implicated in pH homeostasis in plant cell (Palmgren, 2001). It has been shown that during stomatal closure, ABA inhibits the PM H^+-ATPase activity by reducing its phosphorylation level to maintain membrane depolarization and prevent stomatal opening (Zhang et al., 2004; Hayashi et al., 2011). Thus, the *ost2-1* and *ost2-2* dominant mutants of ARABIDOPSIS H^+ATPASE1/OPEN STOMATA2 (AHA1/OST2), show increase in H^+-ATPase activity and strongly ABA-insensitive phenotypes in stomatal closure assays; *abi1-1* and *abi2-1* mutants show the same phenotype with increased H^+-ATPase activity (lack of ABA-mediated inhibition) suggesting regulation by clade A PP2Cs and downstream kinases (Roelfsema et al., 1998; Merlot et al., 2007). Finally, NADPH oxidases, key enzymes for ROS production, are direct targets of SnRK2.3/2.6, CBL1/9-CIPK26 and CPKs, which mediate phosphorylation-dependent activation of NADPH oxidase resulting in ROS production as a second messenger in early steps of ABA signaling (Kwak et al., 2003; Sirichandra et al., 2009; Boudsocq et al., 2010; Drerup et al., 2013).

1.2.3 Secondary messengers in ABA signal transduction

A number of well-characterized second messengers has been described in the ABA signaling pathway, such as Ca^{2+}, reactive oxygen species (ROS), cyclic ADP-ribose (cADP), cyclic GMP (cGMP), nitric oxide (NO), phospholipase D (PLD)-derived phosphatidic acid (PA), inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3/IP_3), inositol hexakisphosphate (IP_6) and sphingosine-1 phosphate (S1P) (Leckie et al., 1998; Schroeder et al., 2001; Coursol et al., 2003; Siegel et al., 2009; Hong et al., 2010; Hubbard et al., 2010; Kim et al., 2010; Lozano-Juste and Leon, 2010b).

Interestingly, several of these messengers lead to changes in cytosolic Ca^{2+} levels, which is essential in early steps of ABA signaling. In fact, ABA is known to induce an increase in [Ca^{2+}]cyt in the guard cells, directly associated with stomatal closure (Pei et al., 2000). The cytoplasmic Ca^{2+} concentrations ([Ca^{2+}]cyt) is maintained at sub-micromolar levels (ranging from 100 to 200 nM), while in response to stress stimuli it can be increased up to several micromolar through opening of plasma membrane and endomembrane-located calcium channels (Hetherington and Brownlee, 2004; Kudla et al., 2010; Dodd et al., 2010).

The spatial and temporal patterns of Ca^{2+} levels, the amplitude of the signal, and the frequency of Ca^{2+} oscillations are designated as "Ca^{2+} signatures" (Webb et al., 1996; Allen et

al., 2001). These diverse Ca^{2+} signatures have been proposed to be subsequently decoded by a large number of Ca^{2+} sensor proteins that effectively bind Ca^{2+} ions and transmit the Ca^{2+} signal by (i) interaction with target proteins and regulation of their activity, (ii) phosphorylation cascades, or (iii) transcriptional regulation (Luan et al., 2002; Sanders et al., 2002). For instance, increased ABA-induced [Ca^{2+}]cyt activates S-type and R-type anion channels, whereas inhibits inward-rectifying K^+ channels and H^+-ATPase activity, leading to stomatal closure (Schroeder and Hagiwara, 1989; Bouche et al., 2005; Kudla et al., 2010; Uraji et al., 2012; Ye et al., 2013). An extended set of Ca^{2+} sensors with varying Ca^{2+} affinities, subcellular localizations and downstream targets confers an additional degree of specificity to Ca^{2+} signaling in response to a wide range of environmental and developmental cues (reviewed in Bouché et al., 2005; Kudla et al., 2010). There are multiple families of Ca^{2+} sensor proteins, which contains either the calcium-binding EF hand domain (e.g. CALMODULINS (CaM), CBL and CDPK) or calcium-dependent lipid-binding domains (C2) (Clapham, 2007).

In addition to Ca^{2+}, ABA induces ROS and NO production, which also play an essential role in ABA signal transduction as second messengers. Elevation of ROS levels, generated by ABA-activated NADPH oxidase, induces Ca^{2+} influx through activation of plasma membrane Ca^{2+}-permeable channels, resulting in transient [Ca^{2+}]cyt elevation. As an example, ROS elevation has been shown to activate Ca^{2+}-permeable channels in root cells, as well as Ca^{2+}-influx channels for stomatal closure in response to ABA (Allen et al., 2000; Pei et al., 2000; Demidchik and Maathuis, 2007). In turn, as positive feedback regulation, [Ca^{2+}]cyt elevations can trigger enhanced ROS production by activation of NADPH oxidase via the above mentioned CPK5, CBL1/9-CIPK26 phosphorylation or direct Ca^{2+} interactions (Boudsocq et al., 2010; Demir et al., 2013; Kimura et al., 2013). Downstream of ROS, NO has also been implicated in ABA signalling (Bright et al., 2006). In guard cells, NO appears to modulate intracellular Ca^{2+} levels through regulation of plasma membrane or endomembrane-located Ca^{2+} channels, resulting in [Ca^{2+}]cyt elevation, inhibition of inward-rectifying K^+ channels and anion channels, which induces stomatal closure (Garcia-Mata et al., 2003). More recently, the ABA-hypersensitive phenotype of NO-deficient triple mutant *nia1nia2noa1-2* has revealed that NO has a negative role in ABA signaling and, probably, its molecular mechanism of action is Ca^{2+}-independent by acting on positive regulators such as PYR/PYLs receptors or SnRK2s (Lozano-Juste and Leon, 2010a) Downstream of ROS and NO, it has been also proposed that cGMP and cADPR act as second messengers through elevation of [Ca^{2+}]cyt in the guard cell (Leckie et al., 1998; Garcia-Mata et al., 2003; Dubovskaya et al., 2011).

Several phospholipids such as phosphoinositides (PIs), phosphatidic acid (PA) and sphingolipids also act as second messengers in the ABA signaling pathway. Thus, inhibition of phosphatidylinositol-3-phosphate (PI3P) and phosphatidylinositol-4-phosphate (PI4P) production prevents NADPH oxidase activation and ABA-induced stomatal closure in guard

cell (Park et al., 2003). In addition, PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C (PI-PLC) hydrolyses PIs, such as PI4P or PI(4,5)P_2, into two well-known second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). For instance, PI-PLC seems to be involved in ABA signaling because depletion of PI-PLC activity causes impaired ABA-induced stomata movements in guard cells and its constitutive over-expression presents impaired seed germination (Sanchez and Chua, 2001; Hunt et al., 2003; Mills et al., 2004). Initially, IP_3 was shown to cause an elevation in [Ca^{2+}]cyt leading to inhibition of K^+-influx channels in Ca^{2+}-dependent manner and stomatal closure (Blatt et al., 1990). However, recent data indicate that in plants, increased levels of IP_3 lead to enhanced sensitivity to ABA, therefore IP_3 might be phosphorylated into inositol hexakisphosphate (IP_6), which is, probably the emerging signaling molecule of PI-PLC signaling. In fact, IP_6 is rapidly formed in response to ABA and it is ~100 times more potent inducer of Ca^{2+} release from intracellular stores than IP_3 (Lemtiri-Chlieh et al., 2003; Gunesekera et al., 2007).

Likewise, a number of studies suggest a positive role for the PHOSPHOLIPASE D (PLD) and its product PA in triggering downstream ABA responses. Thus, PLD and PA induce stomatal closure and prevent stomatal opening (Jacob et al., 1999; Zhang et al., 2004; Mishra et al., 2006; Zhang et al., 2009; Hong et al., 2010; Guo et al., 2012). PA was found to bind and inhibit ABI1 phosphatase activity and promote the tethering of ABI1 to the plasma membrane (Zhang et al., 2004). PA has also been shown to interact with and stimulate NADPH oxidases, as well as the production of ROS for ABA-mediated stomatal closure (Zhang et al., 2009). Finally, SPHINGOSINE KINASE (SPHK) could be another PA target in the ABA pathway. This enzyme produces S1P, which has recently been implicated in ABA signaling. S1P has been reported as a calcium-mobilizing molecule in plants and it is involved in ABA-induced stomatal closure (Ng et al., 2001; Coursol et al., 2003).

1.2.4 Membrane recognition by phospholipid-binding domains

In coordination with other cell membranes, plasma membrane PM acts as an essential site for perceiving, transmitting and responding to external stress signals. Many signaling events are strongly linked to PM as production of key second messengers such as Ca^{2+} and lipids. It is well-known that physical properties of membranes (such as fatty acid composition) and membrane–protein interactions are essential for stress responsive processes. For instance, under drought, salinity, cold stress or ABA, a stress-induced PM Ca^{2+} wave is generated through activation of Ca^{2+} channels and release from intracellular stores (Li et al., 2013b; Choi et al., 2014). Cellular membranes contain a wide range of different lipids, including sphingo-, neutral-, glyco-, and phospholipids, all with unique biophysical properties (Furt et al., 2010; Cacas et al., 2012). These lipids can act as membrane-docking targets for proteins containing the

appropriated lipid-binding domain, leading to its translocation from the cytosol to membrane and further activation of downstream signaling pathways. It has now been widely accepted that PM is an heterogeneous mosaic of small regions, called lipid or membrane rafts, detergent-resistant membranes (DRMs), micro- or nanodomains, defined by a varying crosstalk between different lipids and membrane-resident proteins (reviewed in Lingwood and Simons, 2010; Malinsky et al., 2013; Li et al., 2013b). Thus, formation of these lipid rafts increases the probability of signaling events at the PM, by (i) providing these small platforms for protein–protein interactions that initiate the intracellular signaling cascade and (ii) acting as physical support for some enzymatic activities (Li et al., 2013b; Malinsky et al., 2013).

In the latest years, identification and characterization of PM-related proteins have been a major challenge for scientists. Technological advances in fluorescence microscopy and in mass spectrometry have created the opportunity for a new field of "lipidomics". In the field of ABA signaling, recent studies from *Arabidopsis* DRMs have provided evidence of colocalization of ABA signaling proteins in membrane microdomains, such as NADPH oxidase RbohD, anion channel SLAH3 and CPK21 (Demir et al., 2013; Hao et al., 2014). These studies showed that protein association to membrane microdomains is an important aspect for ABA responses. For example, via ABI1 inhibition, ABA signaling modulates the formation of a SLAH3/CIPK21 complex within lipid microdomains. Thus, ABI1 interacts with CPK21, disrupting the formation of the SLAH3/CIPK21 complex and causing anion channel inactivation (Demir et al., 2013).

It is well-known that phospholipids are ubiquitous components of cell membranes. Some of the more abundant phospholipids include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphoinositides (PI) and phosphatidic acid (PA) (Figure 1.10). The variation in physical properties between their lipid head group and fatty acid chains are important parameters in lipid recognition by peripheral membrane proteins. At least 10 different phospholipid-binding domains have been identified up to date. According to its membrane interaction mechanism, these domains can be classified in two groups: (i) some with high stereospecific recognition of particular membrane components such as specific type of phospholipids and (ii) others with non-specific recognition that involves general physical properties of the membrane such as charge or membrane surface curvature.

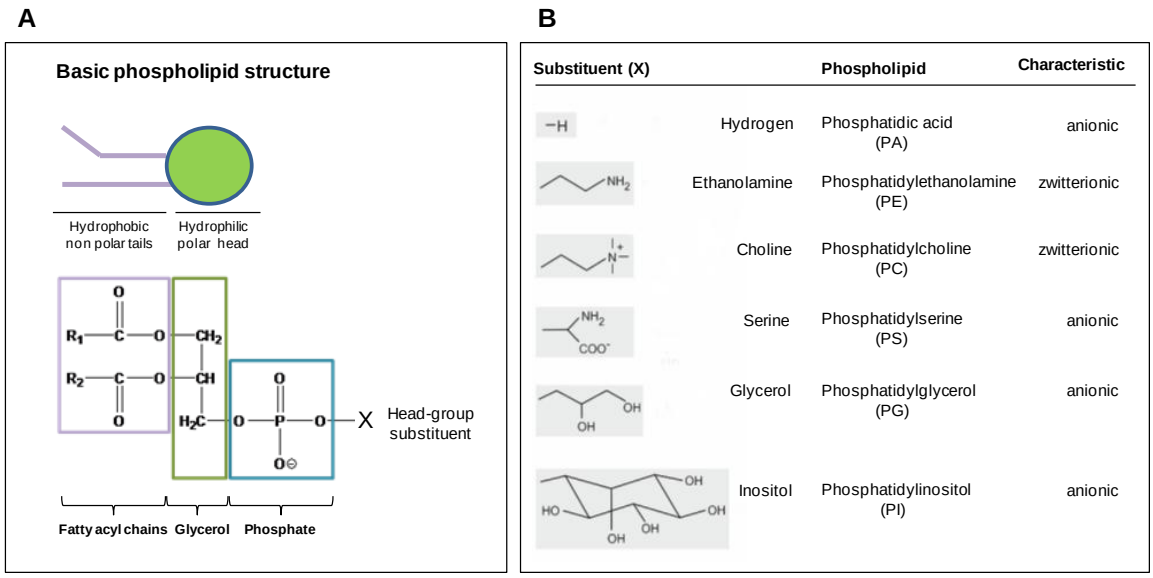

Figure 1.10 General structure of phospholipids and common head groups. (A) Phospholipids contain two fatty acids ester-linked to glycerol at C-1 and C-2, and apolar head group attached at C-3 via a phosphodiester bond. The fatty acyl side chains are hydrophobic and can vary in carbon group length and saturation degree. The head group forms a hydrophilic region and determines the type of phospholipid. This amphipathic property of phospholipids provides the basis for the compartmentalization of cells. (B) The different common polar head groups and charges are indicated. PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

Target-specific binding domains

The peculiar structure of these domains determines type of affinity and specificity for a particular phospholipid head group in the context of electrostatic potential compatibility with the proximal membrane surface. Therefore, these domains employ as usual the membrane-association mechanism with combined head group binding, electrostatic attraction and membrane insertion (reviewed in Lemmon, 2008) (Figure 1.11).

– *C1 domains* (named after "conserved region-1" from PKC) with characteristic Zn^{2+} finger domains of ~ 50 amino acids that contain the signature motif $HX_{12}CX_2CX_{13-14}CX_2CX_4HX_2CX_7C$ (in which C is cysteine, H is histidine and X is any residue). Typical C1 domains bind phorbol esters and DAG, and are responsible for PKC isoforms and DAG activation. A band of hydrophobic side chains encircles the DAG/phorbol ester binding sites and penetrate the polar milieu of the membrane (Lemmon, 2008)

– *FYVE domains* (acronym of Fab1, YOTB, Vac1 and EEA1) are small (70-80 amino acids) zinc fingers domains that homodimerize and specifically recognize PI3P, mostly found in endosome, multivesicular bodies and phagosome. The positively charged binding pocket of FYVE domain coordinates hydrogen bonds with PI3P for initial membrane association and the hydrophobic adjacent region drives final membrane association and orientation of the domain at the membrane interface (Dumas et al., 2001; Gil et al., 2012).

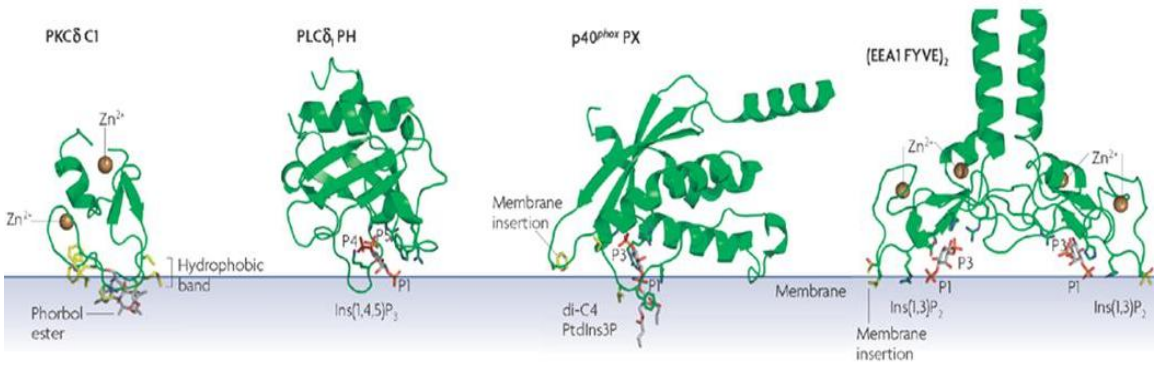

Figure 1.11 Structures of target-specific phospholipid-binding domains. The protein kinase Cδ (PKCδ) C1 domain (Protein Data Bank (PDB) code 1PTR) was solved as a complex with phorbol-1,3-acetate12. The two Zn2+ ions are labelled, as is the 'hydrophobic band' of residues that is thought to penetrate the membrane surface. The likely position of the membrane is approximated by the shaded bar. Pleckstrin homology (PH) domain of phospholipase Cδ1 (PLCδ1) (PDB code 1MAI), which is bound to inositol-(1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) through the 'canonical' binding site. Phox-homology (PX) domain of p40*phox* with bound dibutanoylphosphatidylinositol 3-phosphate (PtdIns3P) (PDB code 1H6H), which shows the phosphoinositide-binding site and membrane insertion loop. A truncated 'Fab1, YOTB, Vac1, EEA1' (FYVE) domain dimer from early endosome antigen-1 (EEA1)41 bound to $Ins(1,3)P_2$ is also shown (PDB code 1JOC), illustrating headgroup binding, membrane insertion and dimerization. Structurally crucial Zn2+ ions are marked. Yellow side chains are those that are likely to penetrate the membrane. The target lipids of these domains are represented by corresponding head groups. From Lemmon, 2008.

- *Pleckstrin homology domain* (PHD) (~100 amino acid) is the most abundant lipid-binding domain with β-sandwich structure, mostly found in pleckstrin and numerous other proteins with membrane associated functions. PH domains are well known effectors of the lipid second messengers such as $PI(3,4,5)P_3$ and $PI(3,4)P_2$. All high-affinity, stereospecific PH domains share a similar phosphoinositide-binding site, in which the basic lysines and arginines play an important role in forming hydrogen bonds with the head group of PIP (Lemmon, 2007; 2008; Moravcevic et al., 2012)
- *Phox- homology domain* (PX) (~130 amino acid) with α+β structure was identified as PI3P-binding domain. Most PX domains are found in sorting nexin proteins, which are important for membrane trafficking. PX domain employ membrane-association mechanism with combined headgroup binding, electrostatic attraction and membrane insertion (Stahelin et al., 2006; Lemmon, 2008; Moravcevic et al., 2012)
- PROPPINs are ~ 500-amino-acid β-propeller proteins that bind $PI(3,5)P_2$ with high affinity and specificity (Baskaran et al., 2012).

Domains with lower target specifity

Domains with low target specificity bind ubiquitous phospholipids of cell membranes, such as, for example, PS (the most abundant acidic phospholipid). The most representative members of these domains are *annexins* with α-helical array structure and *C2 domain* (the second domain of PKC) (Figure 1.12). Such domains allow temporal and spatial specificity in membrane targeting without changing the nature of phospholipid headgroup. Additionally, these

domains undergo membrane association in Ca^{2+}-dependent manner. Thus, transiently elevated cytosolic calcium levels control the timing of their translocation to membranes. Therefore, annexin fold and C2 domain are two major classes of Ca^{2+}-sensing structural motifs able to carry changes in [Ca^{2+}]cyt levels to membrane surfaces. In contrast to target-specific phospholipid-binding domains, these domains lack a basic binding pocket for negatively charged lipids and bound Ca^{2+} has the crucial bridging role in (i) conferring positive electrostatic potential to the membrane-binding protein regions, thus, acting as a nonspecific "electrostatic switch" that attract negatively charged target lipids and (ii) specifically recognizing PS headgroup through the formation of coordination bonds with the carboxyl and/or phosphoryl oxygens (reviewed in Lemmon, 2008). However, it has to be mentioned that, in some cases, these protein domains may have Ca^{2+}-independent membrane association using divers mechanisms, as for example, by forming a positively charged "core domain" for stereospecific recognition of PS (Creutz et al., 1992; Lemmon, 2008). Likewise, due to dual membrane binding site, some C2 domains may also bind simultaneously to PS and other lipids. For example, the structure of PKCα C2 domain is able to interact with two distinct lipids: PS at the Ca^{2+}-binding region and PI(4,5)P through residues located in the β3 and β4 strands that have been named the lysine-rich or polybasic cluster, or the β-groove. Indeed, substitutions in polybasic cluster may impair ability of PKCα to translocate from the cytosol to the plasma membrane in PI(4,5)P-dependent manner (Guerrero-Valero et al., 2009). Likewise, PKCε C2 domain binds PA and this is required for the protein to be translocated to the membrane upon stimulation (Testerink and Munnik, 2005).

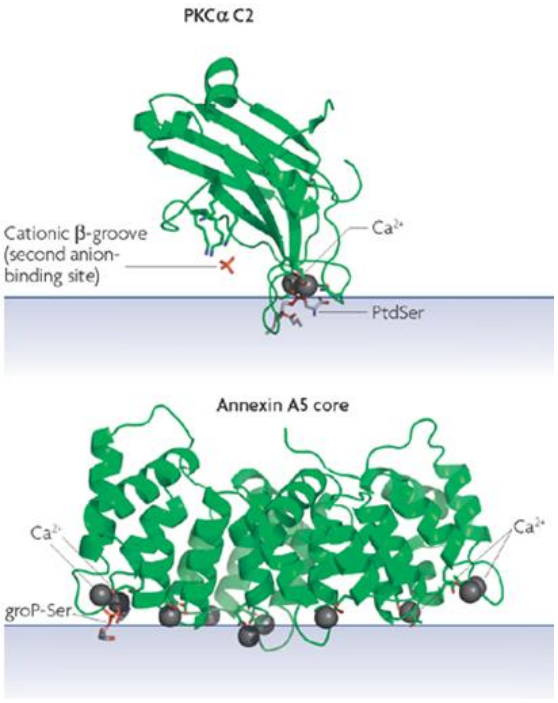

Figure 1.12 Structures of phosphatidylserine-binding domain. The PKCα C2 (Protein Data Bank (PDB) code 1DSY) and annexin A5 core (PDB code 1A8A) domains are intracellular phosphatidylserine (PtdSer)-binding domains. The PKCα C2 domain forms a β-sandwich with two Ca2+ ions that are coordinated at one corner, and make bridging interactions with the bound dicaproyl-phosphatidylserine. Membrane penetration is thought to occur as shown, and the proposed cationic β-groove, at which phosphoinositides are thought to bind C2 domains, is shown. The annexin core from annexin A5 is shown with bound glycero-phosphorylserine (groP-Ser). Ten coordinated Ca2+ ions form bridging interactions between the annexin core and the membrane phospholipids. From Lemmon, 2008.

1.2.4.1 C2 domains as calcium-dependent lipid binding domain

The C2 domain was first identified as the second of four conserved regulatory regions of the Ca^{2+}-dependent PKC and represents the second most abundant lipid binding domain found in eukaryotic proteins, after the PH domain(Cho and Stahelin, 2006; Lemmon, 2008). In general, this domain represents a Ca^{2+}-dependent membrane binding module in eukaryotic membrane-associated proteins and are combined with a wide range of other domains (for example SH2, SH3, PH, WW, EF, PDZ, etc), with different functions (Rizo and Sudhof, 1998). Interestingly, recent structural and functional evolution studies show that C2 domain possibly emerged as part of the general adoption of the Ca^{2+}ions and lipids as second messengers in the cell signaling. Therefore, the C2 domain is apparently an eukaryotic innovation and has not been identified in prokaryotes yet (Clampham, 2007). Examination of phyletic patterns suggests that the C2 domain was greatly diversified in the last eukaryotic common ancestor in about 9 families acquiring different subcellular roles, but all related to membrane functions, as for example lipid-based signal transduction with different enzymatic activities (such as protein kinases and phosphatases, peptidases, ubiquitin E3 ligases and phospholipases), cytoskeletal interactions (actin and tubulin anchoring), membrane repair, vesicular trafficking, localization of small GTPases to membrane and ciliogenesis (Figure 1.13) (Zhang and Aravind, 2010). Indeed, the most studied C2 domains are involved in (i) Ca^{2+}-dependent translocation of soluble proteins to membranes (cytosolic phospholipase A2 ($cPLA_2$), (ii) Ca^{2+} and phospholipid-dependent activation of enzymes (PKC isoforms α, β, γ), (iii) Ca^{2+} and phospholipid-dependent interaction between proteins (rabphilin-3A) and (iv) membrane repair (Synaptotagmin1 (Syt1)), but there are many others with yet not assigned functions (Murray and Honig, 2002).

The three-dimensional structure of C2 domains is extremely well conserved despite the low sequence similarity among the members of the family (Corbalan-Garcia and Gomez-Fernandez, 2014). C2 domains are around 130 amino acid residues and comprise a characteristic β-sandwich fold composed of two antiparallel four-stranded β sheets with highly variable inter-strand regions that might contain one or more α-helix and flexible loops on top and at the bottom of the sandwich. C2 domains display two lipid-binding sites. The first is located in the three variable flexible upper loops which define formation of a concave Ca^{2+}-dependent membrane-binding area or pocket (Ca^{2+}-binding loops or regions (CBL1-3/CBR1-3)) (Figure 1.14). These loops often contain a set of five aspartic residues that reversibly bind two or three Ca^{2+} ions (Figure 1.15). In absence of bound Ca^{2+}, the canonical membrane-binding pocket formed by these three loops is often acidic with negative potential and seldom, if ever, interact

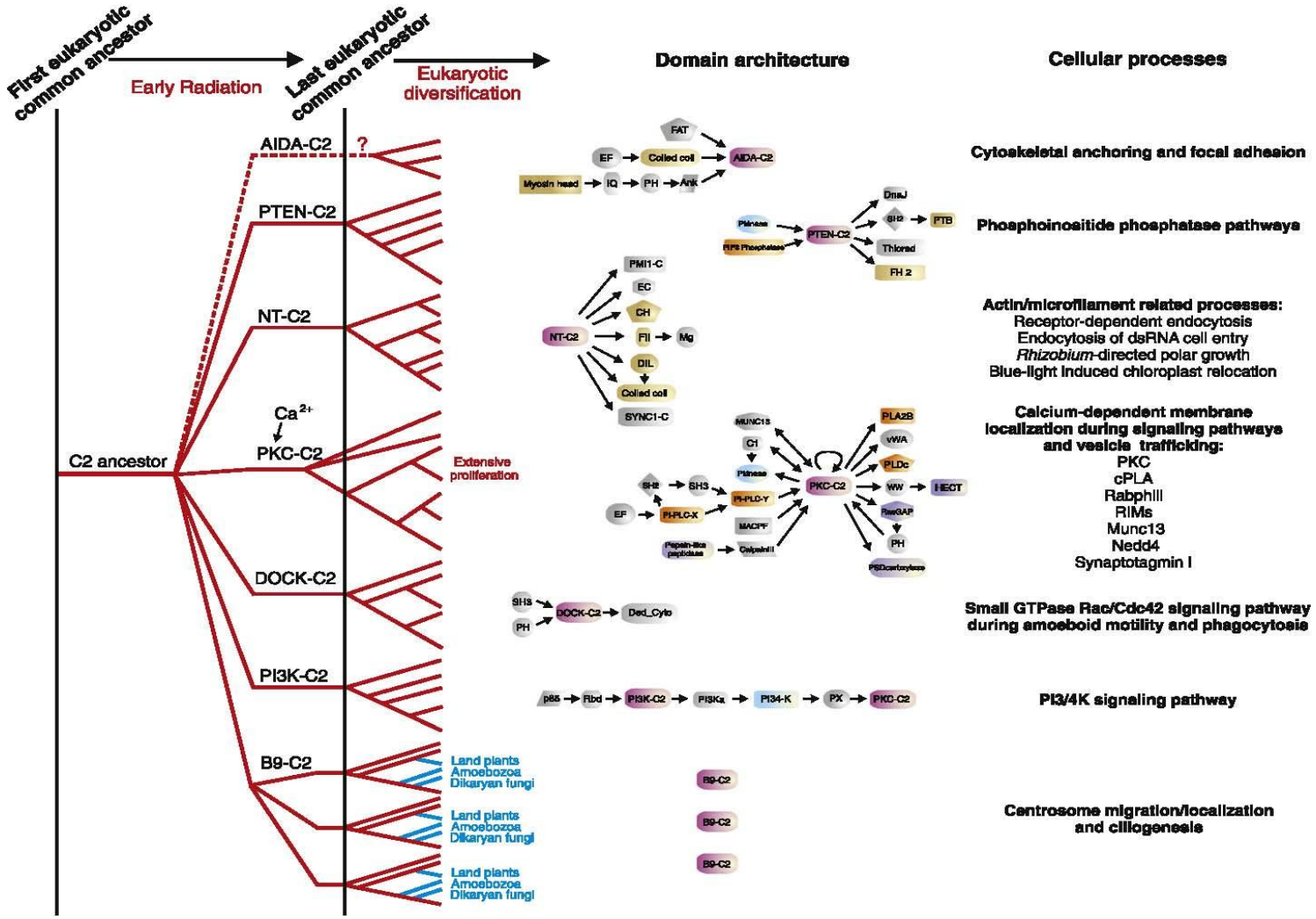

Figure 1.13 Divergent evolution of the C2 domain superfamily in eukaryotes. Duplications of the domain and speciation are indicated by red lines whereas domain loss for B9–C2 domains is indicated by blue lines. Dashed red line with question mark (?) indicates unclear point of origination of AIDA–C2. A domain architecture linkage graph for the seven C2 versions is shown in the next column. The arrows indicate the direction of domain connection in a polypeptide with the arrowhead pointing to the C-terminus. C1, Protein kinase C, C1 domain; CalpainIII, Calpain large subunit, domain III; DnaJ, DnaJ domain; PIP3 phosphatase, including both dual specificity phosphatase, catalytic domain and protein–tyrosine phosphatase; Thiored, is a thioredoxin-like domain called DUF547; FH2, Formin homology 2 domain; HECT, HECT-domain (ubiquitin-transferase); MACPF, MAC/Perforin domain; MUNC13, Munc13 (mammalian uncoordinated) homology domain; p85, PI3-kinase family, p85-binding domain; Papain-like peptidase, calpain family cysteine protease; PI34-K, Phosphatidylinositol 3- and 4-kinase; PI3Ka, Phosphoinositide 3-kinase family, accessory domain; PI-PLC-X, Phosphatidylinositol-specific phospholipase C, X domain; PI-PLC-Y, Phosphatidylinositol-specific phospholipase C, Y domain; Pkinase, Protein kinase domain; PLA2B, Lysophospholipase catalytic domain; PLDc, Phospholipase D C terminal; PSDcarbxylase, Phosphatidylserine decarboxylase (PF02666); PTB, Phosphotyrosine-binding domain (PF08416); PX, PX domain; RasGAP, GTPase-activator protein for Ras-like GTPase; Rbd, PI3-kinase family, ras-binding domain; SH2, Src homology 2 domain; vWA, vWA-like copine domain; and WW, WW domain. From Zhang and Aravind, 2010.

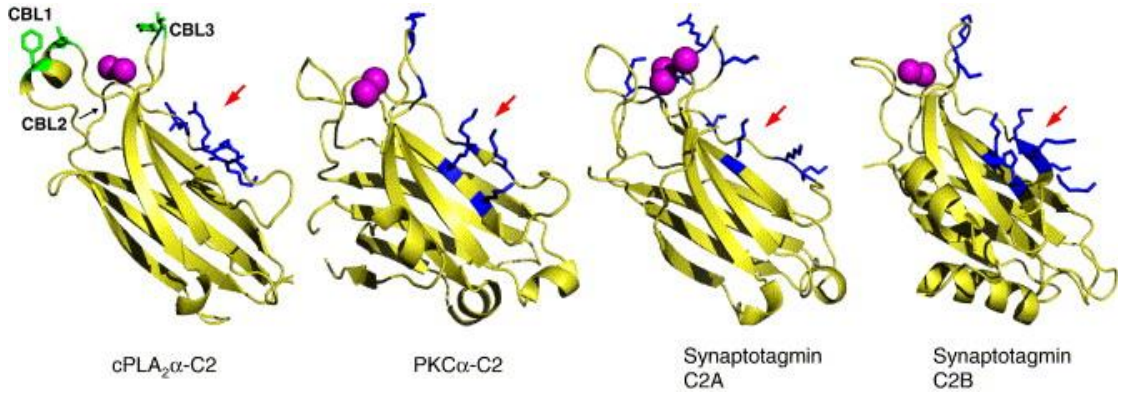

Figure 1.14 Representative structures of C2 domains. Structures of PKCα-C2, cPLA$_2$α-C2, synaptotagmin I-C2A, and synaptotagmin I-C2B are shown in ribbon diagram. Bound Ca^{2+} ions are shown as magenta spheres and three Ca^{2+} binding loops (CBL1–3) are marked. Cationic (blue) and hydrophobic (green) residues in CBL's and the β-groove are shown in stick models. Locations of cationic β-grooves are indicated by red arrows From Cho and Stahelin, 2006.

with membranes of any composition (Murray and Honig, 2002; Cho and Stahelin, 2006; Lemon, 2008). Ca^{2+} binding causes dramatic "electrostatic switch" in the loop regions, reversing from negative to neutral or positive potential and allowing nonspecific electrostatic interactions with the anionic or zwitterionic phospholipids such as PS (Shao et al., 1997; Murray and Honig, 2002). On the other hand, Ca^{2+} binding provides a "bridge" between C2 domain and anionic phospholipids what also cooperatively enhance its complete binding and affinity of C2 domains (Zhang et al., 1998; Davletov et al., 1998b; Verdaguer et al., 1999). Additionally, Ca^{2+} binding can induce intra- or interdomain conformational changes that, in turn, may trigger membrane-protein interactions (Sutton et al., 1995; Grobler et al., 1996; Bittova et al., 1999). It has to be mentioned that, although the major part of known C2 domains exhibit Ca^{2+}-dependent membrane binding, it has also been reported Ca^{2+}-independent membrane C2 domains, such as the C2 domain present in the PTEN tumor suppressor, which preferentially interact with anionic

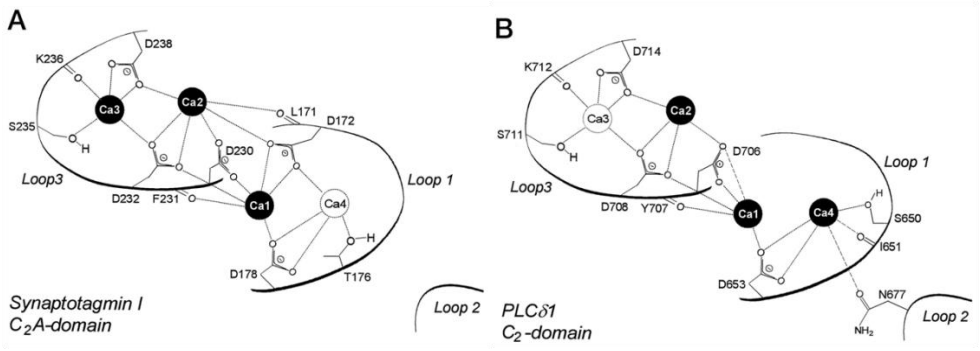

Figure 1.15 Summary of the Ca^{2+}-binding sites in the C_2-domains of synaptotagmin I (*A*) and of PLCδ1 (*B*). The side chains and carbonyl groups involved in binding are indicated. Sites actually observed in the structures are shown by *solid circles*, and potential additional sites in each C_2-domain by*open circles*. For the PLCδ1 C_2-domain, site Ca2 was only occupied in the La^{3+} complex, and the serine 650 side chain (*S650*) was only involved in metal ion coordination in this complex. Although not observed experimentally, a site could exist in the synaptotagmin I C_2A-domain that is analogous to site Ca4 in PLCδ1, and a site analogous to site Ca3 could be formed by the PLCδ1 C_2-domain. From Rizo and Südhof, 1998.

membrane owing to its total positive potential, very similar to that of the Ca^{2+}-bound forms of Ca^{2+}-dependent C2 domains (Murray and Honig, 2002). These differences of Ca^{2+}-binding properties, in spite the fact of high sequence homology among C2 domain orthologs, have been explained by different orientations of critical Ca^{2+}-binding residues, which makes difficult the prediction of Ca^{2+} affinity of C2 domains only by sequence-based analysis (Dai et al., 2004).

The second membrane-binding site is the cationic β-groove or cationic patch, composed by a series of basic and aromatic residues presented on a concave surface of the C2 domain (Figure 1.14). This cationic patch was found to be an additional binding site for anionic phospholipids in a calcium-independent manner. The cationic β-groove is not conserved in terms of primary and tertiary structure and its size or electrostatic potential widely vary among C2 domains (Cho and Stahelin, 2006).

As described above, due to dual membrane binding site, C2 domains present highly variable and relatively low lipid selectivity. For example, lipid selectivity of Ca^{2+}-dependent C2 domains is generally determined by their Ca^{2+}-binding loops. Thus, some Ca^{2+}-dependent C2 domains recognize PS with high affinity, others bind to all anionic phospholipids, and, finally, some show varing lipid selectivity depending on Ca^{2+} concentration. On the other hand, if a C2 domain interacts with the membrane primarily through the cationic β-groove as for example in case of some Ca^{2+}-independent C2 domains, lipid selectivity is determined by their cationic β-groove. Some C2 domains may simultaneously bind multiple membrane components with both Ca^{2+}-binding loops and cationic β-groove. As a result of diversity on the binding mode, lipid selectivity and Ca^{2+} dependence, C2 domains can present different membrane localization with its defined signaling consequences (Cho and Stahelin, 2006; Lemon, 2008).

In contrast to the large number of C2-containing domain proteins characterized in yeast and animals, only very few genes encoding proteins with C2 modules have been functionally characterized in plants. Interestingly, most of these reports describe the involvement of C2-domain proteins in plant responses to abiotic and biotic stresses suggesting their importance in stress responses and signaling. Among them, the most studied are PLC, PLD and PI 3-kinase, all three involved in different lipid signaling cascades and, thereby, in plant stress response (Kopka et al., 1998; Munnik et al., 1998; Wang and Wang, 2001; Kim et al., 2004b; Wang et al., 2009). Additionally, *Arabidopsis* synaptotagmin1 (SYT1) (Yamazaki et al., 2008; Schapire et al., 2008) is required for maintenance of plasma membrane integrity in response to abiotic stress and plant development through a Ca^{2+}-dependent signaling pathway. *Arabidopsis* C2-domain BAP1 acts as a negative regulator of programmed cell death induced by biotic stimuli (Yang et al., 2006; 2008). In the last decade, it has been reported a novel C2 domain family only identified in plants and composed of small single C2-domain-containing proteins, without any additional conserved motif (Kopka et al., 1998). For instance, the first reported plant small single C2-domain-containing protein was Cmpp16-1 from pumpkin, which is involved in RNA

delivery (Xoconostle-Cazares et al., 1999). Later, other small C2-domain-containing proteins were identified, such as rice OsERG1/OsERG3, which is involved in defense responses and membrane translocation in a Ca^{2+}- dependent manner (Kim et al., 2003; Kang et al., 2011); a pepper C2 domain-containing SRC2 protein targeted to the plasma membrane in a Ca^{2+}-dependent manner, which is involved in the abiotic stresses and defense responses, and OsSMCP1, whose overexpression in *Arabidopsis* resulted in enhanced tolerance to abiotic and biotic stresses (Yokotani et al., 2009). These studies suggest that small C2 domain-containing proteins are implicated in response to both biotic and abiotic stresses and, probably their function is to mediate protein-protein interactions with target proteins (Kang et al., 2011). However, very little is known about targets of small C2-domain proteins in plants. Finally, this work has contributed to the identification and characterization of an *Arabidopsis* C2-domain ABA-related (CAR) family of 10 proteins as new interacting partners of PYR/PYLs ABA receptors, which mediate their approaching to the plasma membrane in a Ca^{2+}-dependent manner, and thereby, are involved in abiotic stress responses (Results, chapter II).

2. OBJECTIVES

2. OBJECTIVES

1. Identification of PP2Cs and other possible interacting partners of PYL8 involved in root ABA responses by Yeast Two-Hybrid (Y2H) and Tandem Affinity Purification (TAP) approaches. Functional characterization of these interactions by loss-of function genetic approaches.
2. Identification and characterization of C2-domain ABA-related (CAR) proteins as new interacting partners of PYR/PYLs ABA receptors. Elucidation of the molecular mechanism of PYR/PYL/RCAR-CAR interaction and role of CAR proteins in ABA signaling.

3. CHAPTER I

Identification of PYL8 interacting proteins implicated in root ABA response.

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Identification of PYL8 interacting proteins implicated in root ABA response.

3.1 INTRODUCTION

Over the past decade, significant progress was made on revealing the ABA signaling core components and their mode of action. As described in general introduction, the ABA signaling core is formed by three key components: PYR/PYL/RCAR ABA receptors, the first component, which play a crucial role in hormone perception and ABA-dependent inhibition of downstream PP2Cs, second component and key negative regulators of the pathway, which results in activation of ABA-dependent SnRK2s, third component and, thereby, in activation of ABA signaling pathway (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Gonzalez-Guzman et al., 2012). Thus, PYR/PYL/RCAR receptors are major early ABA signaling components and the crucial step to initiate ABA signaling cascade.

There are 14 PYR/PYL/RCARs family members (Ma et al., 2009; Park et al., 2009). This large number of PYR/PYL/RCAR receptors raises several questions about the relative contribution of each type of receptor in response to ABA and how multiple inputs of perception are integrated into ABA signaling outputs. On one hand, genetic analysis of single *pyr/pyl/rcar* loss-of-function mutants showed that all of them except *pyl8* have normal ABA responses, indicating functional redundancy between PYR/PYL/RCAR receptors. Moreover, gradual inactivation of PYR/PYL/RCAR genes in multiple ABA receptor loss-of-function mutants showed high additive effect in insensitivity to ABA in seed germination, root growth, stomata closure, and expression of ABA responsive genes (Park et al., 2009; Gonzalez-Guzman et al., 2012). These results revealed that receptor family members may contribute quantitatively to ABA response with high level of functional redundancy (Gonzalez-Guzman et al., 2012). On the other hand, genetic analysis of different combinations of multiple *pyr/pyl* mutants clearly showed that function of PYR/PYL/RCAR proteins is not completely redundant and importance of several PYR/PYL/RCAR family members may vary among ABA responses (Gonzalez-Guzman et al., 2012). Moreover, there are other lines of evidence that indicate non redundant function of ABA receptors. Biochemical parameters such as the oligomeric state (dimeric or monomeric), ABA-binding affinity (lower affinity for dimeric receptors and high affinity for

monomeric receptors) or binding affinity of ABA-bound receptors to each target PP2C as well as subcellular localization poin to a particular function of some ABA receptors (Santiago et al., 2009b; Szostkiewicz et al., 2010; Dupeux et al., 2011b; Antoni et al., 2012; Gonzalez-Guzman et al., 2012). Finally, transcriptomic data as well as GUS reporter analysis have shown that the PYR/PYL/RCAR receptors present different expression levels and different spatio-temporal expression patterns in *Arabidopsis*. For instance, the expression of *PYL3* and *PYL10* to *PYL13* is almost undetectable in different whole-genome microarrays, whereas the expression of *PYR1* and the rest of *PYL1* to *PYL9* could be detected in both vegetative and reproductive tissues, although at different levels (Winter et al., 2007; Kilian et al., 2007). Gonzalez-Guzman et al. (2012) analyzed the expression pattern of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5*, and *PYL8* in different tissues. They have reported that all of them are present in guard cells of mature leaves, in the peripheral layer of the embryo (embryo epidermal layer) as well as in the provascular cells within the cotyledons and hypocotyls. In contrast, in the endosperm of 24h germinated seeds only *PYL8* and *PYR1* showed high expression levels. Likewise, root ABA signaling seems to involve types of receptors depending of the root tissue. For instance, *PYR1*, *PYL1*, *PYL2* and *PYL8* were expressed in vascular tissue, *PYL1*, *PYL4*, and *PYL8* were expressed in the columella cells (Gonzalez-Guzman et al., 2012) (Figure 3.1). Interestingly, further detailed comparative analysis of GUS expression of ABA receptors in the whole root, have reveled that PYL8 additionally could be detected in root epidermis and lateral root cap (Antoni et al., 2013).

This differential expression of PYR/PYL/RCAR ABA receptors genes in root and the

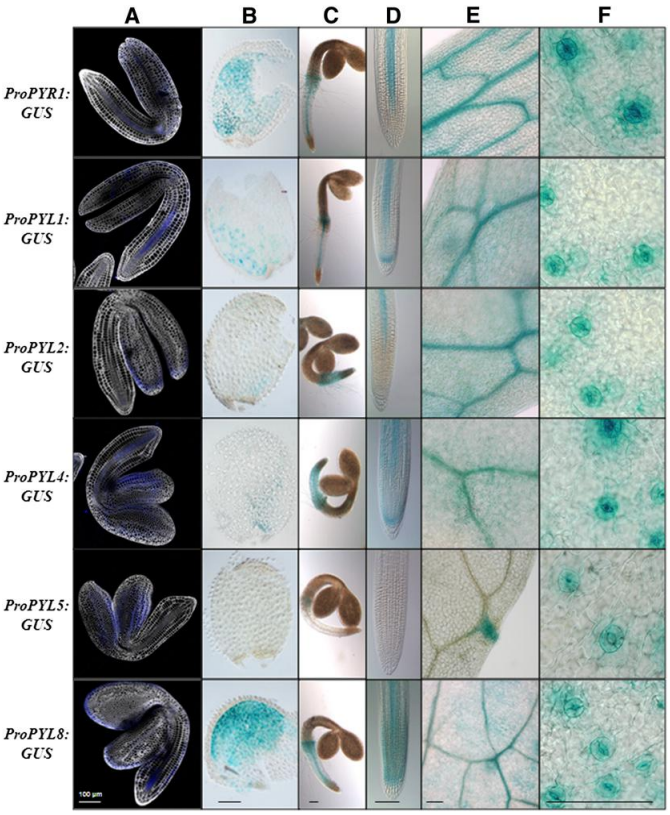

Figure 3.1 Photographs Showing GUS Expression Driven by *ProPYL1*, *ProPYR1*, *ProPYL2*, *ProPYL4*, *ProPYL5*, and *ProPYL8*:*GUS* Genes in Different Tissues. (A) and (C) Embryos dissected from mature seeds imbibed for 24 or 48 h, respectively.(B) Dissected seed coat and endosperm imbibed for 24 h.(D) Primary root from 5-d-old seedlings.(E) and (F) Vascular tissue and guard cells in leaves of 15-d-old seedlings, respectively. Bars =100 μm. From Gonzalez-Guzman et al., 2012

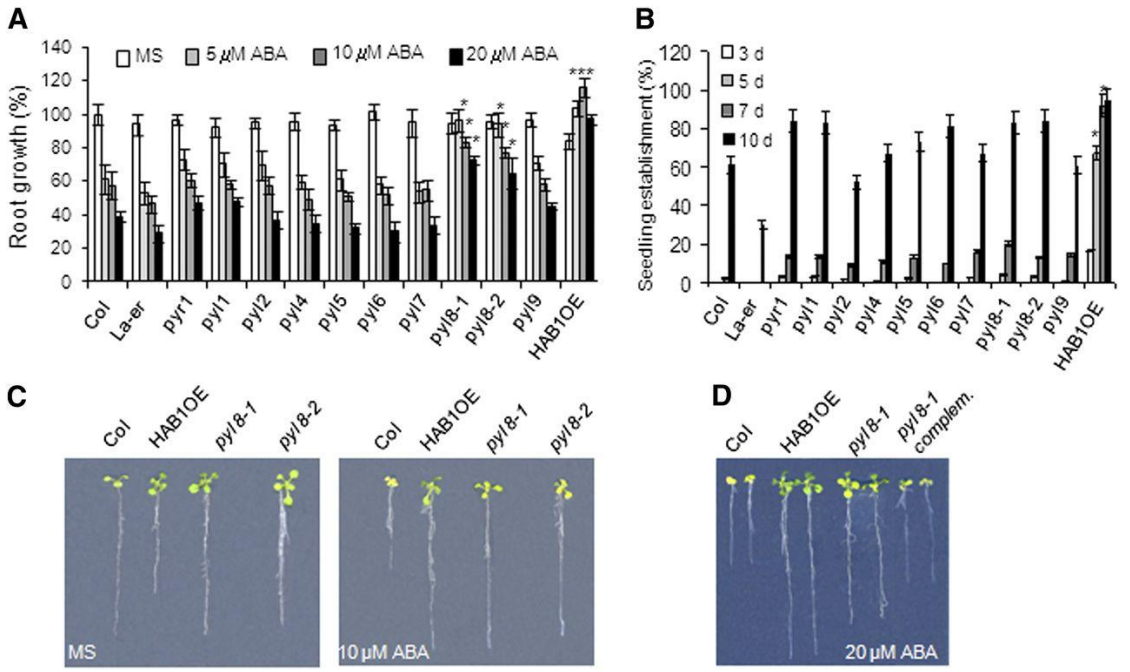

Figure 3.2 PYL8 plays a nonredundant role in root sensitivity to ABA. A, Quantification of ABA-mediated root growth inhibition of *pyr/pyl* mutants compared with the wild type. Data are averages ± se from three independent experiments (n = 15 each). *P < 0.01 (Student's t test) with respect to the wild type in the same experimental condition. B, Seedling establishment of *pyr/pyl* mutants compared with Col and Landsberg *erecta* (La-er) wild types in medium supplemented with 0.5 µm ABA at 3, 5, 7, and 10 d after sowing. Data show the percentage of seeds that germinated and developed green cotyledons. Values are averages ± se for three independent experiments (100 seeds each). *P < 0.01 (Student's t test) with respect to the wild type in the same experimental condition. C, ABA-insensitive phenotypes of *pyl8-1* and *pyl8-2* alleles compared with the Col wild type. Photographs show representative seedlings 10 d after the transfer of 4-d-old seedlings from Murashige and Skoog medium (MS) to plates lacking or supplemented with 10 µM ABA. D, Complementation of the *pyl8-1* allele by introduction of a *35S*:*3HA-PYL8* transgene (*pyl8-1* complemented). The photograph shows representative seedlings 12 d after the transfer of 4-d-old seedlings from Murashige and Skoog medium to plates supplemented with 20 µm ABA.

importance of ABA signaling to control root growth under different environment conditions prompted us to analyze the root ABA sensitivity of 9 single *pyr/pyl* mutants (i.e. *pyr1*, *pyl1*, *pyl2*, *pyl4*, *pyl5*, *pyl6*, *pyl7*, *pyl8* and *pyl9*) as well as several multiple mutant combinations. Unexpectedly, despite the described functional redundancy among PYR/PYL genes, we found that *pyl8* single knockout mutant was the only single mutant that showed reduced ABA-mediated root growth inhibition even at 20 µM ABA (Figure 3.2), whereas as expected, combination of different *pyr/pyl* mutations enhanced this phenotype (Antoni et al., 2013). This genetic evidence together with root expression analyses served to point out relevant ABA receptors in the root, particularly PYL8. Therefore, we decided to investigate the reduced root ABA sensitivity of *pyl8* through identification of PYL8 interacting partners.

Indeed, to address this question, it is important to clarify how PYL8 mediates ABA signaling through interaction with PP2Cs and other possible interaction partners as well as what are the key PYL8-PP2C complexes for PYL8 specificity in ABA root responses. To this end, we decided follow both a biochemical approach for explore genome wide interaction targets of

PYL8 and genetic approach to study functional associations between PYL8 and PP2Cs in ABA response.

Protein-protein interaction studies, such as affinity purification coupled to mass spectrometry (AP-MS) and yeast two-hybrid (Y2H) screening, were the two major platforms used to screen and identify ABA core signaling components (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009a; 2009b; Nishimura et al., 2009). Although both are high-throughput methods used for identification of protein-protein interactions, the advantages of AP-MS are obvious: (i) involves isolation of the bait from the plant sample by affinity purification trying to maintain near-physiological conditions, reflecting *in planta* binding, (ii) allows the study of protein-protein interaction in different plant physiological conditions and (iii) can be used for *in situ* purification of dynamic protein complexes. AP-MS method however has a great disadvantage: is based on the short single step purification of the bait protein, which brings to considerable contaminant background and following high false-positive rate. This often demands integration of complex quantitative tools to discriminate true from false-positive interactions. Moreover, detection of low-abundance proteins during MS is often masked by interference of peptides derived from these contaminating proteins. Recently it was found a solution to this pitfall by using, Tandem Affinity Purification (TAP) approach, an innovative form of AP characterized by much smaller false positive rate. The TAP approach is a highly specific and stringent method for isolate binding partners of a bait protein. It is based on the expression of a bait protein fused to a double affinity tag and a two-step purification process followed by mass spectrometry of protein complex members (Van Leene et al., 2008). The classical *yeast* TAP tag consists of two immunoglobulin G (IgG)-binding domains of *Staphylococcus aureus* protein A or protein G, a specific protease cleavage site for elution by addition of the tobacco (*Nicotiana tabacum*) etch virus (TEV) protease and calmodulin-binding peptide (CBP) or streptavidin-binding peptide (SBP). In plants, despite the development and testing of a large variety of plant-adopted versions of classical TAP tags, only using GS tag (which combines two (IgG)-binding domains of protein G with SBP, separated by two TEV cleavage sites) was possible to produce a 10-fold increase in the efficiency compared to the conventional TAP tag (Figure 3.3). Thus, GS-TAP strategy allows to reduce background levels, making MS analysis much less labor intensive and the identification of genuine protein interactions easier, especially with low abundant complexes (Van Leene et al., 2008; Dedecker et al., 2015; Van Leene et al., 2015).

To gain a comprehensive understanding of the molecular mechanism of PYL8, we have performed a GS-TAP approach in collaboration with the group of Geert De Jaeger (Department of Plant Systems Biology, VIB, Ghent, Belgium) followed by mass spectrometry analysis of PYL8-associated proteins to provide new *in vivo* evidence for the mechanism of action of PYL8 in regulation of ABA signaling.

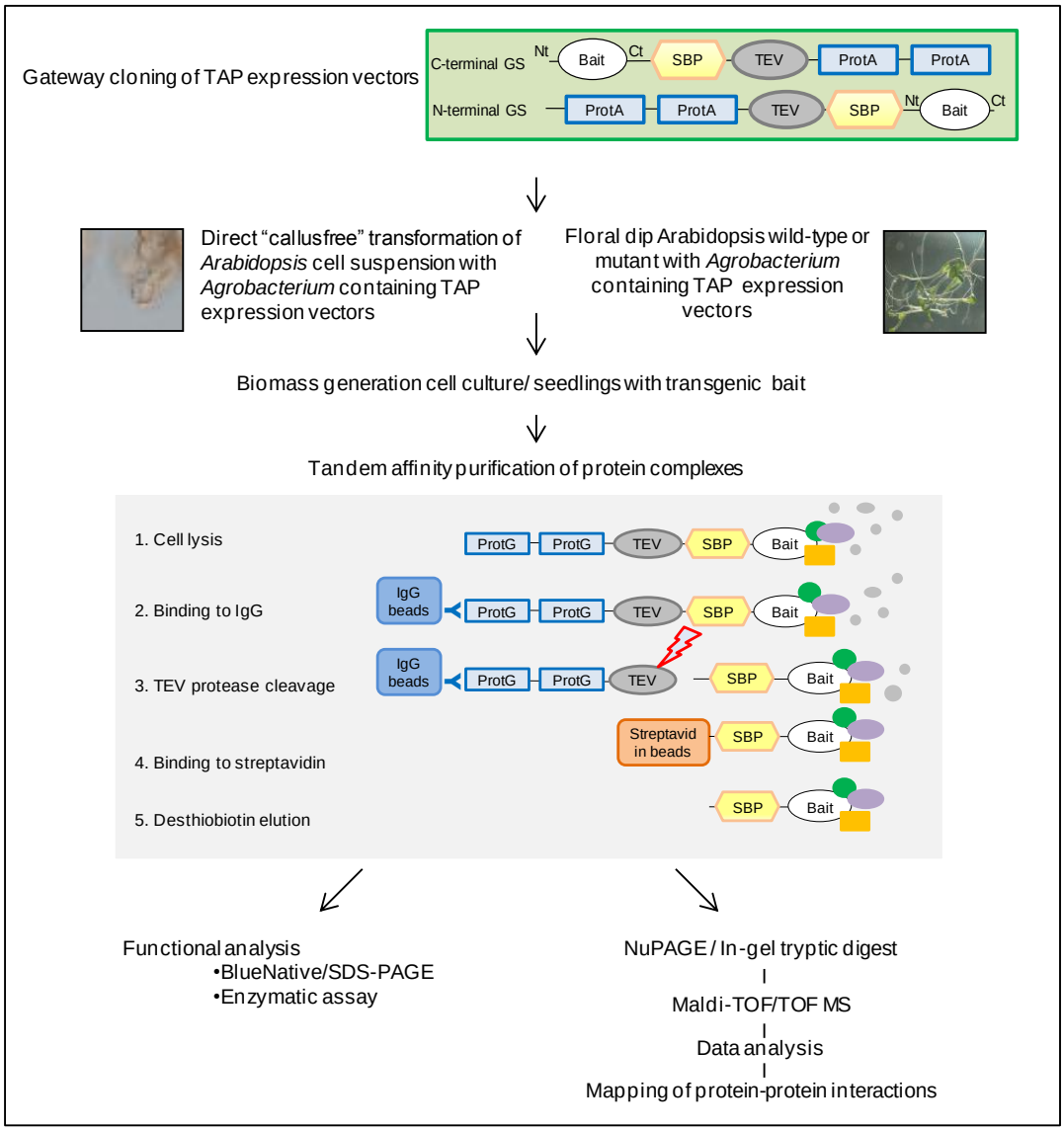

Figure 3.3 Schematic representation of strategy followed to clone, express, purify, and identify tagged proteins and their interacting partners in GS-TAP procedure in *Arabidopsis*. The GS-TAP strategy workflow begins with the GS tagging of a bait protein, which consist in generation of the both N-terminal and C-terminal GS tagged fusion constructs. The GS tag combines two IgG-binding domains of protein G with streptavidin-binding peptide, separated by two tobacco eatch virus cleavage sites. The bait fusion construct is introduced via *Agrobacterium tumefaciens* transformation of *Arabidopsis* cell suspension cultures or by floral dip method in *Arabidopsis* wild-type or mutant plants. Introduction of the GS tag increased the purification yield, the fusion protein accumulation levels and the specificity of the purification, thus lowering the amount of contaminating proteins. Selected transgenic lines of *Arabidopsis* cell suspension cultures or *Arabidopsis* seedlings are used for the subsequent tandem affinity purification, which consists in the next purification steps: (1) cell lysis and protein extraction, (2) isolation of native protein complexes that incorporate the transgenic bait through high-affinity binding to IgG, (3) elution of bound complexes by tag cleavage with TEV protease, (4) eluted protein complexes of interest are trapped through binding to streptavidin-conjugated beads whereas co-eluting non-interacting proteins and the TEV protease are separated from the tagged proteins and their associations in the flow-through, (5) desthiobiotin elution of the complexes. Two-dimensional BN/SDSPAGE followed by immunoblot analysis is used to evaluate the purification procedure. On the other hand, eluted proteins are separated on NuPAGE gels, excised and subjected to tryptic digestion, and analyzed via MALDI-TOF-TOF-MS. The resulting peptides are then clustered to protein sequences and scores assigned to protein identifications. The additional analysis of the data and development of the novel interaction confidence score allow mapping of the obtained protein-protein interactions.

3.2 RESULTS

Genetic evidences of interaction between PYL8 and PP2Cs

The sensitivity of ABA signaling depends on the ABA affinities of the receptors in addition to the affinities of the ABA-bound receptors to target PP2Cs. Biochemically PYL8 is a prototype monomeric ABA receptor that displays strong affinity to ABA with small dissociation constant Kd value (~1 μM). To clarify the role of PYL8-PP2Cs complexes in ABA signaling and to further address the unique function of the PYL8 receptor in root ABA signaling, we firstly performed an indicative interaction analysis of PYL8 with eight group A PP2C proteins using Y2H system. To this end, PYL8 was fused to the Gal4 DNA binding domain (BD domain) while eight PP2Cs, HAB1, HAB2, ABI1, ABI2, PP2CA/AHG3, HAI1/AIPH2, HAI2/AIP1 and AHG1 were fused to the Gal4 activation domain (AD domain). Y2H assay was performed in presence or absence of ABA. As a result, we showed that PYL8 interacts with all eight PP2Cs tested (Figure 3.4A). PYL8 interactions with seven of these PP2Cs (HAB1, HAB2, ABI1, ABI2, PP2CA, HAI1, HAI2) were really ABA-independent, with exception of AHG1, where PYL8 needed the addition of ABA to achieve stronger interaction with the PP2C. This result is consistent with results of interaction of PYL8 obtained by different groups with different PP2Cs in Y2H system such as HAB1 (Santiago et al., 2009b), ABI1 (Nishimura et al., 2010), ABI2 (Ma et al., 2009), HAI1, HAI2 and HAI3 (Bhaskara et al., 2012) as well as in BiFC assays of PYL8 with ABI1 and ABI2 (Szostkiewicz et al., 2010). Previous crystal structures of PYR/PYLs-ABA-PP2Cs ternary complexes confirmed that, in absence of ABA, monomeric PYLs-PP2Cs complexes are less stable and only in presence of ABA, affinity of PYLs to target PP2Cs considerably increase. However, both ABA-dependent and ABA-independent interactions between monomeric PYR/PYL/RCARs receptors and PP2Cs can be observed in Y2H system. It is possible that we are not only testing PYLs-PP2Cs ABA-independent interactions since several fungi produce small amounts of ABA (Nambara and Marion-Poll, 2005). Hence low levels of endogenous ABA from yeast might be sufficient to promote monomeric PYLs–PP2Cs interactions. Next, we performed additional PP2C interaction Y2H assays with other members of the Subclass I of PYR/PYL/RCAR receptors, such as PYL7, PYL9 and PYL10 to compare their PP2C interactions with PYL8 (Figure 3.4B, C and D). For instance, PYL7, PYL9 and PYL10, which similarly to PYL8 have monomeric nature, showed ABA-independent interactions with PP2Cs (with exception of HAB1, ABI2 in case of PYL7, and AHG1, in case of PYL9). However, in difference to PYL8, all tree receptors, PYL7, PYL9 and PYL10, showed less PP2C interaction partner range. Finally, to expanding our understanding of PYL8 specificity, we completed PP2Cs Y2H interaction analysis of PYL1 and PYL4, (members of Subclass III and II, respectively), which have similar root expression

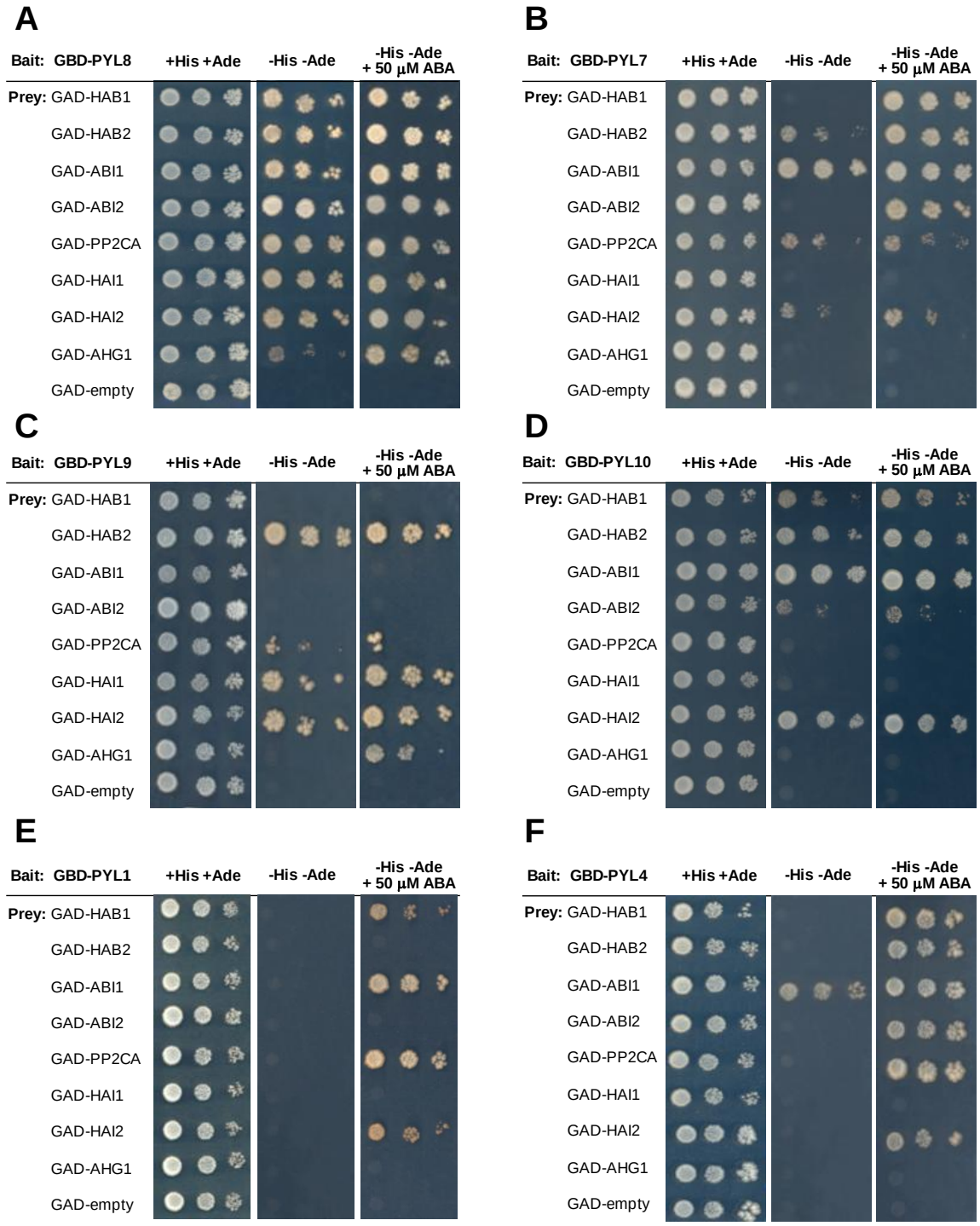

Figure 3.4 Different PP2C interactions of PYL8 and other PYR/PYL/RCAR ABA receptors ABA receptor in Y2H assay. Interaction was determined by growth assay on media lacking histidine and adenine (–His, –Ade), which were supplemented or not with 50 µM ABA (+ABA). Dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of saturated cultures were spotted onto the plates. GAD, GAL4 activation domain; GBD, GAL4 DNA binding domain.

patterns with PYL8 (Figure 3.4E and F). PYL1, as a typical dimeric receptor from subclass III, showed ABA-dependent interactions with HAB1, ABI1, PP2CA and HAI2 (Figure 3.4E). PYL4, which is predicted to be monomeric receptor from Subclass II, needed the addition of exogenous ABA to interact with most PP2Cs, such as HAB1, HAB2, ABI2, PP2CA and HAI2 (with the exception of ABI1) (Figure 3.4F). Overall, these results from Y2H assays suggest that

PYL8 has the wide range of interaction with PP2Cs than other ABA receptors, what is compatible with the putative key role of PYL8 in root ABA signaling.

On the other hand, an additional difference among different PYR/PYL/RCAR family members is their capacity to inhibit PP2C activity at different ABA concentrations. Previously *in vitro* assays have been shown that PYL8 has higher capacity (lower inhibitory concentration 50) to inhibit *in vitro* certain PP2Cs (ABI1, PP2CA, HAI1) than other PYR/PYLs (Santiago et al., 2009b; Antoni et al., 2012). This fact suggested us that *pyl8* phenotype might be due to enhanced *in vivo* activity of PP2Cs. To test this hypothesis, we seek for genetic evidences by crossing *pyl8-1* mutant with the *hab1-1abi1-2pp2ca-1 PP2Cs* triple mutant to generate different mutant combinations of *pyl8-1* with *hab1-1*, *abi1-2*, *pp2ca-1*. Selected PP2Cs belongs to different clade A PP2Cs families and have strong effects on ABA sensitivity. Previous analysis of single, double, and triple combinations of these *pp2c* mutants showed the progressive increase on ABA sensitivity in root growth response. In fact, the triple *hab1-1abi1-2pp2ca-1* triple mutant is extremely sensitive to exogenous ABA in root growth (Rubio et al., 2009). As a result, we show that reduced sensitivity of *pyl8-1* to ABA-mediated inhibition of root growth was notably diminished when *pyl8-1* was combined either with *abi1-2*, *hab1-1* or *pp2ca-1* (Figure 3.5). Therefore, these results are in agreement with the putative function of PYL8 as a master regulator of clade A PP2Cs activity and reveal genetic interaction of PYL8 with different clade A PP2Cs.

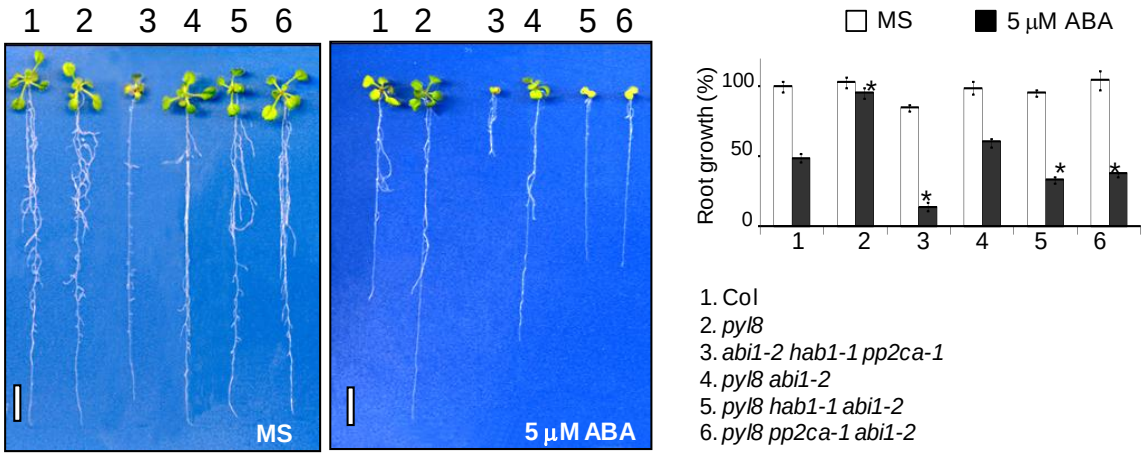

Figure 3.5 Genetic evidence of PYL8 interaction with clade A PP2Cs. A, The reduced sensitivity of *pyl8* to ABA-mediated inhibition of root growth is abrogated by knocking out clade A PP2Cs. Quantification of ABA-mediated root growth inhibition of the indicated genotypes compared with the wild type. Data are averages ± SE from three independent experiments (n = 15 each). * P < 0.01 (Student's t test) with respect to the wild type in the same growth conditions. Photographs show representative seedlings 10-d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 5 µM ABA. Bars = 1 cm.

Identification of PYL8 interacting proteins *in vivo* by TAP approach

To further study the mechanism of action of PYL8 through PP2Cs and other putative targets, we followed TAP biochemical approach to identify PYL8 interacting proteins *in vivo* using *Arabidopsis* suspension cells culture that express a GS tagged PYL8 bait (Van Leene et al., 2008). The GS tag combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two tobacco TEV cleavage sites, and it has proved to be a good tag for TAP approaches (Van Leene et al., 2008). Previously, PYL8 and other PYR/PYL/RCAR ABA-receptors have been recovered *in vivo* as ABI1-interacting proteins using affinity protein complex purifications and subsequent identification by MS analyses (Nishimura et al., 2010). Therefore, it has been demonstrated that a single PP2C interacts *in vivo* with different ABA receptors. We wondered whether a single ABA-receptor might be able to interact *in vivo* with different PP2Cs. To this end, we performed TAP of protein complexes in *Arabidopsis* suspension cells culture that express PYL8 fused to the N- or C-terminal GS tag (GS-PYL8 and PYL8-GS, respectively) as bait, and subsequently, we proceeded to the identification of

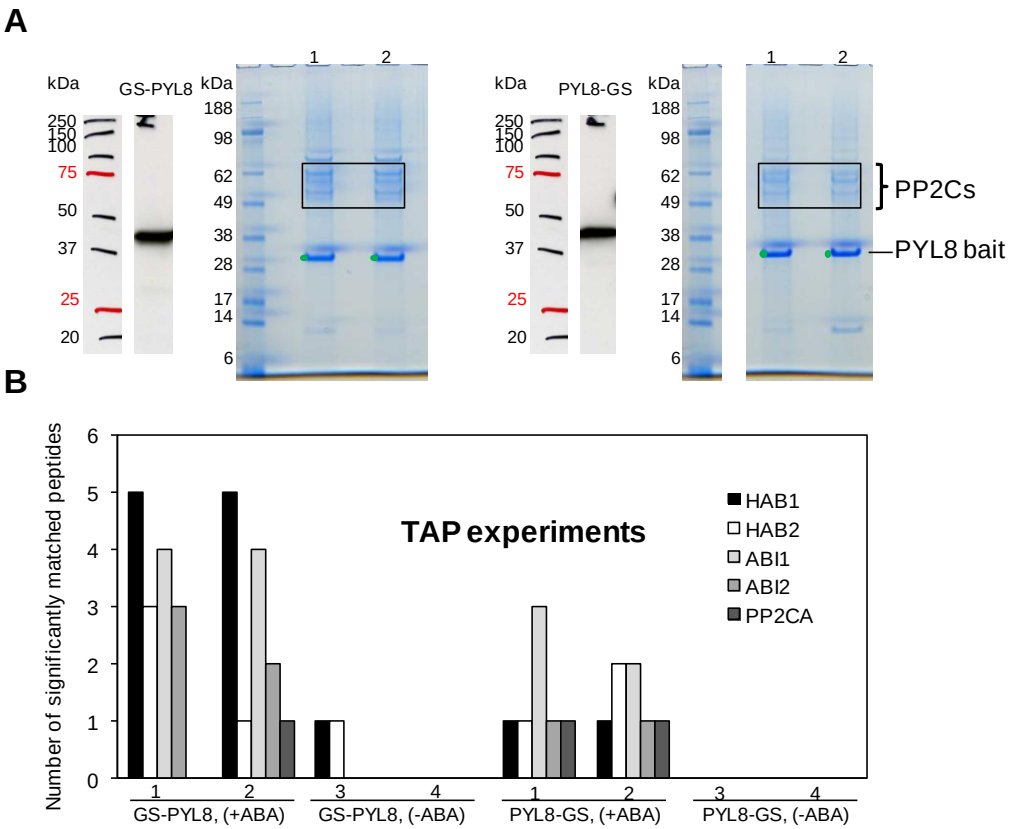

Figure 3.6 Biochemical evidence of PYL8 interaction with clade A PP2Cs. A, Clade A PP2Cs interact in vivo with PYL8 in the presence of ABA. GS-PYL8 and PYL8-GS interact with clade A PP2Cs expressed in Arabidopsis cell suspension cultures. The SDS-PAGE analysis shows the zone were HAB1/HAB2 (two upper bands) and ABI1/ABI2/PP2CA (lower bands) were recovered as interacting partners of PYL8 when extracts and TAP purification buffers were supplemented with 50 µM ABA. C, Quantification of significantly (p>95%) matched peptides of clade A PP2Cs recovered in independent TAP experiments using either GS-PYL8 or PYL8-GS as baits. ABA supplementation (50 µM, +ABA) dramatically increased the recovery of clade A PP2Cs compared to samples lacking ABA supplementation (-ABA). Detailed results of the peptides identified by mass-spectrometrical analyses are provided as table 1.

interacting partners by MS analyses (Table 1). The *Arabidopsis* suspension cells cultures used in TAP experiments were supplemented with ABA to prepare protein extracts but they were not grown in the presence of ABA to avoid growth impairment and overexpression of the other PP2Cs. Recombinant GS-PYL8 or PYL8-GS protein production was confirmed by immunoblot analysis (Figure 3.6A). PYL8-bound complexes were recovered from both cultures (two technical repeats per culture) treated with 50 µM ABA and finally they were eluted from streptavidin beads and analyzed by SDS-PAGE (Figure 3.6A). MALDI-TOF/TOF-MS analysis of these complexes revealed that five clade A PP2Cs, i.e. HAB1, HAB2, ABI1, ABI2 and PP2CA/AHG3, were able to interact with PYL8 in samples supplemented with 50 µM ABA (Figure 3.6A and B; Table 1). Therefore, these results provide evidence that a PYR/PYL ABA receptor can form complexes with several clade A PP2Cs *in vivo* in *Arabidopsis*.

We did not recover any ABA-activated SnRK2 in these complexes, which suggests that the ABA-dependent interaction of PYL8 with the PP2Cs effectively competed *in planta* with the interaction of PP2Cs and SnRK2s or alternatively, that the residual interaction of the SnRK2s (via the ABA box) and PP2Cs was not enough to withstand the double purification of the TAP procedure. When samples were not supplemented with exogenous ABA, only one peptide that significantly matched both to HAB1 and HAB2 (VIQWQGAR, identical sequence in both PP2Cs) could be recovered from PYL8-interacting proteins in one experiment (Figure 3.6B; Table 1). Thus, exogenous ABA supplementation dramatically increased the recovery of PP2Cs as PYL8-interacting proteins in TAP complexes.

Table 1. Summary of results obtained in TAP experiments and protein identification details using the 4800 MALDI-TOF/TOF Proteomics Analyzer (AB SCIEX) Explorer version 6 (AB SCIEX) software package. Column headers for protein and peptide data are explained in Material and Methods

		Identified proteins			PMF data					MSMS data												
Condition	TAP Experiment	Locus	Name	Found in two TAPs	Protein Score	Expect	RMS error (ppm)	Sequence coverage %	Unique Peptides	Total Ion Score	Peptide Number	Start	End	Observed	Mr(Exp)	Mr(Calc)	Delta	Miss	Ions Score	Expect	Peptide	Variable Modification
without ABA	GS-PYL8	AT5G53160	PYL8	2	1830	3,5E-77	4	67	27	1529	1	60	66	910,5151	909,5078	909,5072	0,0001	0	94	5,90E-04	YKPFISR	
											2	53	59	918,4771	917,4698	917,4719	-0,0054	1	74	7,30E-03	RFDQPQK	
											3	154	165	1487,704	1486,6967	1486,7014	-0,0021	1	35	7,30E-03	DETCYFVEALIK	
											4	39	52	1640,9413	1639,934	1639,9311	-0,0007	0	247	9,61E-11	HINAPVHIVWSLVR	
											5	96	109	1679,8955	1678,8882	1678,889	-0,0001	0	174	1,67E-07	LELLDDNEHILSIR	
											6	119	134	1815,9237	1814,9164	1814,9162	-0,0043	0	240	1,53E-10	NYSSIISLHPETIEGR	
											7	135	153	2017,0839	2016,0766	2016,0779	0,02335	0	168	1,10E-06	IGTLVIESFVVDVPEGNTK	
											8	22	38	2032,0254	2031,0181	2030,9956	-0,001	1	148	4,90E-04	HHKHELVDNQCSSTLVK	
											9	117	134	2057,1023	2056,095	2056,0953	0,0003	1	179	1,02E-07	LKNYSSIISLHPETIEGR	
											10	92	109	2153,1211	2152,1138	2152,1124	0,0006	1	169	2,10E-06	STERLELLDDNEHILSIR	
		AT1G72770	HAB1	1	55	0,12	8	16	6	31	1	382	389	957,5246	956,5173	956,5192	-0,0019	0	31	2,00E-02	VIQWQGAR	
		AT1G17550	HAB2	1	59	0,048	3	18	7	31	1	382	389	957,5246	956,5173	956,5192	-0,0019	0	31	2,00E-02	VIQWQGAR	
	PYL8-GS	AT5G53160	PYL8	2	1745	1,8E-78	6	68	29	1421	1	60	66	910,5165	909,5092	909,5072	0,00185	0	85	1,54E-03	YKPFISR	
											2	154	165	1487,7041	1486,6968	1486,7014	-0,004	0	162	3,04E-07	DETCYFVEALIK	
											3	39	52	1640,9432	1639,9359	1639,9311	0,00365	0	234	2,51E-10	HINAPVHIVWSLVR	
											4	96	109	1679,8947	1678,8874	1678,889	-0,0014	0	189	2,10E-07	LELLDDNEHILSIR	
											5	119	134	1815,9232	1814,9159	1814,9162	0,0015	0	266	2,40E-12	NYSSIISLHPETIEGR	
											6	135	153	2017,0792	2016,0719	2016,0779	-0,0063	0	158	2,31E-06	IGTLVIESFVVDVPEGNTK	
											7	117	134	2057,1001	2056,0928	2056,0953	-0,0007	1	174	1,26E-07	LKNYSSIISLHPETIEGR	
											8	92	109	2153,1221	2152,1148	2152,1124	0,00275	1	153	1,50E-05	STERLELLDDNEHILSIR	
with ABA	GS-PYL8	AT5G53160	PYL8	2	1594	4,5E-70	6	63,5	28	1280	1	60	66	910,5192	909,5119	909,5072	0,00305	0	89	9,90E-04	YKPFISR	
											2	154	165	1487,6979	1486,6906	1486,7014	-0,0056	0	85	1,70E+00	DETCYFVEALIK	
											3	39	52	1640,9434	1639,9361	1639,9311	0,00195	0	211	2,50E-07	HINAPVHIVWSLVR	
											4	119	134	1815,9263	1814,919	1814,9162	0,0039	0	254	5,80E-12	NYSSIISLHPETIEGR	
											5	135	153	2017,0812	2016,0739	2016,0779	-0,0035	0	187	3,20E-07	IGTLVIESFVVDVPEGNTK	
											6	117	134	2057,1047	2056,0974	2056,0953	0,0026	1	219	5,80E-08	LKNYSSIISLHPETIEGR	
											7	135	165	3485,7317	3484,7244	3484,7688	-0,025	1	132	2,40E-02	IGTLVIESFVVDVPEGNTKDETCYFVEALIK	
											8	96	109	1679,8933	1678,886	1678,889	-0,0029	0	77	3,20E-07	LELLDDNEHILSIR	
											9	53	59	918,4787	917,4714	917,4719	-0,0005	1	13	1,70E+00	RFDQPQK	
		AT1G72770	HAB1	2	778	2,20089E-33	6	34	23	596	1	382	389	957,5319	956,5246	956,5192	0,00545	0	72	2,46E-02	VIQWQGAR	
											2	390	398	995,5356	994,5283	994,527	-0,0044	0	30	3,09E+00	VFGVLAMSR	Oxidation (M)
											3	258	268	1327,703	1326,6957	1326,6932	0,0026	0	150	1,49E-06	LHFALAEEIER	
											4	200	214	1723,7882	1722,7809	1722,7923	-0,0039	0	82	2,94E-03	SEMEDAFAVSPHFLK	Oxidation (M)
											5	404	420	2079,085	2078,0777	2078,091	-0,0086	0	81	1,02E-02	YLKPYVIPEPEVTFMPR	
											6	404	420	2095,094	2094,0867	2094,086	-0,0045	0	25	5,14E+00	YLKPYVIPEPEVTFMPR	Oxidation (M)
											7	180	199	2307,1404	2306,1331	2306,1365	-0,0013	0	166	9,91E-07	SVYELDCIPLWGTVSIQGNR	
											8	469	488	2152,051	2151,0437	2151,034	0,0097	0	5	6,30E+00	GIDPACQAAADYLSMLALQK	Oxidation (M)
											9	316	348	3373,7205	3372,7132	3372,6654	0,0478	0	1	7,90E+00	VLEAVASETVGSTAVVALVCSSHIVVSNCGDSR	
		AT1G17550	HAB2	2	282	6,60E-04	5	18	13	211	1	382	389	957,5326	956,5253	956,5192	-0,0006	0	23	9,40E+00	VIQWQGAR	
											2	179	198	2310,1287	2309,1214	2309,1184	-0,0042	0	63	3,61E-02	SVYELECIPLWGTISICGGR	
											3	399	420	2550,2473	2549,24	2549,2512	0,0038	0	49	1,76E-01	SIGDQYLEPFVIPDPEVTFMPR	

		Identified proteins			PMF data					MSMS data													
	TAP				Protein	Expect	RMS error	Sequence	Unique	Total	Peptide								Ions				Variable
Condition	Experiment	Locus	Name	Found in two TAPs	Score		(ppm)	coverage %	Peptides	Ion Score	Number	Start	End	Observed	Mr(Exp)	Mr(Calc)	Delta	Miss	Score	Expect	Peptide	Modification
with ABA	GS-PYL8	AT4G26080	ABI1		545	1,822E-22	4	27	20	382	1	297	304	943,5097	942,5024	942,5035	-0,0031	0	64	2,61E-02	VIQWNGAR	
											2	220	227	967,5398	966,5325	966,5287	0,00125	0	37	4,90E-01	ALFNSFLR	
											3	305	313	979,537	978,5297	978,5321	-0,0024	0	55	1,16E-01	VFGVLAMSR	
											4	305	313	995,5238	994,5165	994,527	-0,0096	0	39	9,70E-01	VFGVLAMSR	Oxidation (M)
											5	151	163	1400,6483	1399,641	1399,6402	-0,0013	0	52	1,90E+00	FLQSSSGSMLDGR	Oxidation (M)
											6	125	137	1456,7273	1455,72	1455,718	-0,0052	0	85	2,00E-03	SVPLYGFTSICGR	
											7	319	335	1926,1104	1925,1031	1925,0986	1E-04	1	70	1,22E-02	YLKPSIIPDPEVTAVKR	
											8	164	189	2887,282	2886,2747	2886,2569	0,01155	0	20	2,20E+00	FDPQSAAHFFGVYDGHGGSQVANYCR	
		AT5G57050	ABI2		423	6,7E-17	6	23	12	339	1	295	303	979,5221	978,5148	978,5321	-0,0106	0	66	1,12E-01	VFGVLAMSR	
											2	295	303	995,5343	994,527	994,527	-0,0013	0	15	1,29E+01	VFGVLAMSR	Oxidation (M)
											3	135	147	1408,7443	1407,737	1407,7358	0,0036	0	164	8,98E-07	FLQVSSSSLLDGR	
											4	109	121	1481,7205	1480,7132	1480,7167	0,0002	0	103	2,72E-03	CVPLYGVTSICGR	
											5	148	177	3308,4817	3307,4744	3307,5007	-0,0262	0	4	2,00E+00	VTNGFNPHLSAHFFGVYDGHGGSQVANYCR	
		AT3G11410	PP2CA	1	129	4,5E-09	11	19	5	98	1	277	284	979,5221	978,5148	978,4923	0,0225	0	13	1,50E+00	VIYWDGAR	
											2	58	76	2042,0692	2041,0619	2041,0692	-0,0072	0	74	7,40E-07	ETVVLSTLPGNLDLDSNVR	
											3	249	269	2341,2583	2340,251	2340,255	-0,004	0	11	7,00E-01	NGVAIPLSVDHKPDRPDELIR	
	PYL8-GS	AT5G53160	PYL8	2	1488	3,5E-61	6	62	25	1219	1	60	66	910,5187	909,5114	909,5072	0,0029	0	89	1,30E-03	YKPFISR	
											2	39	52	1640,9457	1639,9384	1639,9311	0,0066	0	257	2,44E-12	HINAPVHIVWSLVR	
											3	117	134	2057,0959	2056,0886	2056,0953	-0,0017	1	238	1,41E-09	LKNYSSIISLHPETIEGR	
											4	92	109	2153,1199	2152,1126	2152,1124	0,00555	1	185	4,70E-08	STERLELLDDNEHILSIR	
											5	119	134	1815,9219	1814,9146	1814,9162	-0,0007	0	249	3,97E-11	NYSSIISLHPETIEGR	
											6	135	153	2017,0959	2016,0886	2016,0779	0,0107	0	99	1,70E-09	IGTLVIESFVVDVPEGNTK	
											7	135	165	3485,8274	3484,8201	3484,7688	0,0514	1	7	1,20E+00	IGTLVIESFVVDVPEGNTKDETCYFVEALIK	
											8	96	109	1679,8943	1678,887	1678,889	-0,0019	0	93	7,70E-09	LELLDDNEHILSIR	
		AT1G72770	HAB1	2	195	5,E-05	9	18	12	115	1	382	389	957,5255	956,5182	956,5192	0,00265	0	8	2,11E+01	VIQWQGAR	
											2	258	268	1327,7076	1326,7003	1326,6932	0,0113	0	94	3,35E-03	LHFALAEEIER	
											3	404	420	2095,105	2094,0977	2094,086	0,0118	0	5	4,40E+00	YLKPYVIPEPEVTFMPR	Oxidation (M)
											4	180	199	2307,1384	2306,1311	2306,1365	-0,0038	0	7	2,19E+01	SVYELDCIPLWGTVSIQGNR	
		AT1G17550	HAB2	2	241	1,E-04	6	13	9	208	1	382	389	957,5283	956,521	956,5192	-0,0008	0	32	1,90E+00	VIQWQGAR	
											2	257	267	1267,6614	1266,6541	1266,6568	-0,004	0	66	6,20E-01	IHSALAEEIER	
											3	179	198	2310,1409	2309,1336	2309,1184	0,0064	0	84	1,71E-02	SVYELECIPLWGTISICGGR	
											4	399	420	2550,2246	2549,2173	2549,2512	-0,0339	0	13	7,50E-01	SIGDQYLEPFVIPDPEVTFMPR	
											5	399	420	2566,2476	2565,2403	2565,2461	-0,0085	0	20	5,16E+00	SIGDQYLEPFVIPDPEVTFMPR	Oxidation (M)
		AT4G26080	ABI1	2	454	5,E-15	6	28	18	318	1	297	304	943,5164	942,5091	942,5035	0,00305	0	58	6,70E-02	VIQWNGAR	
											2	220	227	967,5356	966,5283	966,5287	-0,0004	0	12	1,57E+00	ALFNSFLR	
											3	305	313	979,5364	978,5291	978,5321	-0,0077	0	41	7,20E-01	VFGVLAMSR	
											4	305	313	995,5297	994,5224	994,527	-0,004	0	32	1,62E+00	VFGVLAMSR	Oxidation (M)
											5	125	137	1456,7272	1455,7199	1455,718	0,00655	0	106	2,34E-04	SVPLYGFTSICGR	
											6	319	335	1926,1021	1925,0948	1925,0986	-0,0042	1	91	2,91E-03	YLKPSIIPDPEVTAVKR	
											7	164	189	2887,2651	2886,2578	2886,2569	-0,0044	0	7	4,60E+00	FDPQSAAHFFGVYDGHGGSQVANYCR	
		AT5G57050	ABI2	2	128	3,E-01	4	13	4	55	1	135	147	1408,7354	1407,7281	1407,7358	-0,0006	0	65	2,99E-02	FLQVSSSSLLDGR	
											2	109	121	1481,7166	1480,7093	1480,7167	-0,0074	0	19	2,50E-01	CVPLYGVTSICGR	
		AT3G11410	PP2CA	2	159	1,70E-02	6,5	14,5	8	112	1	277	284	979,5063	978,499	978,4923	0,00485	0	33	1,55E+00	VIYWDGAR	
											2	58	76	2042,0632	2041,0559	2041,0692	-0,0122	0	72	1,72E-02	ETVVLSTLPGNLDLDSNVR	
											3	294	315	2475,2612	2474,2539	2474,2693	-0,0154	0	6	3,90E+00	AIGDNYLKPYVIPDPEVTVTDR	

3.3MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4ºC for 3 days. Then, seeds were sowed on Murashige-Skoog (1962) (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 µE m^{-2} sec^{-1}. The T-DNA insertion line *pyl8-1* have been described previously (Park et al., 2009; Lackman et al., 2011; Gonzalez-Guzman et al., 2012). The *abi1-2 abi2-2 hab1-1 pp2ca-1* quadruple mutant was generated by crossing two triple pp2c mutants described in Rubio et al., (2009). The *pyl8-1* allele was crossed with the *abi1-2 hab1-1 pp2ca-1* triple mutant to generate different combinations of *pyl8* and *pp2c* knockout alleles.

Root growth assays.

Seedlings were grown on vertically oriented MS plates for 4 to 5 days. Afterwards, 20 plants were transferred to new MS plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10-d to produce image files suitable for quantitative analysis of root growth using the NIH Image software ImageJ v1.37.

Yeast Two-Hybrid assay

The full-length coding sequences of PYR/PYL/RCAR ABA receptors from *Arabidopsis* were amplified by PCR using the primers described in Table 2 and cloned into the pCR8/GW/TOPO entry vector (Invitrogen), recombined by LR reaction into the pGBKT7GW vector to generate in-frame fusions with the GAL4 DNA-binding domain (GBD). The pGADT7-HAB1/HAB2/ABI1/ABI2/PP2CA/HAI1/HAI2/AHG1 constructs fused to the GAL4 activation domain (GAD) was previously described (Santiago et al., 2009b; Vlad et al., 2010; Pizzio et al., 2013; Gonzalez-Guzman et al., 2014). For Y2H assays, pGBKT7 constructs where PYR/PYL/RCARs acted as baits were faced with the PP2Cs PGADT7 constructs as preys, following protocol similar to those described previously (Saez et al., 2008).

Tandem Affinity Purification (TAP)

Cloning of transgenes encoding GS-tagged fusions under control of the constitutive cauliflower tobacco mosaic virus 35S promoter and transformation of *Arabidopsis* cell suspension cultures were carried out as previously described (Van Leene et al., 2007). Briefly, the GS tag combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two tobacco etch virus (TEV) cleavage sites. In GS-PYL8, the two IgG-binding domains of protein G are placed in front of the streptavidin-binding peptide; in PYL8-GS, the order of these domains is opposite. Tandem affinity purification of protein complexes was done using the GS tag (Buerckstuemmer et al., 2006) followed by protein precipitation and separation, according to Van Leene et al. (2008). For the protocols of proteolysis and peptide isolation, acquisition of mass spectra by a 4800 MALDI TOF/TOF Proteomics Analyzer (AB SCIEX), and MS-based protein homology identification based on the TAIR genomic database, we refer to Van Leene et al. (2010). Experimental background proteins were subtracted based on approximately 40 TAP experiments on wild type cultures and cultures expressing TAP-tagged mock proteins GUS, RFP and GFP (Van Leene et al., 2010).

Protein Identification via MALDI/TOF/TOFTM

Results obtained in TAP experiments and peptide identification were obtained with 4800 MALDI TOF/TOFTM Proteomics analyzer (AB SCIEX) and the GPS explorer v3.6 (AB SCIEX) software package with search engine Mascot version 2.2 (Matrix Science) and database TAIR10. Four TAP experiments (two samples each) were performed using N-terminal (Nm) GS-tagged PYL8, two in the presence of 50 μM supplemented ABA and two in the absence of exogenous ABA. Additionally, four TAP experiments were performed using C-terminal (Cm) SG-tagged PYL8, in the same conditions as above. The table shows the significantly (p>95%) matched peptides recovered in the experiments. Column headers for Protein and Peptide data are explained below. **Protein score:** The score calculated by the Mascot search engine for each protein. This score is based on the probability that peptide mass matches are non-random events. If the Protein Score is equal to or greater than the Mascot®Significance Level calculated for the database search, the protein match is considered to be statistically non-random at the 95% confidence interval. Protein score = -10*Log(P), where P is the probability that the observed match is a random event. **Expect**: Protein score expectation value. **RMS error (ppm):** RMS error of the set of matched mass values, in ppm. **Sequence coverage %:** Percentage of protein sequence covered by assigned peptide matches. **Unique peptides**: The number of peptides with unique sequences matching the selected protein. **Total Ion Score**: A score calculated by weighting Ion Scores for all individual peptides matched to a given protein. **Peptide Number:** Peptide index number within the list of peptides associated with a given

protein. **Start**: The starting position of the peptide in the protein. **End:** The ending position of the peptide in the protein. **Observed**: The observed monoisotopic mass of the peptide in the spectrum (m/z). **Mr (Exp)**: The experimental mass of the peptide calculated from the observed m/z value. **Mr (Calc)**: The theoretical mass of the peptide based on its sequence. **Delta (Da)**: The difference between the theoretical (Mr (Calc)) and experimental (Mr (Exp)) masses, in daltons. **Miss**: Number of missed Trypsin cleavage sites. **Ions score**: The Ions Score is calculated by the Mascot search engine for each peptide matched from MS/MS peak lists. This score is based on the probability that ion fragmentation matches are non-random events. If the Ion Score is equal to or greater than the Mascot® Significance Level calculated for the database search, the peptide match is considered to be statistically non-random at the 95% confidence interval. Ions score = -10*Log(P), where P is the probability that the observed match is a random event. **Best Ions score:** The highest individual Ion Score for a given protein identification. **Expect**: Ions score expectation value. **Peptide:** The amino acid sequence of the selected peptide. **Variable Modification**: Variable modification type on the peptide.

Accession numbers

The *Arabidopsis* Genome Initiative locus identifiers for *PYR1, PYL1, PYL2, PYL3, PYL4, PYL5, PYL6, PYL7, PYL8, PYL9, PYL10, PYL11, PYL12* and *PYL13* are, *At4g17870, At5g46790, At2g26040, At1g73000, At2g38310, At5g05440, At2g40330, At4g01026, At5g53160, At1g01360, At4g27920, At5g45860, At5g45870 and At4g18620*, respectively.

Table 2. List of oligonucleotides used in this work

PCR	Primer name	Sequence 5'→ 3'
pyl8-1	FwMEANpyl8	ATGGAAGCTAACGGGATTGAG
	RvESRVpyl8	TTAGACTCTCGATTCTGTCGT
T-DNA	LB3SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC
abi1-2	Fabi1-2	AGGAAACCCTTATTGAAATTC
	Rabi1-2	CTCTGTTCTGCTGATCATCT
T-DNA	LBpROK2	GCCGATTTCGGAACCACCATC
hab1-1	FHAB1	AACTGCTGTTGTTGCCTTG
	RHAB1	GGTTCTGGTCTTGAACTTTCT
T-DNA	LBpROK2	GCCGATTTCGGAACCACCATC
pp2ca-1	FDNPP2CA	AATTCTGTTACGGAAGCAGAGA
	RPP2CA	GTCGACTTAAGACGACGCTTGATTATTC
T-DNA	LBpROK2	GCCGATTTCGGAACCACCATC
TAP constructs	Primer name	Sequence 5'→ 3'
	FattBPYL8	AAAAAGCAGGCTCCACCATGGAAGCTAACGGGATTGAG
	RPYL8StopattB	AGAAAGCTGGGTCTTAGACTCTCGATTCTGTCG
	RPYL8attB	AGAAAGCTGGGTCTGAGACTCTCGATTCTGTCG
Y2H	Primer name	Sequence 5'→ 3'
PYL1	FNco5g46790	ACCATGGCGAATTCAGAGTCCTC
	RBamHI46790	GGATCCTTACCTAACCTGAGAAGAGTTGT
PYL4	FNco2g38310	ACCATGGAGCACGTGGAGCTTTCCCAC
	R2g38310	CGCACGAATTCACAGAGACATCTTCTTCTT

PYL7	FPYL7	ACCATGGAGATGATCGGAGGAGAC
	RPYL7	TCAAAGGTTGGTTTCTGTATGATTC
PYL8	FCDS5g53160	ATGGAAGCTAACGGGATTGAG
	RCDS5g53160	TTAGACTCTCGATTCTGTCGT
PYL9	FwNcoIPYL9	ACCATGGTGGACGGCGTTGAAGGC
	RVPYL9	TCACTGAGTAATGTCCTGAGA
PYL10	FPYL10	ACCATGGACGGTGACGAAACAAAGAAG
	RPYL10	TCATATCTTCTTCTCCATAGATTC

4. CHAPTER II

Identification and characterization of C2-domain CAR proteins as new plasma membrane interacting partners of PYR/PYL/RCAR ABA receptors, which positively regulate abscisic acid sensitivity

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Identification and characterization of C2-domain CAR proteins as new plasma membrane interacting partners of PYR/PYL/RCAR ABA receptors, which positively regulate abscisic acid sensitivity

4.1 INTRODUCTION

Abscisic acid (ABA) elicits plant responses through binding to soluble PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptors, which constitute a 14-member family. PYR/PYL/RCAR receptors perceive ABA intracellularly and as a result, form ternary complexes with clade A PP2Cs, thereby inactivating them (Park et al., 2009; Ma et al., 2009). This allows the activation of downstream targets of the PP2Cs, such as the SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASES (SnRKs) subfamily 2, i.e. SnRK2.2/D, 2.3/I and 2.6/OPEN STOMATA1 (OST1)/E, which are key players in the regulation of transcriptional response to ABA and stomatal aperture (Umezawa et al., 2009; Fujita et al., 2009; Vlad et al., 2009; Fujii and Zhu, 2009). Additional targets of clade A PP2Cs have been described, such as SnRK1, SnRK3s/calcineurin B-like (CBL)-interacting protein kinases (CIPKs), calcium-dependent protein kinases (CDPKs/CPKs), ion transporters such as the K+ channel AKT1 and AKT2 or the slow anion channel 1 (SLAC1) and SLAC1 homolog 3 (SLAH3), and transcriptional regulators such as bZIP transcription factors and chromatin-remodeling complexes (Guo et al., 2002; Cherel et al., 2002; Lee et al., 2007; Saez et al., 2008; Lee et al., 2009; Geiger et al., 2009; Brandt et al., 2012; Antoni et al., 2012; Lynch et al., 2012; Pizzio et al., 2013; Rodrigues et al., 2013). Some of these interactions have been shown to be modulated by PYR/PYL/RCAR receptors (Geiger et al., 2010; 2011; Brandt et al., 2012; Pizzio et al., 2013; Rodrigues et al., 2013). Therefore, clade A PP2Cs act as key negative regulators of ABA signaling and as a hub for regulation of different environmental responses.

Genetic evidence about the function of PYR/PYLs indicates they play a major role in quantitative regulation of ABA responses, affecting both seed and vegetative responses to ABA (Park et al., 2009; Nishimura et al., 2010; Gonzalez-Guzman et al., 2012; Antoni et al., 2013). Analysis of their gene expression patterns together with their biochemical and genetic characterization have served to establish common and divergent properties of PYR/PYL ABA receptors (Dupeux et al., 2011b; Hao et al., 2011; Gonzalez-Guzman et al., 2012). Analyses of combined *pyr/pyl* mutants indicate that PYR/PYL function is partially redundant; however, PYL8 plays a nonredundant role to regulate root sensitivity to ABA (Antoni et al., 2013).

Structural and biochemical studies also reveal several divergences among PYR/PYLs, particularly with respect to oligomeric structure and perception of chemical agonists (Dupeux et al., 2011a; Hao et al., 2011; Okamoto et al., 2013). Activation of dimeric receptors by the ABA agonist quinabactin, which also activates to some extent PYL5 and PYL7, is enough to elicit both seed and vegetative responses to ABA (Okamoto et al., 2013; Cao et al., 2013).

Regulation of cellular processes involves intermolecular interactions that alter the location and/or activity of signaling proteins and cellular membranes are a platform for intracellular communication involving lipid-protein and protein-protein complexes (Cho and Stahelin, 2005; Scott and Pawson, 2009). PYR/PYL proteins are intracellular ABA receptors localized both at the cytosol and nucleus; however, detailed knowledge of their subcellular localization or putative transient interactions with membrane systems of the cell is currently lacking. Cytosolic proteins can reside partially in vesicles as peripheral proteins or transiently interact with membranes for trafficking or signaling purposes driven by protein modules that recognize specific features of proteins or membranes (Cho and Stahelin, 2005; Seet et al., 2006; Lemmon, 2008). In addition to the plasma membrane, eukaryotic cells possess an elaborate membrane system with multiple intracellular membranes, e.g. at the nucleus, organelles, endocytic and secretory pathways (Mellman and Emr, 2013; Voeltz and Barr, 2013). Thus, lipid bilayers take part in a myriad of processes in the plant cell and cytosolic/nuclear proteins can interact transiently with membranes for signaling, transport or other purposes (Cho et al., 2005; Voeltz and Barr, 2013). PYR/PYL ABA receptors, together with clade A PP2Cs and ABA-activated SnRK2s, play a key role in the regulation of ion transporters and membrane-associated enzymes that generate second messengers involved in ABA signaling (Geiger et al., 2009; Sirichandra et al., 2009; Sato et al., 2009; Lee et al., 2009; Cutler et al., 2010; Geiger et al., 2011); however, it is not understood how PYR/PYL proteins (or PP2Cs/SnRK2s) can reach the proximity of cellular membranes beyond random diffusion. It is possible that auxiliary proteins might be involved in approaching transiently receptor, phosphatase or kinase complexes next to cellular membranes, where early ABA signaling events take place. Recently, it was shown that ABA signaling modulates through ABSCISIC ACID INSENSITIVE1 (ABI1) and PYL9 the association of the signaling and transport complex CPK21/SLAH3 within plasma membrane domains, which is reminiscent of animal lipid rafts (Demir et al., 2013). These results imply that PYL9 must be able to inhibit ABI1 in the proximity of lipid nanodomains to allow the activation of SLAH3 by CPK21 (Demir et al., 2013).

In order to identify putative regulatory proteins of PYR/PYL receptors in *Arabidopsis thaliana*, e.g. auxiliary proteins that might regulate their subcellular localization or activity, we performed a Yeast Two-Hybrid (Y2H) screening using PYL4 as bait. PYL4, a representative member of the PYR/PYL family, shows high expression levels in different tissues, and its inactivation is required to generate strongly ABA-insensitive combined *pyr/pyl* mutants (Park et

al., 2009; Gonzalez-Guzman et al., 2012). The search for new interacting partners of PYL4 resulted in the discovery of a family of small proteins containing a lipid-binding C2 domain, named CAR proteins for C2-domain ABA-related protein, which interact with PYR/PYLs and positively regulate ABA sensitivity. The C2 domain comprises approximately 130 residues and was first identified in protein kinase C (PKC), located between the C1 domain and the PKC catalytic domain (Nishizuka, 1988). The C2 domains of classical PKCs bind to phospholipid membranes in a calcium-dependent manner and are involved in targeting PKC activity to cell membranes in response to extracellular signals (Guerrero-Valero et al., 2007). C2 domains share functional characteristics with annexins, which also bind phospholipids in a calcium-dependent manner, but they are structurally unrelated (Lemmon et al., 2008). C2 domains are usually found in a large variety of eukaryotic proteins, where the C2 module is combined with a wide range of other modules encoding different enzymatic activities involved in intracellular signal transduction and membrane trafficking (Zhang and Aravind, 2010). The C2 domain acts in these proteins as a Ca^{2+}-activated module that promotes targeting to membranes of the catalytic activity encoded in another region of the polypeptide. However, small C2-domain proteins, such as CAR proteins, that lack additional catalytic domains have also been identified in plants (Kim et al., 2003; Yokotani et al., 2009; Wang et al., 2009). Finally, the C2 domain and the EF-hand motif are the two most frequently occurring calcium sensors, and at least 123 proteins contain C2 domains in *Arabidopsis* (http://smart.embl-heidelberg.de/). However, not all C2 domains are able to bind calcium and some of them have diverged evolutionary into Ca^{2+}-independent lipid-binding variants (Cho and Stahelin, 2006).

4.2 RESULTS

Identification of CAR proteins as interacting partners of PYR/PYLs

We performed a Y2H screening in the absence of ABA using PYL4 as a bait. As a result, we found a PYL4-interacting protein, At5g37740, whose binding to PYL4 was not ABA-dependent (Figure 4.1A). Analysis using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) revealed that At5g37740 belongs to a branch of the C2-domain superfamily represented by single-C2 domain proteins that lack additional catalytic domains (Figure 4.2). Other PYL receptors, such as PYL1, PYL6 or PYL8 were also able to interact in Y2H assays (minus Histidine, minus Adenine) with this small C2-domain protein (Figure 4.1A). Interaction with PYR1 in Y2H assay was only detected under less demanding conditions (minus Histidine). Deletion of the N-terminal region of PYL4, PYL6 and PYL8 severely impaired the interaction with At5g37740 (Figure 4.1B). This N-terminal deletion did not affect the binding of Δ1-47 PYL6 or Δ1-27 PYL8 to HAB1, which reflects that this region

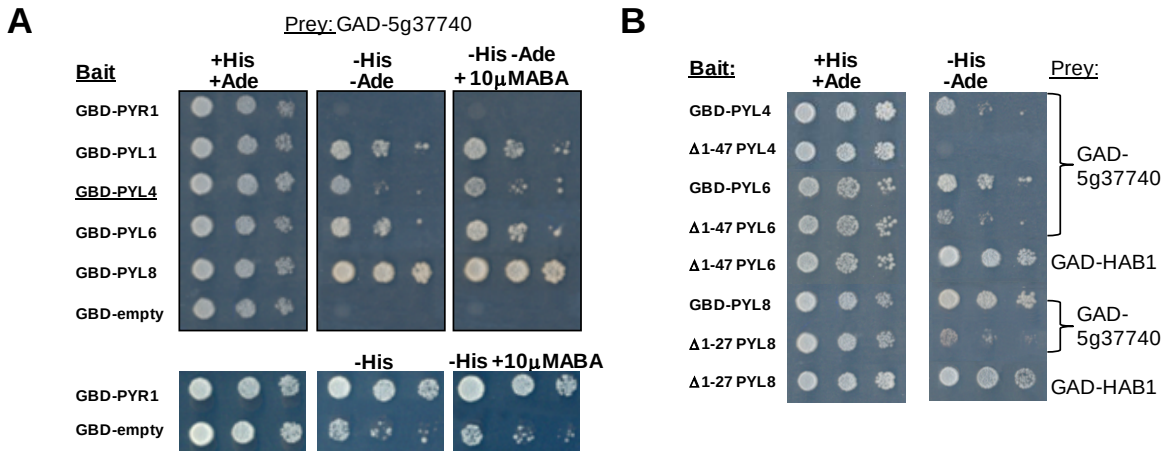

Figure 4.1 PYL4 and Other PYR/PYL Receptors Interact with CAR1 in Y2H Assays. (A) ABA-independent interaction between CAR1 and different PYR/PYLs. Interaction was determined by growth assay on media lacking His and adenine (−His and −Ade), which were supplemented or not with 10 µM ABA. Dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of saturated cultures were spotted onto the plates. GAD, GAL4 activation domain; GBD, GAL4 DNA binding domain.(B) Deletion of the N-terminal region of PYL4, PYL6, and PYL8 impairs the interaction with CAR1 but not with HAB1 (assayed for Δ1-47 PYL6 and Δ1-27 PYL8).

is not involved in the formation of the receptor-ABA-phosphatase complex (Melcher et al., 2009; Miyazono et al., 2009; Dupeux et al., 2011a). We named At5g37740 as CAR1 and BLAST search at TAIR revealed a CAR gene family composed of 10 members (CAR1 to CAR10) in *Arabidopsis* (Figure 4.3). The CAR family was also found in other plant species such as tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) (Figure 4.4). *Arabidopsis* CAR proteins range between 165 to 185 amino acid residues and estimated molecular mass of 18-20 kDa (Figure 4.3).

C2 domains are usually found in mammals as regulatory modules of different polypeptides that also include a catalytic domain, such as the typical PKC-C2, phosphatidylinositol 3-kinase-C2 or phospholipase A2-C2 combinations (Zhang and Aravind, 2010). Thus, C2 domains are able to translocate to membrane compartments the associated catalytic activity in response to Ca^{2+} peaks. Analysis of the *Arabidopsis* genome reveals combinations of the C2 domain with different catalytic domains, such as phospholipase D, lysine decarboxilase, phosphoribosylanthranilate transferase, endonucleases, inositol 1,4,5-trisphosphate phosphatases or phospholipase C (Figure 4.2). However, the *Arabidopsis* CAR family hereby identified represents a plant-specific C2 domain family of small proteins not associated with catalytic domains. Therefore, we suggest that CAR proteins as well as other short C2 proteins lacking additional domains might function through interaction with lipids or other proteins and display a dual function as a calcium-dependent phospholipid binding protein and as protein-protein interaction module.

Bimolecular fluorescence complementation (BiFC) assays were used to test the CAR1-PYL4 interaction in plant cells. To this end, *35S:CAR1-YFP^N* (also harboring the 35S CAMV promoter and the N-terminal yellow fluorescent protein gene) and *35S:YFP^C-PYL4* constructs

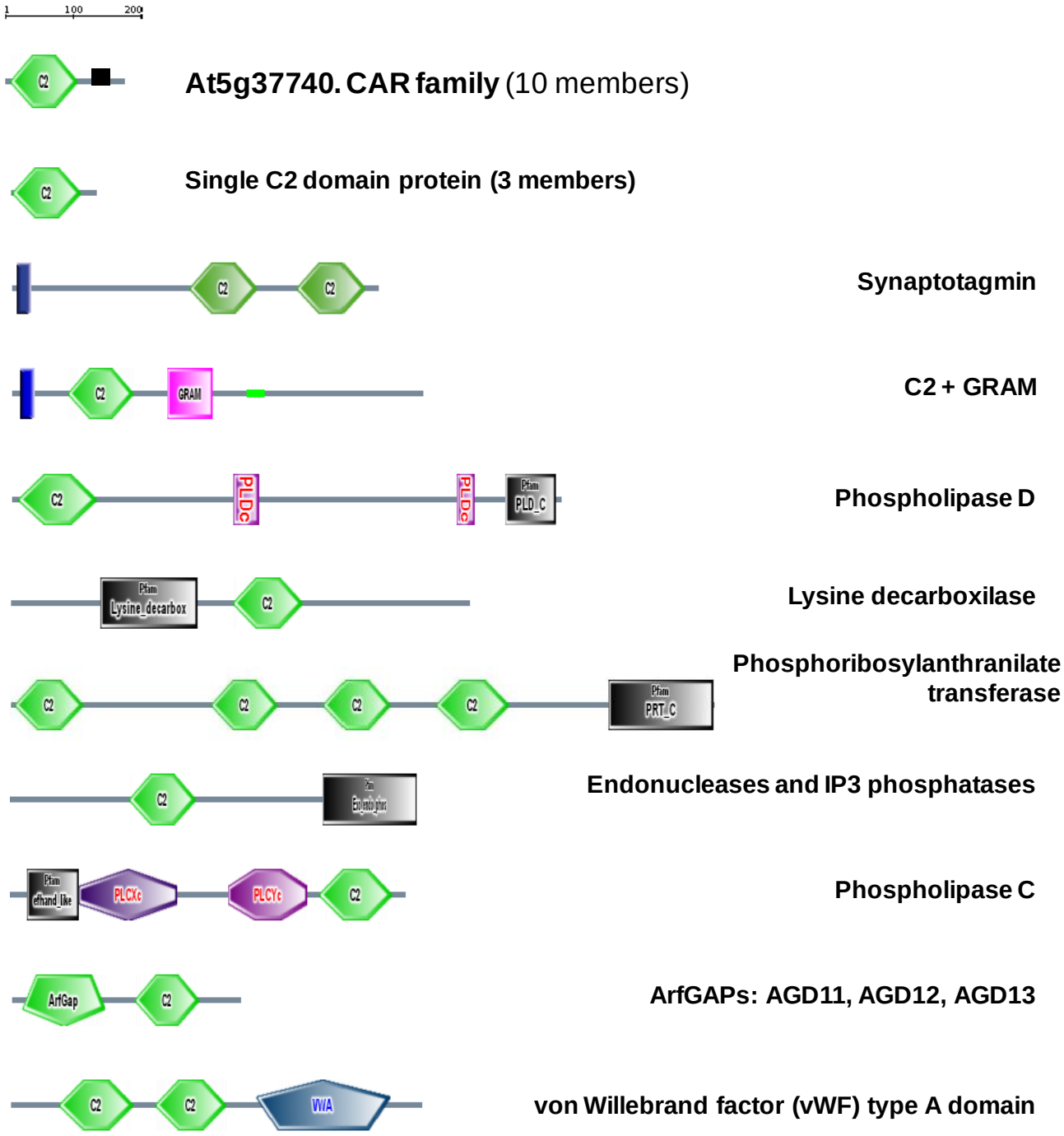

Figure 4.2 Scheme of some representative proteins harboring C2 domains in *Arabidopsis*. A total of 123 proteins containing one or several C2 domains were identified in TAIR using the SMART tool (http://smart.embl-heidelberg.de/) (Schultz et al., 1988). The C2 domain was found either in small proteins that only contain the C2 fold or associated to different catalytic domains. Some C2 proteins such as synaptotagmins or uncharacterized proteins that combine C2 with the phospholipid-binding GRAM domain also contain a hydrophobic transmembrane domain (dark blue rectangle). Compared to the canonical C2 domain from PKC (130 amino acid residues), the CAR family presents an insertion at the C-terminus (indicated by a black box).

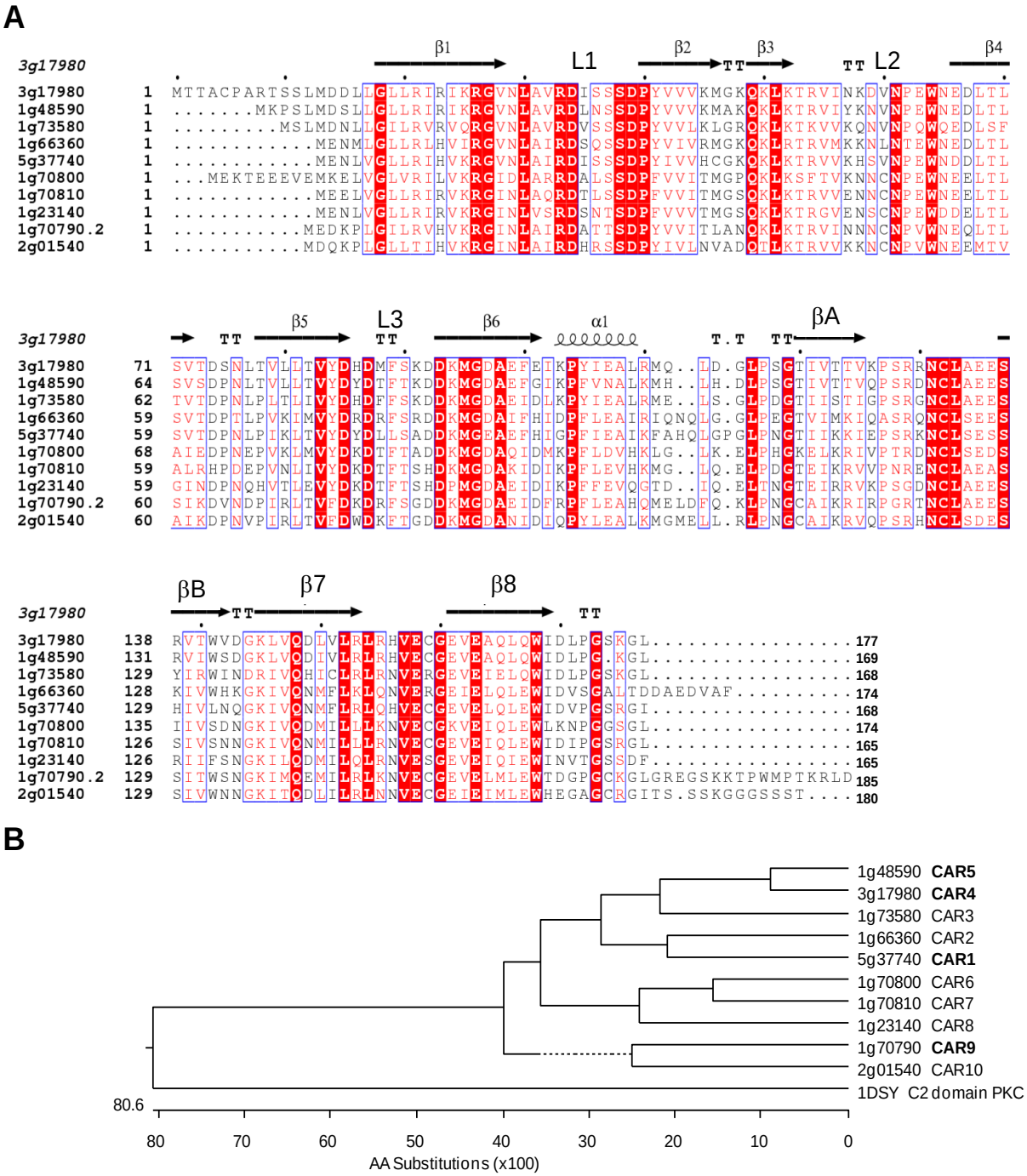

Figure 4.3 Amino acid sequence alignment and cladogram of *Arabidopsis* CAR proteins. (A) A family composed by 10 members was revealed by BLAST search at the TAIR database. An alignment was generated using Clustal W program and Genedoc software. The secondary structure of At3g17980 was added to the alignment using ESPript 2.2. The a-helix starting at residue 103 of At3g17980 and the bA and bB sheets represent a singular CAR insertion into the standard C2 fold described previously for PKC or PLC. The loops involved in Ca2+ coordination are indicated as L1, L2 and L3. (B) Cladogram of the Arabidopsis CAR family. A tree resulting from the previous alignment was generated using PhyloDraw software.

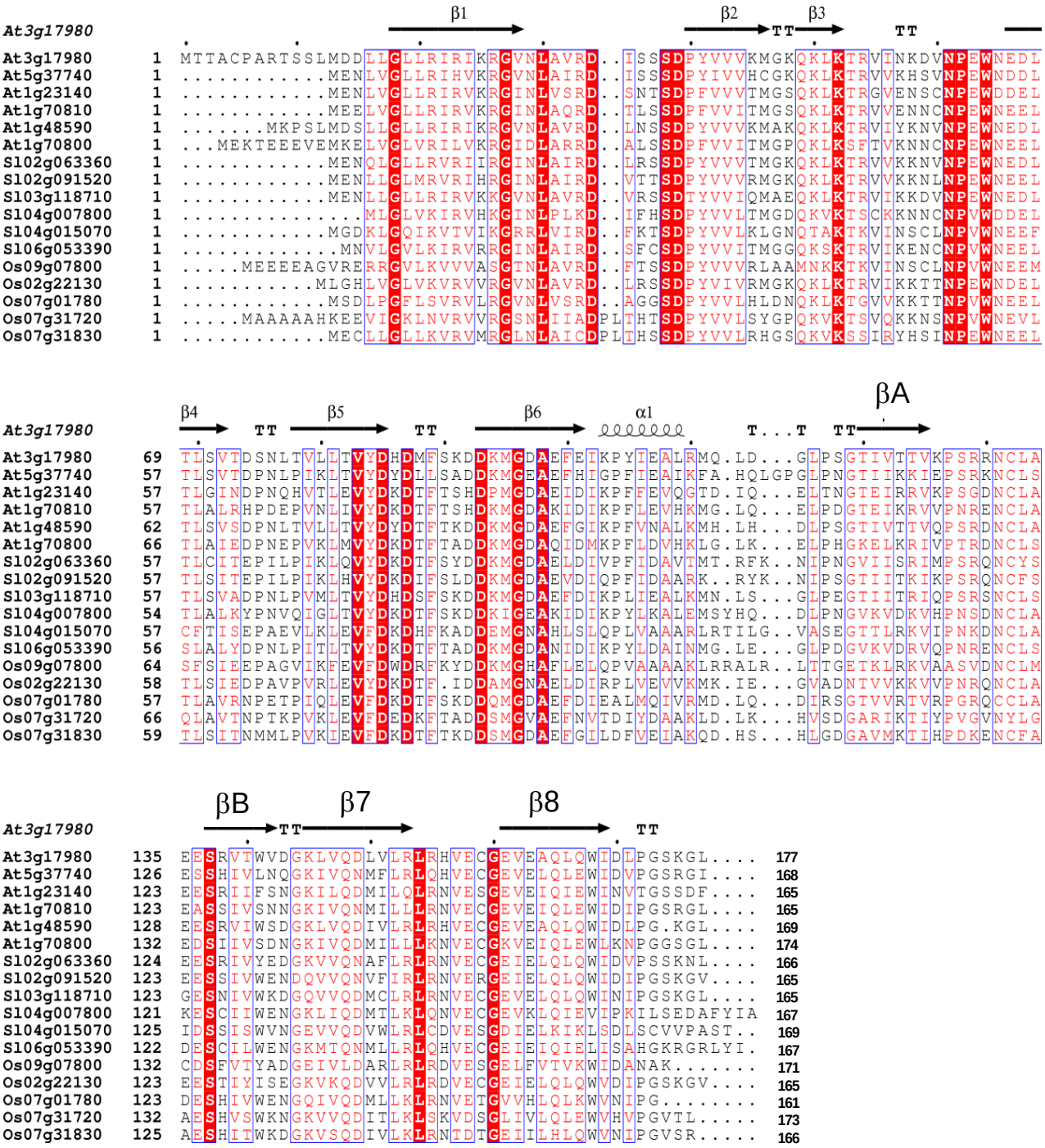

Figure 4.4 Amino acid sequence alignment of representative members of the CAR family in *Arabidopsis*, tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*).

were delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration (Agroinfiltration) and, as a result, fluorescence was observed in the nucleus or visualized as a thin layer that could reflect plasma membrane or cytosolic localization (Figure 4.5A). As a marker of plasma membrane localization, we used the red fluorescence emitted by orange/red fluorescent protein OFP-TM23, a modified version of OFP (orange fluorescent protein) containing a transmembrane domain that results in plasma membrane targeting (Batistic et al., 2012). Therefore, we co-expressed CAR1-YFP^N, YFP^C-PYL4 and OFP-TM23 into leaf cells of tobacco by Agroinfiltration. Next, we followed the protocol described by French et al., (2008) in order to perform statistical analysis of the putative co-localization of the fluorescent markers. We found that Pearson-Spearman correlation coefficients (r_P and r_S, respectively) indicated co-localization of OFP-TM23 and reconstituted YFP proteins; therefore, a significant amount of the CAR1-PYL4 interaction was localized to the plasma membrane (Figure 4.5B). In contrast, GFP did not show co-localization with OFP-TM23 when both proteins were co-expressed in tobacco cells (Figure 4.5A and B). We also found that other PYR/PYLs, such as PYR1, PYL1, PYL6 and PYL8, also interacted with CAR1 in BiFC assays (Figure 4.5C). In contrast to the interaction observed with YFP^C-PYR/PYLs, the YFP^C-$OST1_{1-280}$ protein was not able to interact with CAR1 (Figure 4.5C). We wondered whether other members of the CAR family were able to interact with PYR/PYLs. As a result, we found that CAR4 was able to interact with PYR1, PYL1, PYL4, PYL6 and PYL8 using BiFC assays, whereas it did not interact with YFP^C-$OST1_{1-280}$ (Figure 4.5C). Although the expression levels of the YFP-tagged proteins varied in the different Agroinfiltrations of tobacco plants, we could confirm by immunoblot analyses the expression of the receptors and CAR proteins in all the BiFC experiments (Figure 4.5C).

Finally, we co-expressed in tobacco epidermal cells HA-tagged PYR/PYLs and either CAR1-GFP or CAR4-GFP proteins through Agroinfiltration in order to conduct co-immunoprecipitation (coIP) experiments (Figure 4.5D). To avoid precipitation of the membrane fractions -not mediated by antibody- where CAR1-PYL4 interaction occurred, we used the soluble nuclear fractions where this interaction also occurs (Figure 4.5A and C). To this end, we precipitated nuclear CAR-GFP proteins using α-GFP (green fluorescent protein) and tested the simultaneous presence of PYR/PYLs using α-HA. As a result, we could detect coIP of either CAR1-GFP or CAR4-GFP and the five receptors assayed to various extents (Figure 4.5D). The recovery of co-immunoprecipitated CAR1-GFP and HA-PYL6 was apparently higher than that of the other interactions; however we also recovered more CAR1-GFP in the α-GFP precipitated. We also found a consistently higher recovery of co-immunoprecipitated CAR4-GFP and HA-PYL1/PYL4/PYL6 compared to HA-PYR1. Expression of HA-PYL8 was lower than that of the other HA-tagged PYLs; therefore, the initial input was not comparable to that of the other PYLs.

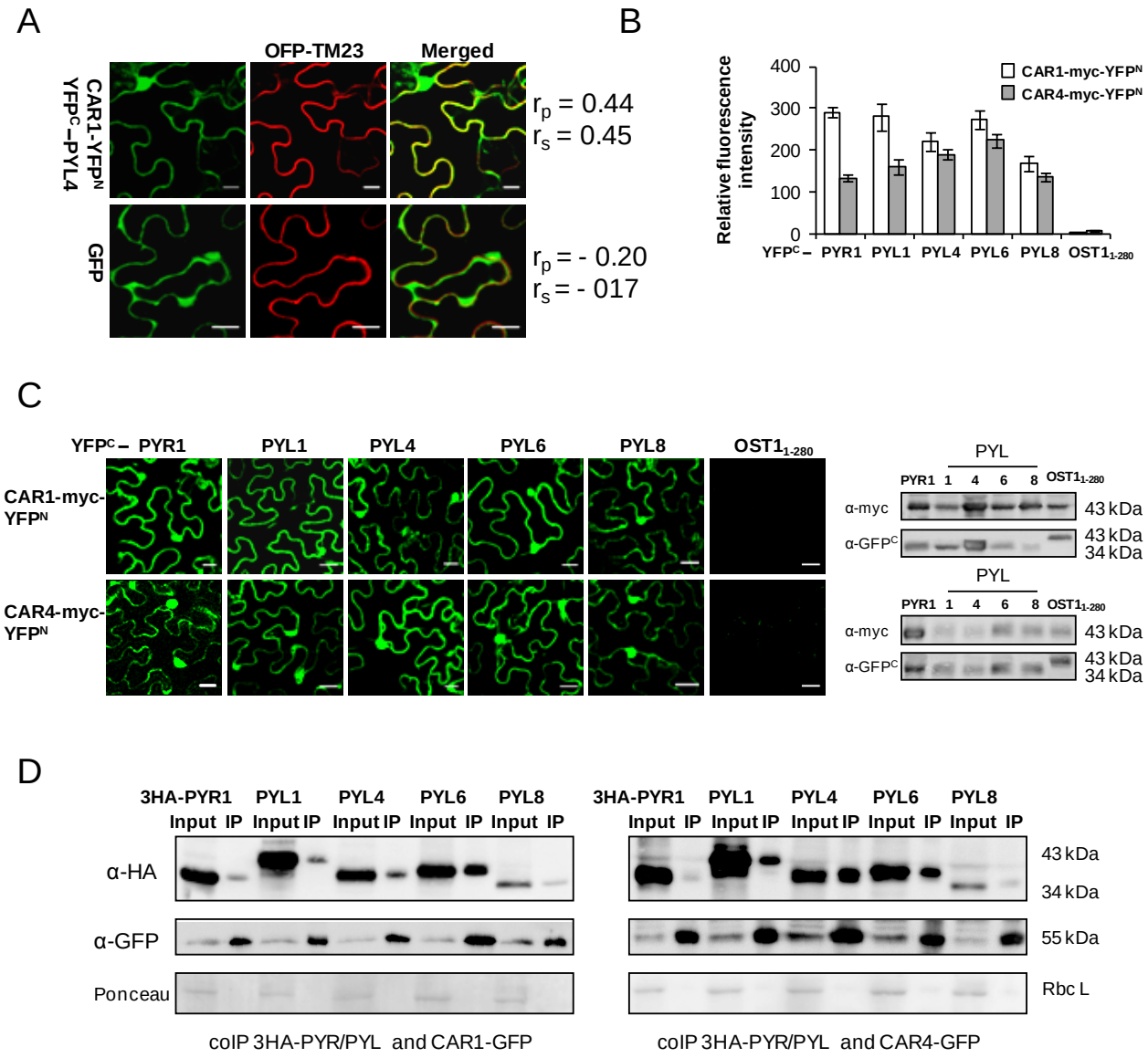

Figure 4.5 BiFC and coIP assays reveal interactions between CAR1/CAR4 and PYR/PYLs in *N. benthamiana* epidermal cells. (A) CAR1 and PYL4 interact in the plasma membrane and nucleus of *N. benthamiana* cells. Confocal images show transiently transformed *N. benthamiana* epidermal cells coexpressing CAR1-YFP^N/YFP^C-PYL4-interacting proteins and the plasma membrane marker OFP-TM23 (top row) or GFP and OFP-TM23 (bottom row). BiFC interaction of CAR1-YFP^N and YFP^C-PYL4 was observed, and this interaction colocalizes with the plasma membrane marker OFP-TM23 (Merged). Epifluorescence confocal images of epidermal *N. benthamiana* leaves infiltrated with the indicated constructs were merged to quantitatively estimate the colocalization of YFP/GFP and OFP fluorescence. Statistical analysis of the colocalization of CAR1-YFP^N/YFP^C-PYL4-interacting proteins and OFP-TM23 was done using Pearson's (r_P) and Spearman's (r_S) correlation factors (French et al., 2008). The degree of colocalization between the two fluorescent signals was analyzed using Zeiss software. Bars = 20 µm. (B) Relative fluorescence intensity of the BIFC interactions detected for either CAR1-YFP^N or CAR4-YFP^N and the indicated constructs. Each BiFC experiment was scanned and measured in 25 randomly chosen microscopic fields (n = 25) and repeated three times. (C) BiFC assays show both nuclear and nonnuclear interactions of CAR1/CAR4 and PYR/PYLs in *N. benthamiana* epidermal cells coinfiltrated with *Agrobacterium* suspensions containing the indicated constructs and the silencing suppressor p19. Immunoblot analyses (at right) confirm the expression of myc-tagged CAR1 (top)/CAR4 (bottom) and YFP^C-tagged PYR/PYL proteins in *N. benthamiana* epidermal cells. (D) coIP assays demonstrate the interaction of CAR1-GFP or CAR4-GFP and PYR/PYLs. Nuclear protein extracts obtained from *N. benthamiana* leaves infiltrated with *Agrobacterium* suspensions harboring the indicated constructs were analyzed using anti-HA or anti-GFP antibody. Input levels of epitope-tagged proteins in crude protein extracts (20 µg of total protein) were analyzed by immunoblotting. Immunoprecipitated (IP) CAR1-GFP and CAR4-GFP proteins were probed with anti-HA antibodies to detect coIP of HA-tagged PYR/PYLs. Ponceau staining from the large subunit of Rubisco (Rbc L) is shown as a loading control.

Subcellular localization of CAR and PYL4 proteins

In order to further explore the subcellular localization of individual CAR and PYL4 proteins, we expressed CAR1-GFP, CAR4-GFP, CAR5-GFP and GFP-PYL4 fusion proteins in tobacco epidermal cells by Agroinfiltration. In addition to their nuclear localization, CAR-GFP proteins decorated the perimeter of the cell, which could reflect plasma membrane or cytosolic localization (Figure 4.6A). As a marker of plasma membrane localization, we used the cyan fluorescence emitted by the reconstituted super cyan fluorescent protein (SCFP) of the $SCFP^{C}$-CIPK24/CBL1-$SCFP^{N}$ interaction, which has been reported previously to be localized at the plasma membrane as a peripheral protein (Waadt et al., 2008). We co-expressed individual

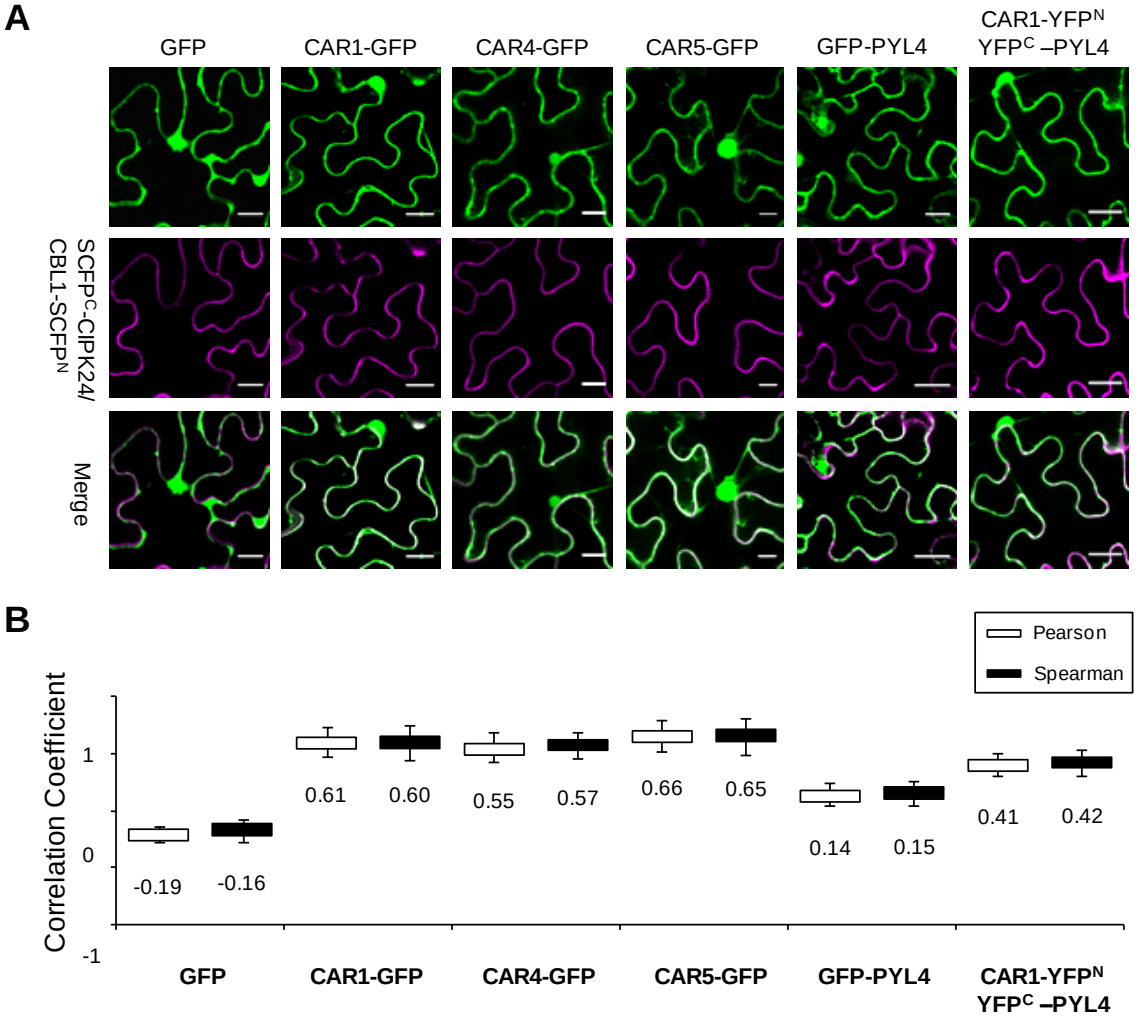

Figure 4.6 CAR-GFP Fusion Proteins Localize to Nucleus, Plasma Membrane, and Cytosol upon Transient Expression in *N. benthamiana*. (A) Confocal images of transiently transformed *N. benthamiana* epidermal cells coexpressing GFP, GFP-tagged CAR/PYL4 proteins or the CAR1-YFP^{N}/YFP^{C}-PYL4-interacting proteins, and the plasma membrane marker resulting from the $SCFP^{N}$-CIPK24/CBL1-$SCFP^{C}$ interaction. The degree of colocalization between the two fluorescent signals was analyzed using merged images and Zeiss software (ZEN Lite 2012). The magenta color of the reconstituted SCFP is a pseudocolor generated from the original cyan fluorescence. Bars = 20 µm.(B) Pearson-Spearman correlation coefficients indicate the colocalization of CAR-GFP proteins or the CAR1-YFP^{N}/YFP^{C}-PYL4 interaction and the plasma membrane marker. Epifluorescence confocal images of epidermal leaves coinfiltrated with the indicated constructs were merged to quantitatively estimate the colocalization of GFP/YFP and SCFP fluorescence (French et al., 2008). At least 10 single-scanned cell images per experiment were collected and analyzed using the same conditions of laser intensity, pinhole size, and gain levels.

CAR-GFP proteins with this marker and performed statistical analysis of the putative co-localization of the fluorescent markers (fluorescence emission spectra in the green range for GFP and cyan range for SCFP). As a result, we found co-localization of both fluorescent markers (Pearson-Spearman correlation coefficients in the range 0.55-0.66; Figure 4.6B). Therefore, a significant fraction of CAR-GFP proteins was localized to plasma membrane. In contrast, GFP alone did not show co-localization with reconstituted SCFP (Pearson-Spearman correlation coefficients below zero). GFP-PYL4 alone not show a substantial localization in plasma membrane; however, when YFP^C-PYL4 was co-expressed with CAR1-YFP^N it showed partial co-localization with the plasma membrane marker used in this experiment (reconstituted SCFP) (Figure 4.6A and B). Independent evidence for the localization in plasma membrane of CAR proteins came from data mining in the results published by Demir et al. (2013). In this work, proteins associated with detergent-resistant membranes from leaf plasma membrane preparations were identified using mass spectrometry. Two CAR proteins, *Arabidopsis* CAR6 and tobacco CAR2 homolog, were identified in dataset S2 and S3, respectively, further confirming the presence of CAR proteins in plasma membrane.

Finally, we examined the subcellular localization of CAR1-GFP, CAR4-GFP, CAR5-GFP and GFP-PYL4 proteins by standard biochemical techniques (Figure 4.7A). First of all, we followed a fractionation technique to separate nuclei (pelleted at 1000g) from the soluble non-nuclear fraction (Saez et al., 2008; Antoni et al., 2012). Compared to GFP, the CAR-GFP proteins were enriched in the nuclear fraction. Additionally, we submitted the soluble fraction to a centrifugation of 100000g to separate the soluble cytosolic fraction from the pelleted microsomal fraction (Antoni et al., 2012). We found that in contrast to GFP, which was localized mostly to the cytosolic soluble fraction, the CAR proteins were localized mostly to the microsomal fraction (Figure 4.7B). GFP-PYL4 also showed a dual nuclear and non-nuclear localization, although a lower percentage of nuclear protein was found compared to CAR-GFP proteins. In contrast to CAR-GFP proteins, most of the soluble non-nuclear fraction of GFP-PYL4 was localized to cytosol, although a significant amount was localized to the microsomal fraction (Figure 4.7B). Overexpression of CAR1 together with PYL4 in coinfiltration experiments increased the presence of PYL4 in membranes relative to cytosolic location, although to a modest extent (Figure 4.7C). This result suggests that membrane recruitment of PYL4 by CAR1 affects only to a fraction of the total receptor pool.

CAR proteins show a α1βAβB CAR-signature extra-domain inserted into a canonical C2 fold

In order to obtain molecular insights into CAR proteins, the group of Armando Albert (Instituto de Química Física Rocasolano, Consejo Superior de Investigaciones Científicas, Madrid, Spain) solved the X-ray structure of CAR4 in complex with Ca^{2+} by molecular

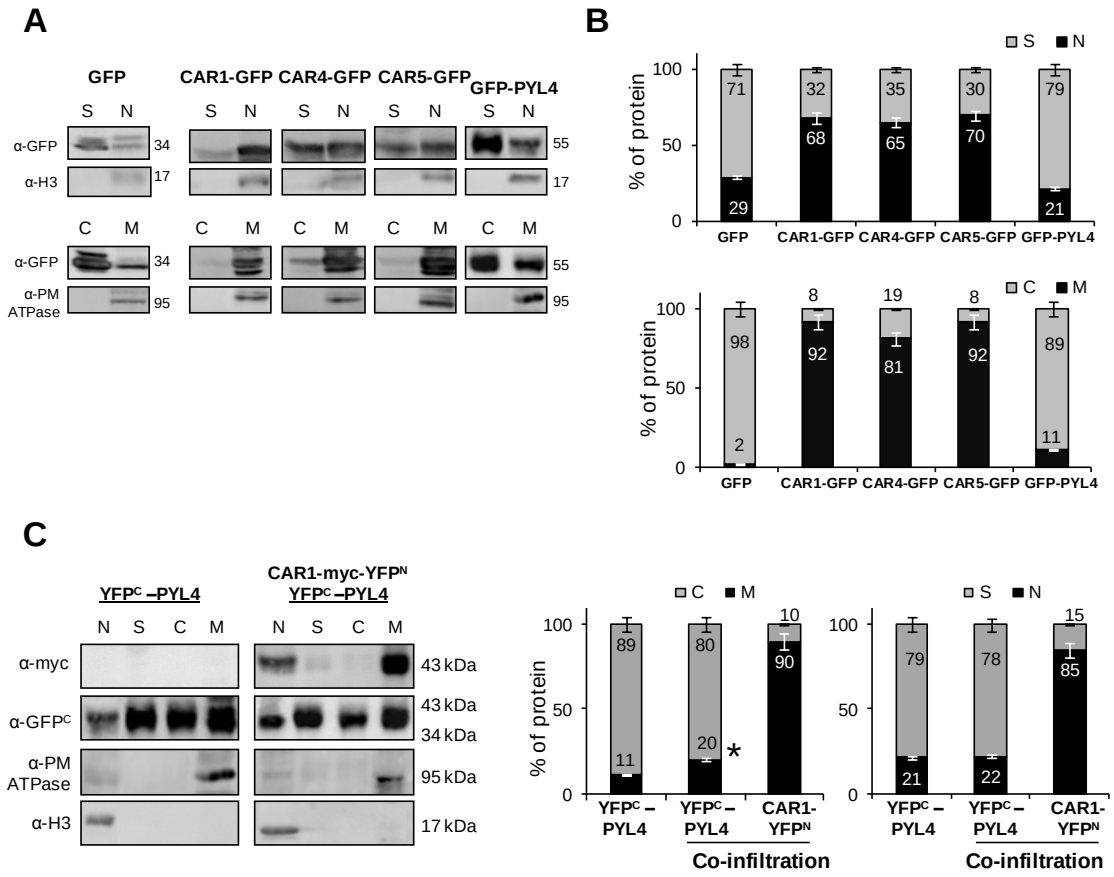

Figure 4.7 CAR-GFP Fusion Proteins Localize to Nucleus, Plasma Membrane, and Cytosol upon Transient Expression in *N. benthamiana*. (A) Biochemical fractionation and immunoblot analyses of protein extracts prepared from *N. benthamiana* leaves infiltrated with *Agrobacterium* harboring the indicated constructs. Protein extracts from the different fractions were analyzed by immunoblotting using anti-GFP, anti-Histone3 (H3), and anti-plasma membrane (PM) H^+-ATPase antibodies. The positions of the molecular mass standards (kD) are indicated. C, cytosolic fraction; M, microsomal fraction; N, nuclear fraction; S, nonnuclear soluble fraction.(B) Quantification of the subcellular localization of GFP and GFP-tagged proteins transiently expressed in *N. benthamiana* epidermal cells. Immunoblot signals obtained in (A) were captured using the image analyzer LAS3000, and quantification of the protein signal was done using Image Guache version 4.0 software .(C) Overexpression of CAR1 together with PYL4 in coinfiltration experiments increases the PYL4 presence in membranes. Biochemical fractionation, immunoblot analyses, and quantification of protein extracts prepared from *N. benthamiana* leaves infiltrated with *Agrobacterium* harboring the indicated constructs were performed.

replacement at 1.6 Å resolution (Figure 4.8). The overall structure of CAR4 is almost identical to that found for other C2 domains. CAR4 folds as a compact beta sandwich that is composed by two 4-stranded beta-sheets with type II domain topology (Rizo and Sudhof, 1998). In addition, it contains an extra-domain insertion consisting of 43 amino acids that connect the two 4-stranded beta-sheets (β3β2β5β6 with β4β1β8β7) and folds as an alpha helix followed by a beta hairpin (α1βAβB) (Figure 4.8, see CAR4 topology). This insertion is conserved among the members of the CAR family (Figure 4.3 and 4.4) and represents a unique CAR-signature when the fold is compared with other known families of C2 domains using the PDBeFold structure similarity service (http://www.ebi.ac.uk/msd-srv/ssm/; Krissinel and Henrick, 2004). The CAR-signature extra-domain is situated in the protein face opposite to loops L1 and L3, which bind Ca^{2+} ions that bridge C2 proteins to membranes (Figure 4.8; Verdaguer et al., 1999). The

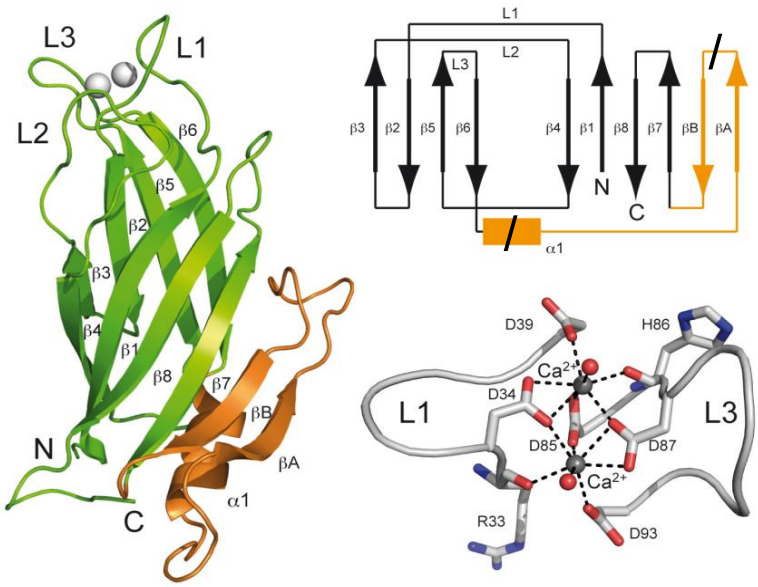

Figure 4.8 Crystal structure, Ca^{2+} coordination and topology of CAR4. A ribbon representation of the CAR4 crystal structure showing overall fold together with a scheme of the topology and a detailed representation of the calcium binding sites. The α1βAβB extradomain is highlighted in orange. The part of this region deleted in the $CAR4^{\Delta ED}$ mutant is indicated in the topological scheme.

crystallographic analysis revealed two CAR4 molecules in the asymmetric unit. The structures of these independent molecules are nearly identical (Cα backbone root-mean square deviation, RMSD 0.14 Å; Emsley et al., 2010).

CAR proteins show Ca^{2+}-dependent phospholipid binding activity and recruit PYR/PYLs to membrane

C2 domains display a well defined calcium-dependent lipid-binding site that relies on the unspecific interaction of the phosphate moiety of phospholipids with the calcium ions coordinated in the conserved L1 and L3 loops, and the specific interaction of the phospholipid headgroup with amino acid residues from the loops that conform the cup-shaped calcium-binding site (Verdaguer et al., 1999). The crystallographic analysis shows that CAR4 binds two calcium atoms at this site (Figure 4.8). They are coordinated with conserved Asp residues in loops L1 (D34 and D39) and L3 (D85 and D87), which would make it possible that Ca^{2+} bridges the C2 domain to phospholipids (Perisic et al., 1998; Verdaguer et al., 1999; Guerrero-Valero et al., 2009). Since the Ca^{2+}-dependent phospholipid binding is a hallmark of many C2 domains, we tested whether CAR proteins were able to bind negatively charged phospholipid vesicles (25% phosphatidyl serine/75% phosphatidyl choline) in a Ca^{2+}-dependent manner. The vesicle pelleting assay is a standard method to detect lipid binding of peripheral proteins and it is summarized in Figure 4.9A (Cho et al., 2001). This assay was performed in the absence or presence of different Ca^{2+} concentrations using CAR1 and CAR4, as well as the $CAR1^{D22A\ D27A}$ mutants, which contain double Asp to Ala mutations in the loops L1 and L3, respectively. As a result, we could observe that Ca^{2+} promoted binding of CAR1 and CAR4 to phospholipid vesicles (Figure 4.9B). We calculated a half-maximal calcium concentration of 1.3 and 7.7 μM for in vitro phospholipid binding of CAR1 and CAR4, respectively, and a certain cooperative effect for Ca^{2+} binding according to Hill coefficient value (n_H) (Figure 4.9B, top of the graph).

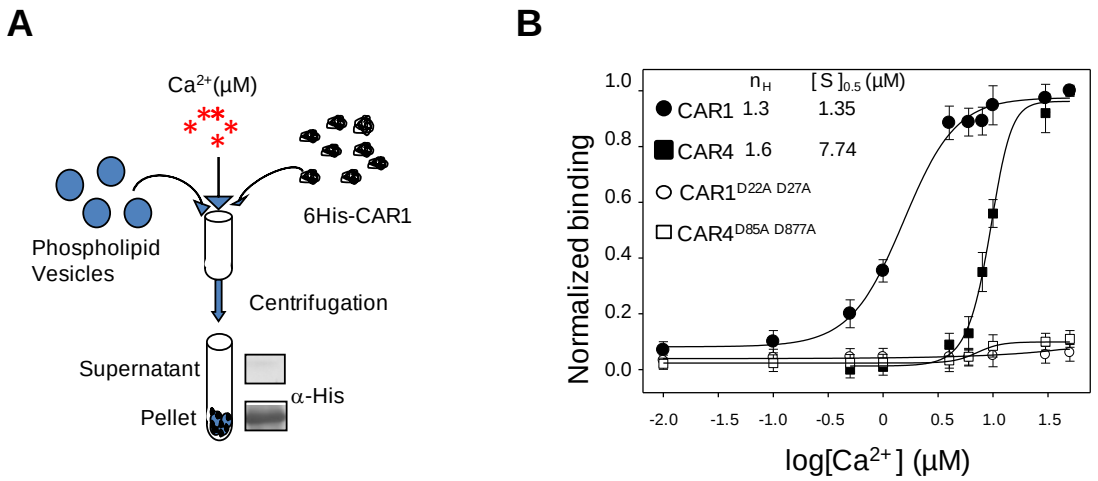

Figure 4.9 Calcium-dependent phospholipid-binding of CAR proteins. (A) Scheme of the biochemical assay to detect Ca^{2+}-dependent protein-phospholipid interaction through pelleting of phospholipid vesicles and immunoblot analysis. Phospholipid vesicles were composed by 25% phosphatidylserine (PS) and 75% phosphatidylcholine (PC). The vesicles were precipitated by centrifugation and bound proteins were revealed by SDS-PAGE and immunoblot analysis using a-His antibody. (B) CAR1, CAR4, $CAR1^{D22A\ D27A}$ and $CAR4^{D85A\ D87A}$ proteins were incubated with phospholipid vesicles in the presence of increasing concentrations of Ca^{2+} to determine the half-maximal binding for the ion. Introduction of two Asp to Ala mutations into the amino acid residues 22 and 27 of CAR1 or 85 and 87 of CAR4 abolished phospholipid-binding. Hill coefficient (n_H) and calcium concentration leading to half-maximal binding $[S]_{0.5}$ are indicated in the inset of the graphic

Transient increases in Ca^{2+} to μM levels have been described in different signaling pathways, so the values found for CAR proteins are into the physiological range of Ca^{2+} signaling (Swanson et al., 2011). Additionally, the limited analysis of two members of the CAR family suggests that different CAR proteins might sense and respond differentially to an increase of intracytosolic Ca^{2+} levels as has been described in different C2 domains of classical PKCs type α, β and γ (Guerrero-Valero et al., 2007). Finally, we confirmed that coordination of Ca^{2+} ions by L1 and L3 loops is crucial for phospholipid binding, since the $CAR1^{D22A\ D27A}$ and $CAR4^{D85A\ D87A}$ mutations abolished phospholipid binding (Figure 4.9B).

One of the receptors that interacted well with CAR proteins in BiFC and coIP assays was PYL6 (Figures 4.5). Only a residual presence of PYL6 in the pellet was detected upon co-incubation with CAR1 and phospholipid vesicles lacking calcium (Figure 4.10, lane 6). In contrast, co-incubation of PYL6 with CAR1 and phospholipid vesicles in the presence of Ca^{2+} promoted the recruitment of PYL6 to membranes (Figure 4.10, lane 3). Such an effect could be

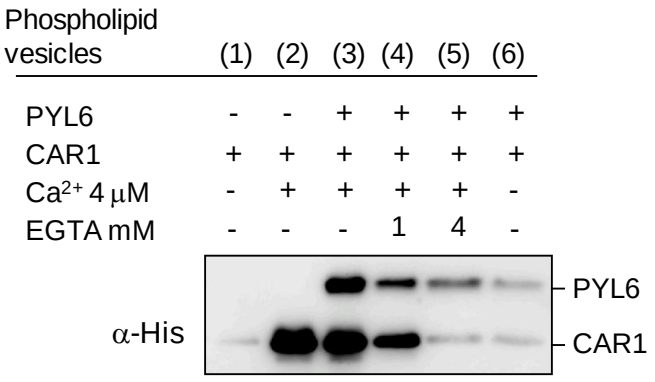

Figure 4.10 Ca^{2+} and CAR1-dependent recruitment of PYL6 to phospholipid vesicles. Ca^{2+} and CAR1-dependent vesicle pelleting of PYL6 can be reversed by EGTA-treatment. Pelleted vesicles bound to PYL6 and CAR1 were EGTA-treated, and analysed by SDS-PAGE and immunoblot.

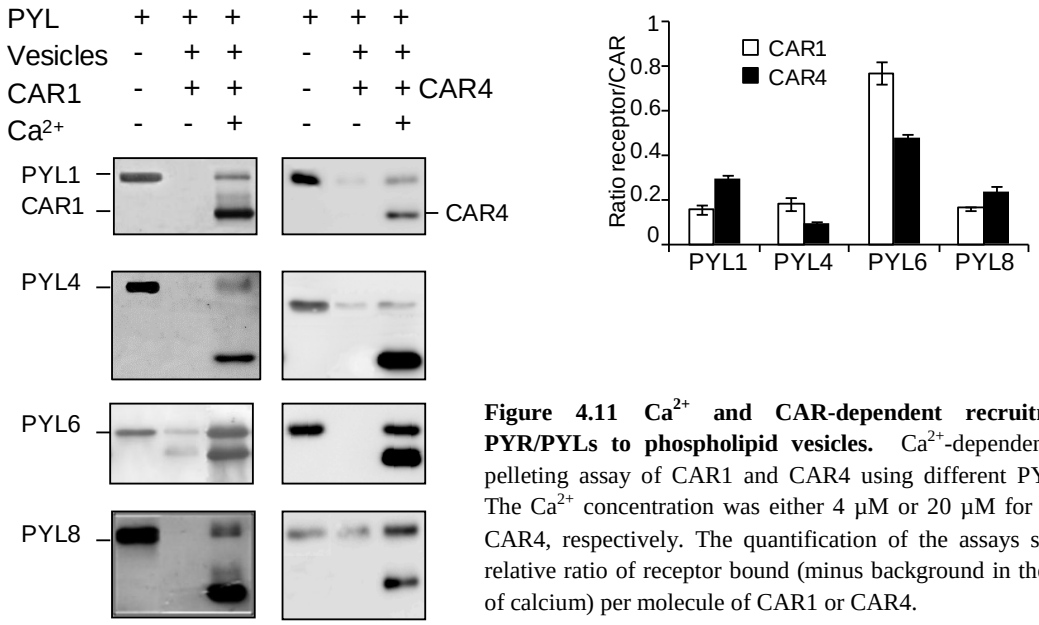

Figure 4.11 Ca^{2+} and CAR-dependent recruitment of PYR/PYLs to phospholipid vesicles. Ca^{2+}-dependent vesicle pelleting assay of CAR1 and CAR4 using different PYR/PYLs. The Ca^{2+} concentration was either 4 µM or 20 µM for CAR1 or CAR4, respectively. The quantification of the assays shows the relative ratio of receptor bound (minus background in the absence of calcium) per molecule of CAR1 or CAR4.

reversed by treating the pelleted vesicles with increasing concentrations of EGTA, a chemical acting as Ca^{2+} chelating agent (Figure 4.10, lanes 4 and 5). These results indicate that binding of PYL6 to phospholipid vesicles was dependent both on Ca^{2+} and CAR1, and the Ca^{2+}-dependent recruitment of PYL6 to membranes by CAR1 was reversible, excluding unspecific effects, such as protein aggregation or protein insolubility induced by the CAR1-PYL6 interaction.

We also tested whether other PYR/PYL receptors could be recruited to phospholipid vesicles by either CAR1 or CAR4 in a Ca^{2+}-dependent manner. PYL1, PYL4, PYL6 and PYL8 were recruited to phospholipid vesicles by CAR1, whereas PYL1, PYL6 and PYL8 were recruited by CAR4 (Figure 4.11). The starting receptor:CAR ratio was 1:1 in the in vitro assay and after performing the vesicle pelleting assay, we measured the ratio of receptor to CAR protein in the pelleted vesicles to estimate the affinity of each receptor to either CAR1 or CAR4. Clearly, PYL6 was efficiently recruited both by CAR1 and CAR4. The other receptors were less efficiently recruited, ranging between 0.2 and 0.3 molecules of receptor bound per molecule of CAR protein. Taken together, these assays reveal that CAR1 and CAR4 proteins were able to selectively bridge the interaction of PYR/PYL proteins with phospholipid vesicles in a Ca^{2+}-dependent manner.

Finally, we generated a CAR4 internal deletion (named Δextra-domain, abbreviated as ΔED) affecting amino acid residues 107-140 to partially eliminate the extra-domain insertion of CAR4 (Figure 4.8) and we tested its effect on the interaction with PYL1 and PYL6 (Figure 4.12). As a result, we found that $CAR4^{\Delta ED}$ did not interact with either PYL1 or PYL6 in BiFC assays, in spite of being synthesized at roughly similar levels to the corresponding wt control (Figure 4.12, right panel). This result suggests that the extra-domain present in CAR proteins is required for interaction with PYL ABA receptors.

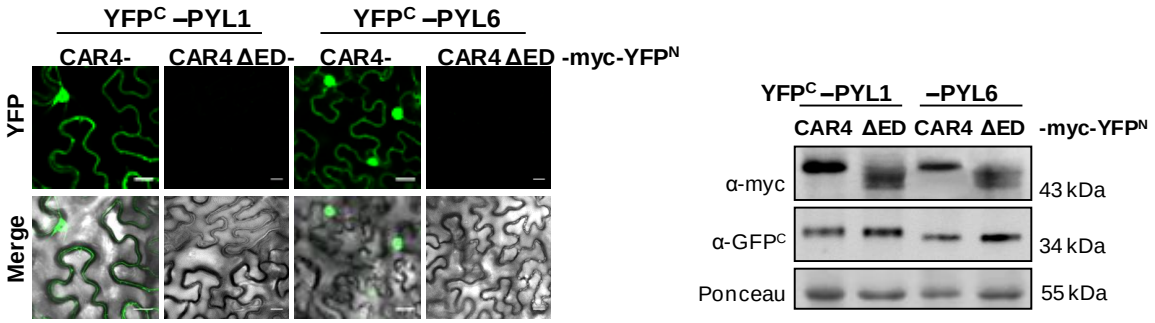

Figure 4.12 A CAR4$^{\Delta ED}$ mutant is unable to interact with PYL1/PYL6 ABA receptors. A BiFC assay was performed in tobacco epidermal cells co-infiltrated with *Agrobacterium* suspensions containing the indicated constructs and the silencing suppressor p19. Immunoblot analyses (right panels) confirm the expression of myc-tagged CAR4/CAR4 $^{\Delta ED}$ and YFP^{C}-tagged PYL1/PYL6 proteins in tobacco epidermal cells. Bars correspond to 20 µm.

Triple mutants impaired in *CAR* genes show reduced sensitivity to both ABA-mediated inhibition of seedling establishment and root growth

Both gain-of-function and loss-of function approaches were followed in order to investigate whether *CAR* genes affect ABA signaling. First, we analyzed ABA sensitivity of *35S:CAR1* lines with respect to ABA-mediated inhibition of seedling establishment and shoot growth (Figure 4.13). *35S:CAR1* lines showed enhanced sensitivity to ABA-mediated inhibition

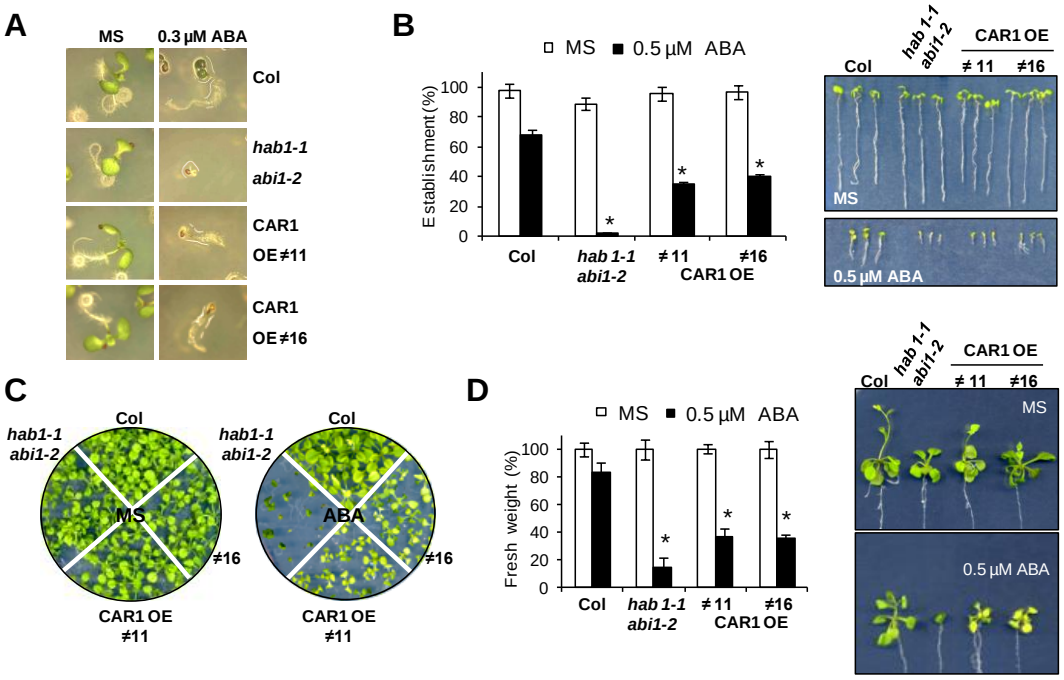

Figure 4.13 Overexpression of CAR1 leads to enhanced ABA-mediated inhibition of seedling establishment and shoot growth. (A) Photographs of Col wt, *hab1-1abi1-2* ABA-hypersensitive mutant (Saez et al., 2006) and two CAR1-overexpressing (OE) lines (#11 and 16) grown for 4 d on MS medium either lacking or supplemented with 0.3 µM ABA.(B) Quantification of ABA-mediated inhibition of seedling establishment of Col wt compared with *hab1-1abi1-2* double mutant and two CAR1 OE lines. Approximately 100 seeds of each genotype were sown on MS plates lacking or supplemented with 0.5 µM ABA and scored for the presence of green expanded cotyledons 7 d later. The photographs show representative seedlings removed at 7 d from MS plates lacking or supplemented with 0.5 µM ABA and rearranged on agar plates. (C) *35S:CAR1* lines show enhanced sensitivity to ABA-mediated inhibition of vegetative growth. Photographs of Col wt, *hab1-1abi1-2* double mutant and two CAR1 OE lines grown for 12 d on MS medium lacking ABA or 21 d in medium supplemented with 0.5 µM ABA. (D) Quantification of fresh weight after 21 d growth in medium lacking or supplemented with 0.5 µM ABA. * indicates $p<0.05$ (Student´s t test) when comparing data of *35S:CAR1* lines and *hab1-1abi1-2* mutant to Col wt plants in the same assay conditions.

of seedling establishment and shoot growth compared to wt (Figure 4.13). Thus, establishment at 0.3-0.5 μM ABA was impaired in *35S:CAR1* lines compared to wt (Figure 4.13A and B). Those seedlings from *35S:CAR1* lines that were able to establish in 0.5 μM ABA showed a clear impairment of shoot growth compared to wt after 20 d growth in medium supplemented with ABA (Figure 4.13C). Likewise, seedlings from *35S:CAR1* lines transferred from MS plates to plates supplemented with 0.5 μM ABA showed reduced fresh weight after 20 d growth compared to Col wt (Figure 4.13D).

Next we identified loss-of-function knockout mutants for different members of the *CAR* family that were available from T-DNA mutant collections. At the beginning of this work, we identified T-DNA homozygous mutants for the loci At5g37740 (*car1*), At3g17980 (*car4*), At1g48590 (*car5*) and At1g70790 (*car9*) (Figure 4.14). Since some functional redundancy might be expected among CAR proteins as was observed previously for PYR/PYL proteins, we generated different double and triple mutants impaired in *CAR* genes and analyzed their sensitivity to ABA (Figure 4.15). ABA-mediated inhibition of seedling establishment assays revealed both a reduced sensitivity to ABA in the combined mutants compared to wt and functional redundancy among *CAR* genes since generation of triple mutants was required to obtain robust phenotypes (Figure 4.15A and B). Inhibition of root growth in the single and double mutants was slightly lower than in wt, whereas triple mutants showed a significant ABA insensitivity compared to wt (Figure 4.15C and D).

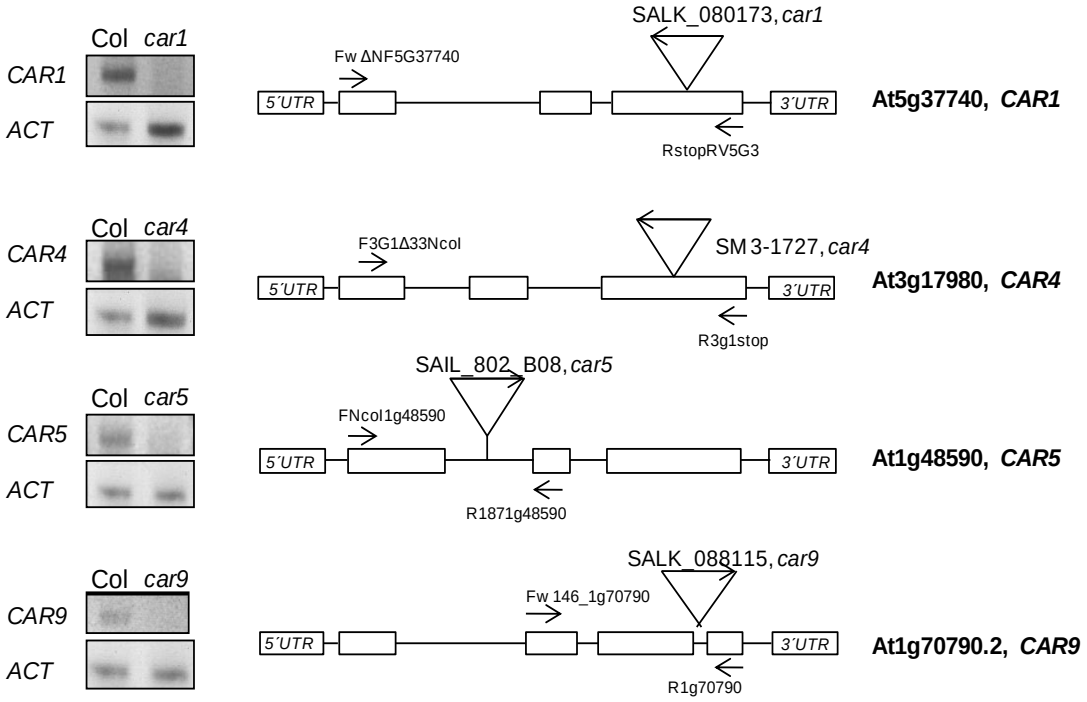

Figure 4.14 Scheme of *CAR1, CAR4, CAR5 and CAR9* genes and location of the corresponding T-DNA insertion in *car* mutants. Position of the primers used for genotyping and RT-PCR analyses are indicated by arrows. Left, RT-PCR analyses of mRNAs extracted from either Col wt seedlings (10 d-old) or the corresponding *car* mutant are shown for each gene; the upper box corresponds to the analysis of each *CAR* gene and the lower box to the *ACTIN* control of cDNA. UTR, untranslated region.

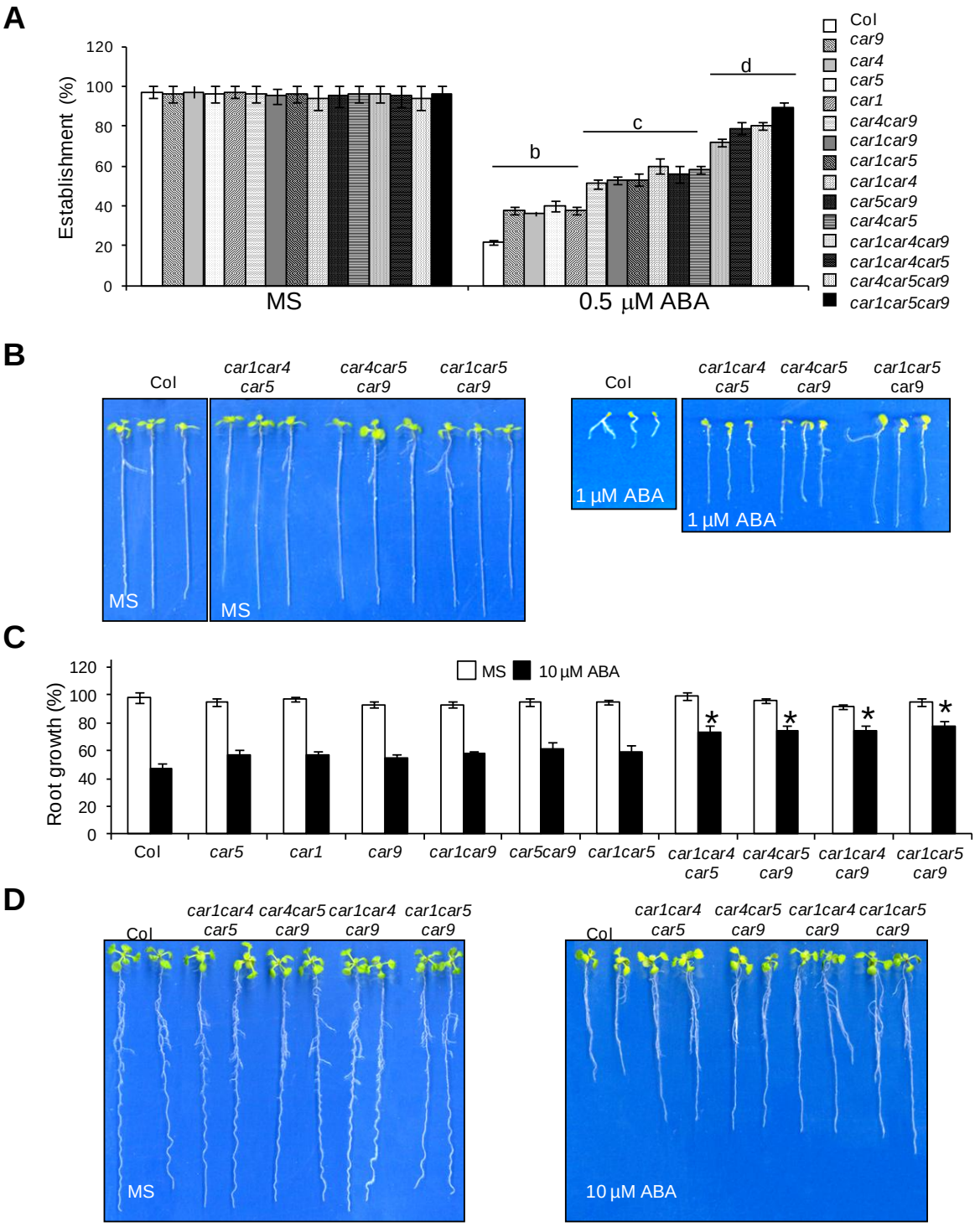

Figure 4.15 Triple *car* mutants show reduced sensitivity to ABA-mediated inhibition of seedling establishment and root growth. (A) Quantification of ABA-mediated inhibition of seedling establishment of Col wt compared with single, double and triple *car* mutants. Approximately 100 seeds of each genotype were sown on each plate and scored for the presence of green expanded cotyledons 5 d later. The letters denote significant differences among the different genetic backgrounds ($p<0.05$, Fisher´s least significant difference test). (B) Photographs of Col wt and triple *car* mutants grown for 7 d on MS medium either lacking or supplemented with 1 µM ABA. (C) Quantification of ABA-mediated root growth inhibition of Col wt compared with single, double and triple *car* mutants. * indicates $p<0.05$ (Student´s t test) when comparing data of *car* mutants to Col wt plants in the same assay conditions. (D) Photographs of representative seedlings 10 d after the transfer of 4 d old seedlings from triple *car* mutants from MS medium to plates lacking or supplemented with 10 µM ABA.

Finally, we selected one of the *car* triple mutants that showed reduced sensitivity to ABA and compared it with a triple mutant impaired in three ABA receptors, i.e *pyr1 pyl4 pyl8*, abbreviated as 148 (Figure 4.16). Seedling establishment of the *car1car5car9* triple mutant was less sensitive to ABA-mediated inhibition than wt (Figure 4.16A and B). The percentage of seedlings that established at 0.5 µM ABA was similar in *car1car5car9* and 148, however further development of the seedlings was less inhibited in 148 compared to *car1car5car9* (Figure 4.16B). The root length of the seedlings that were established in 0.5 µM ABA was larger in *car1car5car9* compared to wt (Figure 4.16C). Interestingly, 0.5 µM ABA supplementation enhanced the root length of *car1car5car9* seedlings compared to medium lacking ABA (Figure 4.16C and D), an effect that was previously described in some combined *pyr/pyl* ABA-insensitive mutants (Gonzalez-Guzman et al., 2012; Antoni et al., 2013). To further study the genetic interaction between C2 and PYR/PYL proteins, we performed a cross between the *car1car5car9* and 148 triple mutants. We were able to recover a *car5car9pyr1pyl4pyl8* pentuple mutant (*CAR1* and *PYL8* show linkage in the lower arm of

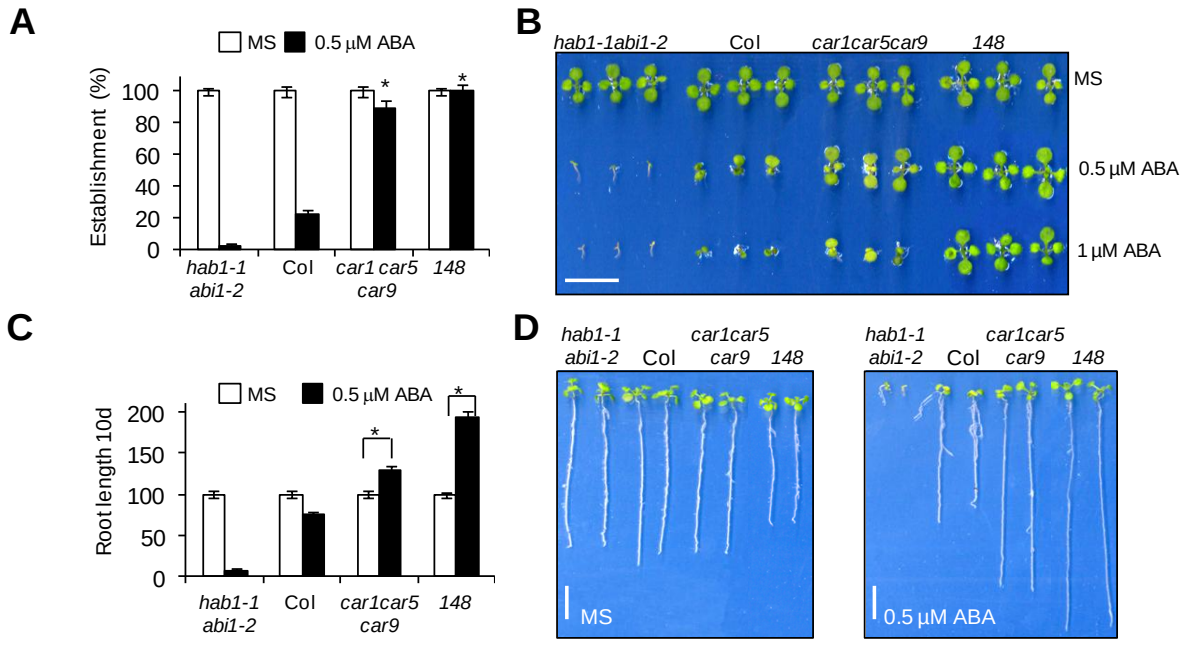

Figure 4.16 ABA sensitivity of the *car1car5car9* triple mutant compared to *pyr1pyl4pyl8* triple mutant. (A) Quantification of ABA-mediated inhibition of seedling establishment of Col wt compared with *car* and *pyr/pyl* triple mutants. Approximately 100 seeds of each genotype were sown on MS plates lacking or supplemented with 0.5 µM ABA and scored for the presence of green expanded cotyledons 5 d later. * indicates $p<0.05$ (Student´s t test) when comparing data of *car* and *pyr/pyl* triple mutants to Col wt plants in the same assay conditions. (B) Photographs of Col wt, *car1car5car9* and *pyr1pyl4pyl8* triple mutants grown for 7 d on MS medium either lacking or supplemented with 0.5 or 1 µM ABA. (C) ABA supplementation improves root growth of *car1car5car9* triple mutant. Quantification of root length in 10-d-old seedlings of experiment described in (A). * indicates $p<0.05$ (Student´s t test) when comparing data obtained in medium lacking or supplemented with 0.5 µM ABA. (D) The photographs show representative seedlings removed at 10 d from MS plates lacking or supplemented with 0.5 µM ABA and rearranged on agar plates.

chromosome 5), and root growth assays in medium supplemented with 20 µM ABA showed that the pentuple mutant was less sensitive to ABA-mediated inhibition of primary root growth than the 148 triple mutant (Figure 4.17A and B). Therefore, both *CAR* and *PYR/PYL* genes additively regulate root sensitivity to ABA. Lateral root growth is also dependent on ABA since endodermal ABA signaling promotes lateral root quiescence during salt stress and accordingly, ABA-insensitive mutants show reduced inhibition of lateral root growth induced by NaCl (Duan et al., 2013). According to this notion, the 148 triple mutant was more resistant to salt-induced inhibition of lateral root growth than wt. Lateral roots of *car1car5car9* also showed a lower sensitivity to NaCl compared to wt, which was additive with the 148 phenotype when the *car5car9pyr1pyl4pyl8* pentuple mutant was assayed (Figure 4.17C). Therefore, taken together, these results indicate that CAR proteins regulate ABA sensitivity both in primary and lateral roots. Reporter gene analysis of the *CAR1* promoter showed predominant expression of *CAR1* in

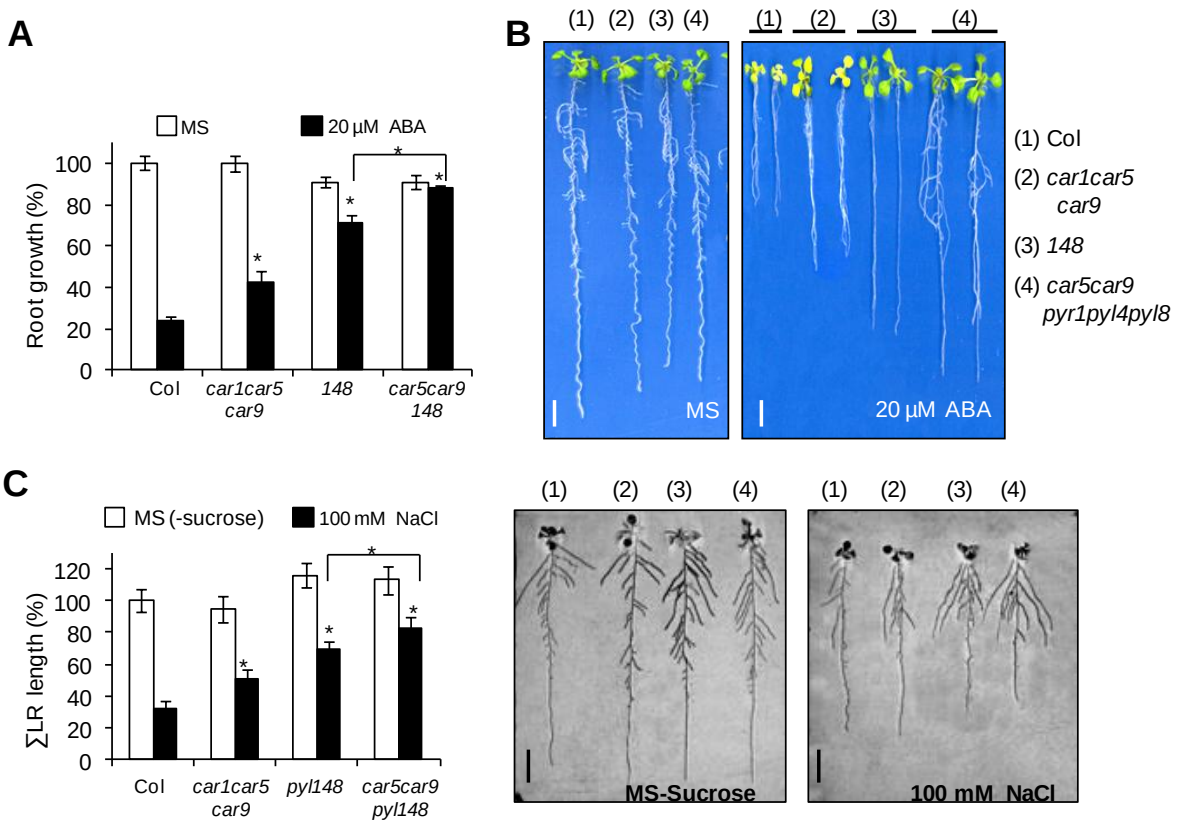

Figure 4.17. Additive effect of the *car5car9pyr1pyl4pyl8* pentuple mutant. (A) Reduced sensitivity to ABA-mediated inhibition of root growth in *car5car9pyr1pyl4pyl8* pentuple mutant compared to other genetic backgrounds. Seedlings were grown on vertically oriented MS plates for 4 d. Afterwards, 20 plants were transferred to new MS plates lacking or supplemented with 20 µM ABA. Quantification of ABA-mediated root growth inhibition was performed after 20 d. * indicates $p<0.05$ (Student´s t test) when comparing data of mutants to Col wt plants in the same assay conditions. (B) The photographs show representative seedlings removed at 20 d from MS plates lacking or supplemented with 20 µM ABA and rearranged on agar plates. (C) Reduced inhibition of lateral root (LR) growth by NaCl in *car5car9pyr1pyl4pyl8* pentuple mutant compared to other genetic backgrounds. Inhibition of lateral root growth by NaCl was assayed in MS plates lacking sucrose and supplemented or not with 100 mM NaCl. * indicates $p<0.05$ (Student´s t test) when comparing data of mutants to Col wt plants in the same assay conditions or *car5car9pyr1pyl4pyl8* pentuple to *pyr1pyl4pyl8* triple mutant. Representative seedlings were removed at 15 d and rearranged on agar plates.

the vascular bundle of the primary root as well as in the cortex of the upper part of the root (Figure 4.18). In lateral roots, *CAR1* expression was also detected in epidermis and root tips.

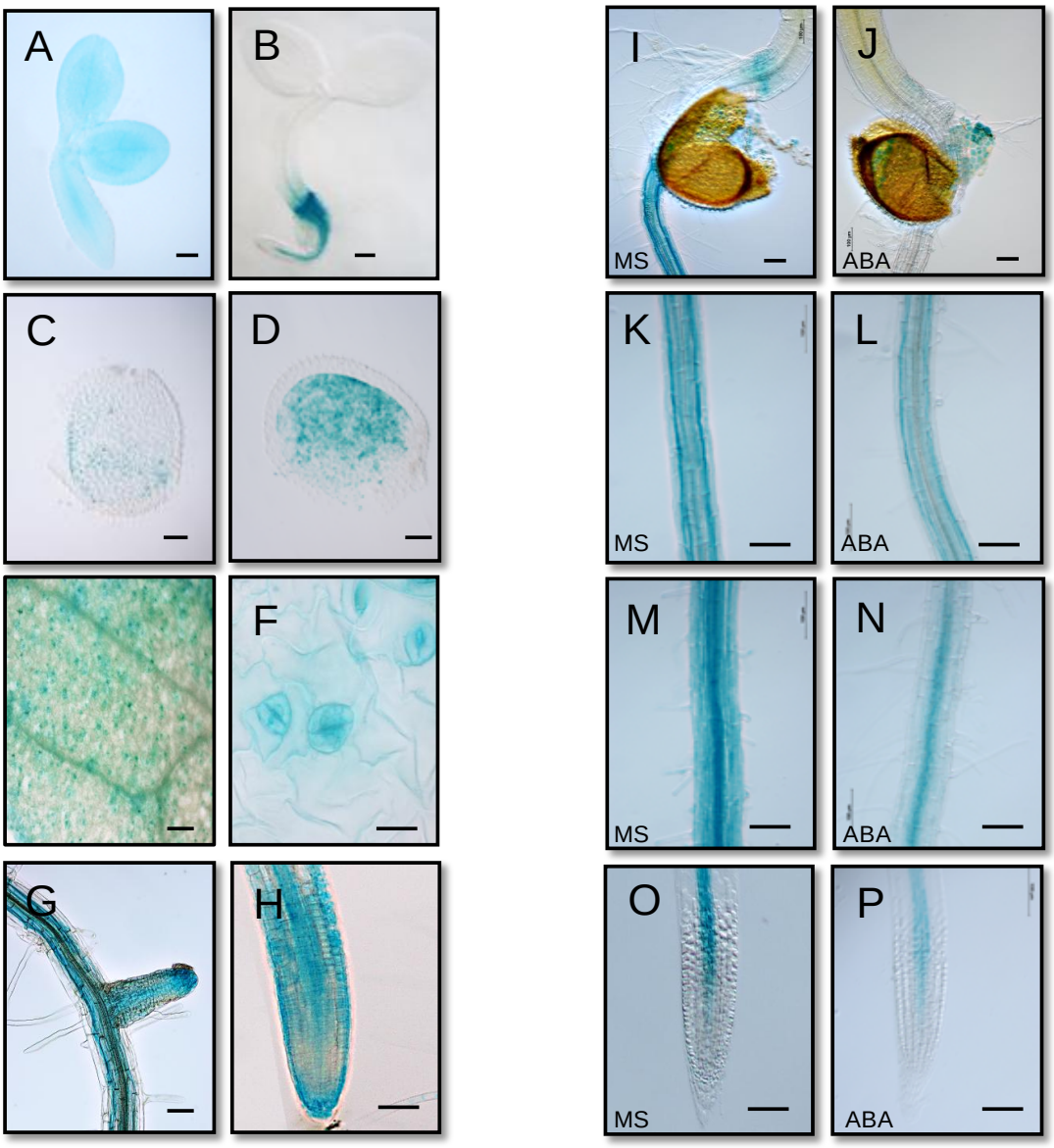

Figure 18. Photographs showing GUS expression driven by ProCAR1:GUS gene in different tissues and developmental stages. Bar = 100µm. Generation of ProCAR1:GUS lines and imaging of histochemical GUS staining was performed as described in Gonzalez-Guzman et al., (2012). A fragment comprising 2 kb 5´ upstream of the ATG start codon of the CAR1 gene was amplified by PCR and cloned into pMDC163 destination vector.
(A), (B). Embryos dissected from mature seeds imbibed for 24 or 48 h, respectively.
(C), (D). Dissected seed coat and endosperm imbibed for 4 or 48 h, respectively.
(E), (F). Vascular tissue and guard cells in leaves of 8-d-old seedlings, respectively.
(G), (H). Emerged lateral roots from 8-d-old seedling.
(I)–(P). Primary root from 5-d-old seedlings that were mock or 10 µM ABA-treated for 10 h. ABA treatment attenuates GUS expression.
(I), (J). Staining of the root-hypocotyl junction.
(K), (L). Staining of mature root zone shows CAR1 expression in cortex.
(M)-(P). Staining of the elongation root zone and root meristem shows predominant expression of CAR1 in vascular tissue.

4.3MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants plants were routinely grown under greenhouse conditions (40%–50% relative humidity) in pots containing a vermiculite:soil (1:3) mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% (v/v) ethanol for 20 min, followed by commercial bleach (2.5% [v/v] sodium hypochlorite) containing 0.05% (v/v) Triton X-100 for 10 min and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Seeds were sowed on MS plates composed of MS basal salts, 0.1% (v/v) MES, 1% (v/v) Suc, and 1% (w/v) agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 mE m^{-2} sec^{-1}.

CAR knock-out insertion lines *car1* (SALK_080173.54.40.X), *car4* (SM 3-1727), *car5* (SAIL_802_B08), and *car9* (SALK_088115.56.00.X) were obtained from the Nottingham *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk). To confirm and identify homozygous T-DNA individuals, seedlings of each insertion line were grown individually and DNA from each plant was extracted and submitted to PCR-mediated genotyping using the primers described in Table 3.

In order to generate *35S:3HA-CAR1* overexpressing lines, the *CAR1* coding sequence was cloned into pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the gateway compatible pALLIGATOR2 vector (Bensmihen et al., 2004). Used primer sequences are described in Table 2. The pALLIGATOR2-CAR1 construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere et al., 1985) by electroporation and used to transform Columbia wild type plants by the floral dip method (Clough and Bent, 1998). T1 transgenic seeds were selected based on GFP visualization and sowed in soil to obtain the T2 generation. Homozygous T3 progeny was used for further studies and expression of HA-tagged protein was verified by immunoblot analysis using anti-HA-peroxidase (Roche).

In order to generate *ProCAR1:GUS* lines, a fragment comprising 2 kb 5´ upstream of the ATG start codon plus the first 30 bp of the CAR1 coding sequence was amplified by PCR and cloned into pCR8/GW/TOPO T/A. Next, it was recombined by Gateway LR reaction into pMDC163 destination vector (Curtis and Grossniklaus, 2003). Used primer sequences are described in Table 3. The pMDC163-based construct carrying ProCAR1:GUS gene was transferred to *Agrobacterium tumefaciens* pGV2260 (Deblaere et al., 1985) by electroporation and used to transform Col wild-type plants by the floral dipping method. Seeds of transformed plants were harvested and plated on hygromycin (20 mg/mL) selection medium to identify T1

transgenic plants, and T3 progenies homozygous for the selection marker were used for further studies. Imaging of GUS within germinating embryos was performed as previously described (Truernit et al., 2008).

Seed germination and seedling establishment assays.

After surface sterilization of the seeds, stratification was conducted in the dark at 4ºC for 3 d. Approximately 100 seeds of each genotype were sowed on MS plates supplemented with different ABA concentrations per experiment. To score seed germination, radical emergence was analyzed at 72 h after sowing. Seedling establishment was scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves at 5 or 7 d.

Root and shoot growth assays.

Seedlings were grown on vertically oriented MS plates for 4 to 5 days. Afterwards, 20 plants were transferred to new MS plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10-d or 20-d to produce image files suitable for quantitative analysis of root growth using the NIH software ImageJ v1.37. As an indicator of shoot growth, fresh weight was measured after 21 d. Inhibition of lateral root growth by NaCl was assayed in MS plates lacking sucrose and supplemented or not with 100 mM NaCl. After 10 d, plates were scanned as described above and total lateral root growth per plant (n=30) was measured.

Yeast Two-Hybrid assay

Yeast Two-Hybrid Screening was performed as previously described in Saez et al., 2008. Briefly, an oligo(dT) primed cDNA library prepared in plasmid pACT2 using mRNA from an *Arabidopsis* cell suspension was kindly provided by Dr. K. Salchert (Saez et al., 2008). The library was shuttled to yeast AH109 by co-transformation with pGBKT7-PYL4. Yeast transformants were pooled and clones able to grow in the absence of exogenous ABA in medium lacking histidine and adenine were selected. Yeast plasmids were extracted, sequenced and retransformed in yeast cells to recapitulate the phenotype. *Arabidopsis* ABA receptors were fused by Gateway recombination to the GAL4 DNA-binding domain (GBD) in pGBKT7GW. N-terminal deletions of PYL4, PYL6 and PYL8 were generated using the primers described in Table 3. The CAR1 prey was fused to the GAL4 activation domain (GAD) in pACT2 vector.

Transient protein expression in *Nicotiana benthamiana*

Agrobacterium infiltration of tobacco leaves was performed basically as described by Voinnet et al., (2003). Constructs to investigate the subcellular localization of CAR and PYL4 proteins

were done in pMDC83 and pMDC43 vectors, respectively. The constructs encoding the plasma membrane markers OFP-TM23 and $SCFP^{C}$-CIPK24/CBL1-$SCFP^{N}$ were reported in Batistic et al., (2012) and Waadt et al., (2008), respectively. To investigate the interaction of CAR and PYR/PYL proteins in planta, we used the pSPYNE-35S and $pYFP^{C}43$ vectors (Walter et al., 2004; Belda-Palazon et al., 2012). The coding sequences of *CAR1*, *CAR4* and *CAR5* were doubly digested *Bam*HI-*Eco*RV and cloned into *Bam*HI-*Sma*I pSPYNE-35S. The coding sequences of PYR1, PYL1, PYL4, PYL6 and PYL8 were recombined by LR reaction from pCR8 entry vector to $pYFP^{C}43$ destination vector. The different binary vectors described above where introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere et al., 1985) by electroporation and transformed cells were selected in LB plates supplemented with kanamycin (50 mg/L). Then, they were grown in liquid LB medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM morpholinoethanesulphonic (MES) acid-KOH pH 5.6 containing 10 mM MgCl2 and 150 mM acetosyringone to an OD600 nm of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1 (pCH32 35S:p19) expressing the silencing suppressor p19 of tomato bushy stunt virus (Voinnet et al., 2003) so that the final density of *Agrobacterium* solution was about 1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old *Nicotiana benthamiana* plants. Leaves were examined 48-72 h after infiltration using confocal laser scanning microscopy.

Confocal Laser Scanning Microscopy

Confocal imaging was performed using a Zeiss LSM 780 AxioObserver.Z1 laser scanning microscope with C-Apochromat 40x/1.20 W corrective water immersion objective. The following fluorophores, which were excited and fluorescence emission detected by frame switching in the single or multi-tracking mode at the indicated wavelengths, were used in tobacco leaf infiltration experiments: SCFP (405 nm/464-486 nm), GFP (488 nm/500-530 nm), YFP (488 nm/529-550 nm) and OFP (561 nm/575-600 nm). Pinholes were adjusted to 1 Air Unit for each wavelength. Post-acquisition image processing was performed using ZEN (ZEISS Efficient Navigation) Lite 2012 imaging software and ImageJ (http://rsb.info.gov/ij/).

Epifluorescence confocal images of epidermal tobacco leaves co-infiltrated with the constructs described in the text were merged to quantitatively estimate co-localization of fluorescent markers (French et al., 2008). Statistical analyses for fluorescence colocalization were performed through determination of the linear Pearson's and nonlinear Spearman's correlation coefficients between fluorescent signals. Nuclear fluorescent signals of GFP, reconstituted YFP, CAR-GFP and GFP-PYL4 proteins were not taken into account for the co-localization analysis.

To quantify relative fluorescence intensities of BiFC experiments, all images were captured using the same laser, pinhole and gain settings of the confocal microscope to maintain high reproducibility of the dates (laser 2.0%, pinhole diameter 34 μm, master gain 740, digital gain 1.00, digital offset 0.00), as well as zoom factor (1.2) covering 2-3 living cells/image. As negative controls in the interaction assays, Agrobacterium expressing either CAR1-myc-YFP^N or CAR4-myc-YFP^N were co-infiltrated with YFP^C-$OST1_{1-280}$ (Vlad et al., 2009). Image quantification of relative fluorescence intensities was carried out using ImageJ software by measuring the fluorescence intensity corrected for mean background fluorescence subtracted from corresponding areas showing no green fluorescence. Each BiFC experiment was scanned and measured in 25 randomly chosen microscopic fields (n=25) and repeated tree times.

Biochemical fractionation, protein extraction, analysis and immunoprecipitation

Constructs to express GFP or HA-tagged proteins were generated in pMDC43/83 or pALLIGATOR2 vectors, respectively. Generation of PYL4 and PYL8 constructs in pALLIGATOR2 has been described previously (Antoni et al., 2013; Pizzio et al., 2013) and similar constructs were done for PYR1, PYL1 and PYL6. The different binary vectors described above where introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) and used for infiltration of tobacco leaves. Protein extracts for immunodetection experiments were prepared from tobacco leaves 48-72 h after infiltration. Plant material (~100 mg) for direct Western blot analysis was extracted in 2X Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.001% bromophenol blue), proteins were run in a 10% SDS-PAGE gel and analyzed by immunoblotting.

Cytosolic and microsomal fractionation of GFP or HA-tagged proteins was performed as described previously (Antoni et al., 2012). The microsomal fractionation procedure used a lysis buffer supplemented with 25 mM $CaCl_2$ (Antoni et al., 2012). Nuclear fractionation was performed as described previously (Saez et al., 2008; Antoni et al., 2012) and the soluble nuclear fraction was used for immunoprecipitation experiments. Soluble proteins from the nuclear fraction were immunoprecipitated using super-paramagnetic micro MACS beads coupled to monoclonal anti-GFP antibody according to the manufacturer´s instructions (Miltenyi Biotec). Purified immunocomplexes were eluted in Laemmli buffer, boiled and run in a 10% SDS-PAGE gel. Proteins immunoprecipitated with anti-GFP antibody were transferred onto Immobilon-P membranes (Millipore) and probed with anti-HA-peroxidase to detect coIP of HA-tagged receptors. Immunodetection of green fluorescent protein (GFP) fusion proteins was performed with an anti-GFP monoclonal antibody (clone JL-8, Clontech) as primary antibody and ECL anti-mouse-peroxidase (GE Healthcare) as secondary antibody. Antibodies were used to a 1:10000 dilution. Detection was performed using the ECL advance western

blotting chemiluminiscent detection kit (GE Healthcare). Image capture was done using the image analyzer LAS3000 and quantification of the protein signal was done using Image Guache V4.0 software.

Phospholipid binding assays

Calcium-dependent protein binding to phospholipid vesicles was assessed as described in Schapire et al., (2008). A mixture of phosphatidyl serine / phosphatidyl choline 25/75 w/w (Sigma Aldrich) was prepared in chloroform and dried under a stream of nitrogen to obtain a thin layer. The dried lipids were resuspended in buffer A (100 mM NaCl, 50 mM HEPES pH 6.8 and 4 mM EGTA) and mixed by vortexing for 20 minutes. We pelleted the large multilamellar vesicles by 20 min centrifugation at 16000 g, next they were resuspended in 1 ml buffer A supplemented or not with the indicated calcium concentration. Free calcium concentrations were calculated using the WEBMAXC program (http://www.stanford.edu/~cpatton/maxc.html). The vesicles (circa 100 µg phospholipids) were used immediately after preparation and mixed with the indicated His-tagged recombinant proteins (5 µg). Next they were incubated with gentle shaking (250 rev/min) on a platform shaker. The vesicles and the bound proteins were pelleted by centrifugation for 10 min at 16000g at 4 ºC and pellets were washed twice with 0.5 ml of buffer A. Proteins that were bound to the vesicles were revealed by immunoblot analysis using anti-His antibody and ECL anti-mouse-peroxidase (GE Healthcare) as secondary antibody. Detection was performed using the ECL advance western blotting detection kit (GE Healthcare). Quantification of the binding was determined using the Image-J software and mathematical analysis of calcium binding was performed based on non-linear least-squares fitting to the 3-parameter Hill´s equation using Sigma Plot 12 software.

Protein preparation and crystallization

CAR4 was obtained from cultures of *Escherichia coli*, purified to homogeneity and crystallized as described (Diaz et al., 2011). Briefly, *CAR4* coding sequence was cloned into the pETM11 vector and the overexpressed His-tagged protein was purified to homogeneity in a single chromatographic step. Prior crystallization CAR4 protein was dialyzed to a buffer containing 20 mM Tris-HCl pH 8.5, 200 mM NaCl, 0.1 mM $CaCl_2$. The stock protein was concentrated to 8.0 mg/ml. CAR4 prismatic crystals were grown in 0.01 M $LiCl_2$, 0.1 M MES pH 6, 20% *(w/v)* PEG 6K. The crystals were mounted in a fibre loop and soaked in cryoprotectant consisting of mother liquor containing 20% (*w/v*) PEG 400 and flash cooled in liquid nitrogen. *CAR1* coding sequence was cloned into pCOLADuet-1 (Novagen) through *Bam*HI digestion and the overexpressed His-tagged protein was purified to homogeneity in a single chromatographic step. $CAR1^{D22A\ D27A}$, $CAR4^{D85A\ D87A}$ and $CAR4^{\Delta ED}$ mutants were

generated using the PCR-overlap extension procedure using the oligonucleotides described in Supplemental Table 3. CAR1$^{D22A\ D27A}$ and CAR4$^{D85A\ D87A}$ were cloned into pETM11, whereas CAR4$^{\Delta ED}$ was cloned into pSPYNE-35S. PYR/PYL proteins were prepared as described previously (Santiago et al., 2009b).

Accession numbers

The atomic coordinates and structure factor amplitudes of CAR4 in complex with calcium have been deposited in the Protein Data Bank (www.pdb.org), PDB ID (4v29). *Arabidopsis* Genome Initiative (AGI) locus identifiers for *CAR1*, *CAR2*, *CAR3*, *CAR4*, *CAR5*, *CAR6*, *CAR7*, *CAR8*, *CAR9* and *CAR10* are At5g37740, At1g66360, At1g73580, At3g17980, At1g48590, At1g70800, At1g70810, At1g23140, At1g70790 and At2g01540, respectively. AGI identifiers for *PYR1*, *PYL1*, *PYL4*, *PYL6* and *PYL8*, are At4g17870, At5g46790, At2g38310, At2g40330 and At5g53160, respectively.

Table 3. List of oligonucleotides used in this work

PCR	Primer name	Sequence 5'→ 3'
car1	FΔN5g37740	ATCGTCGTTCACTGCGGTAAAC
	RStpRVBHI5g37740	GGATCCGATATCCTAAATACCCCTTGAACCCGG
T-DNA	LBpROK2	GCCGATTTCGGAACCACCATC
car4	FΔN3g17980	ACCATGGAGGACATCAGTAGCAGCGATCCT
	Rstop3g17980	TCATAGACCCTTGGAGCCAGGGA
T-DNA	Spm3	ACCGTCGACTACCTTTTTTCTTGTAGTG
car5	FNco1g48590	ACCATGGAACCTTCTTTAATGGATAG
	R187_1g48590	CAAGTCTTCATTCCACTCAGGA
T-DNA	LB3SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC
car9	F146_1g70790	GTAATCCAGTGTGGAACGAACA
	R1g70790	TTAGTCCAATCGTTTTGTCG
T-DNA	LBpROK2	GCCGATTTCGGAACCACCATC
pyl8-1	FwMEANpyl8	ATGGAAGCTAACGGGATTGAG
	RvESRVpyl8	TTAGACTCTCGATTCTGTCGT
T-DNA	LB3SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC
pyl4	FPYL4	ACCATGGTTGCCGTTCACCGTCCTT
	RPYL4	TCACAGAGACATCTTCTTCTTGC
T-DNA	LB3SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC
pyr1	FNc4g17870	ACCATGGCTTCGGAGTTAACACCA
	R4g17870	TCACGTCAC CTGAGAACCACT
Transgenic lines	Primer name	Sequence 5'→ 3'
CAR1	FwBHI5g37740	GGATCCATGGAGAATCTTGTAGGTCT
	RStpRVHI5g37740	GGATCCGATATCCTAAATACCCCTTGAACC
pCAR1::GUS	FProm5g37740new	TCAACCACTAGTAGACCACAAATG
	RProm5g37740	–AATTCGAAGAAGACCTACAAGATT
BiFC	Primer name	Sequence 5'→ 3'
CAR1	FwBHI5g37740	GGATCCATGGAGAATCTTGTAGGTCT
	RNStpRVHI5g37740	GGATCCGATATCCGAAATACCCCTTGAACC

CAR4	FATGNco3g17980	ACC ATG GCA ACG GCG TGT CCG GCG
	RnostopRV3g17980	GATATCTGATAGACC CTTGGAGCCAGGG
CAR5	FNco1g48590	ACCATGGAACCTTCTTTAATGGATAG
	RnostopRV1g48590	GATATCTGATAGACCCTTTCCGGGAAGATC
Mutants	Primer name	Sequence 5'→ 3'
$CAR1^{D22A\ D27A}$	FwD22A D27A	GCCATTAGA**gcc**ATCTCAAGCAGT**gct**CCTTACATC
	RvD22A D27A	GATGTAAGG**agc**ACTGCTTGAGAT**ggc**TCTAATGGC
$CAR4^{D85A\ D87A}$	FwD85A D87A	ACGGTGTAT**gct**CAC**gcc**ATGTTTAGC
	RvD85A D87A	GCTAAACAT**ggc**GTG**agc**ATACACCGT
$CAR4^{\Delta ED}$	FwEAL	GAGATAAAGCCCAGTGGTGTCGACGGCAAGCTCGTCCAGGAT
	RvVTW	GACGAGCTTGCCGTCGACACCACTGGGCTTTATCTCGAACTCAGCATCGCC
Y2H	Primer name	Sequence 5'→ 3'
PYR1	FNc4g17870	ACCATGGCTTCGGAGTTAACACCA
	R4g17870	TCACGTCAC CTGAGAACCACT
PYL1	FNco5g46790	ACCATGGCGAATTCAGAGTCCTC
	RBamHI46790	GGATCCTTACCTAACCTGAGAAGAGTTGT
Δ1-47 PYL4	FGPN2g38310	ACCATGGGTCCTAATCAGTGTTGCTCC
	R2g38310	CGCACGAATTCACAGAGACATCTTCTTCTT
PYL6	FNcoI2g403	ACCATGGCGAATTCAGAGTCCTC
	R2g040330	GGATCCTTACCTAACCTGAGAAGAGTTGT
Δ1-47 PYL6	FNc2g403	ACCATGGAGCACGTGGAGCTTTCCCAC
	R2g040330	GGATCCTTACCTAACCTGAGAAGAGTTGT
Δ1-27 PYL8	FwDNNcoPYL8	ACCATGGTGGATAATCAGTGTAGCTCT
	RvESRVpyl8	TTAGACTCTCGATTCTGTCGT

5. GENERAL DISCUSSION

5. GENERAL DISCUSSION

Identification of PYL8 interacting proteins implicated in root ABA response.

Previous analyses of loss-of-function mutants indicated that the single *pyl8* mutant displayed reduced ABA-mediated inhibition of root growth, indicating a non-redundant role of PYL8 in regulating root sensitivity to ABA. Several reasons might explain this non-redundant role of PYL8 in root ABA response. One of them is the root expression pattern of PYL8, which shows some specificity with respect to other PYR/PYL receptors. For instance, the expression of PYL8 was detected in columella cells (together with PYL1 and PYL4), vascular tissue (together with PYR1, PYL2, PYL4 and PYL9), and epidermis of the primary root and lateral root cap (Gonzalez-Guzman et al., 2012). Biochemically PYL8 is a monomeric receptor with high affinity for ABA (Kd~ 1µM) from Subclass I. Thus, based on *in vivo* Y2H PP2C interaction analysis, we were able to found some differences between PP2C interactions of PYL8 compared to other ABA receptors. For instance, we have shown by Y2H assay that PYL8 display broader ABA-independent PP2C interaction range (8 of 9 PP2Cs) than other members of Subclass I with the similar monomeric nature, such as PYL7, PYL9 and PYL10, or other ABA receptors with overlapped expression pattern such as dimeric PYL1 from Subclass III and monomeric PYL4 from Subclass II (Figure 3.4). Moreover, these interactions were corroborated by TAP approach. For instance, we have shown that at least five clade A PP2Cs (HAB1, HAB2, ABI1, ABI2, PP2CA) interacted *in vivo* with PYL8 in an ABA-dependent manner (Table 1, Figure 3.6). We cannot exclude that other clade A PP2Cs that are strongly induced by ABA (HAI1, HAI2, HAI3) but otherwise expressed at low levels could also interact with PYL8. Previously, it has been demonstrated that a single PP2C interacts *in vivo* with different ABA receptors (Nishimura et al., 2010). In this work, we demonstrated that a single ABA-receptor could interact *in vivo* with different PP2Cs. Indeed, our TAP experiments, together with those of Nishimura et al. (2010) using YFP-ABI1 as a bait, provide evidence that multiple interactions among PP2Cs and PYR/PYLs occur *in vivo*, generating a regulatory network that offers a wide sensitivity and combinatorial possibilities to modulate ABA signaling. Mathematical modeling will be required to provide quantitative insights on the complex combinatory of the PP2C-ABA-PYL interactions.

The combined data of wide PP2C interaction range and previously observed *in vitro* high capacity of PYL8 to inhibit PP2Cs in ABA-dependent manner compared to other ABA receptors, indicate that PYL8 might have a more prominent role in controlling PP2Cs activity in the root tissue than other ABA receptors. Moreover, we showed reduced ABA-mediated

inhibition of root growth of *pyl8* or *pyr/pyl* combined mutants, which suggests that *pyl8* phenotype is due to higher clade A PP2C activity (Figure 3.2 A and C). For instance, lack of PYL8 function likely leads to a globally enhanced activity of PP2Cs or diminished capacity to inhibit them, and our analysis of root ABA sensitivity of *pyl8 pp2c* mutants (Figure 3.5) supports this idea. Thus, we corroborated by genetic approaches a prominent role of the PYL8 function based on its multiple interactions with PP2Cs that leads to high sensitivity and combinatorial possibilities to modulate ABA signaling in roots.

Recently, Duan et al. (2013) identified the endodermis as the key tissue layer required for ABA-mediated growth repression of the lateral roots during salt stress. Additionally, they proposed that ABA signaling may be active in multiple tissue layers of the root during salt stress and that certain functions, such as growth regulation, are controlled by tissue-specific ABA signaling pathways (Duan et al., 2013). For instance, our results, focused on primary root growth, suggest that PYL8, in difference of other ABA receptors, may regulate ABA perception through ABA signaling pathway at the epidermis root tissue level, which probably also may be determinant for its function in root signaling.

In the last decade, although biochemical and structural investigations clearly revealed ABA-dependent molecular mechanisms by which PYR/PYL/RCARs interact and inhibit clade A PP2Cs (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009b; Yin et al., 2009), some other results pointed the ABA-responsive nature of PYR/PYL/RCAR proteins to be questioned: (i) observed constitutive ABA-independent interactions of PYR/PYL/RCARs with PP2Cs in Y2H and *in planta* bimolecular fluorescence complementation (BiFC) experiments as well as ABA-independent co-purification of 9 from 14 ABA receptors with YFP-ABI1 from *Arabidopsis* plants (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b; Szostkiewicz et al., 2010; Nishimura et al.,2010); (ii) *in vitro* ABA-independent inhibition of PP2Cs by subclass of PYLs, represented by PYL10 (Hao et al., 2011) and (iii) ABA-independent and constitutive inhibition of PP2CA by PYL13 and no binding of PYL13 to ABA because of changes in conserved amino acid residues involved in hormone binding (Zhao et al., 2013; Li et al., 2013a). Based on these results of ABA-independent interaction and inhibition of PP2Cs, some groups have speculated of existence of called constitutive pathway with constitutively active ABA receptors (Zhang et al., 2015). However, until now, this hypothesis remains still controversial. Fuchs et al. (2013) found no evidences of PYL13 as ABA-irresponsive receptor and confirm that PYL13 is *a bona fide* ABA receptor, whose function is based on interaction and inhibition of PP2Cs, such as ABI1, ABI2 and PP2CA, in ABA-dependent manner (Fuchs et al., 2013). Recently, Li et al. (2015) have provided detailed biochemical evidence that PYL10 inhibits PP2C activity only in presence of ABA and the previously reported ABA-independent activity was an artifact due to the presence of BSA in the commercial buffers (Li et al., 2015). Likewise, our TAP experiments show that

the monomeric receptor PYL8 only in presence of ABA binds PP2Cs *in vivo*. In a previous experiment, ABA receptors were coimmunoprecipitated with YFP-ABI1both in the absence and presence of exogenous ABA (Nishimura et al., 2010). However, in our TAP experiments we observed a dramatic increase in the recovery of PP2C peptides by exogenous addition of ABA. Thus, without ABA supplementation, GS-PYL8 only recovered one PP2C peptide in one experiment (none with PYL8 tagged at the C-terminus), compared to 28 peptides when 50 µM ABA was supplemented (Figure 3.6, Table 1). These results suggest that *in vivo* PYL8 only shows a weak/transient interaction with clade A PP2Cs when ABA levels are low, further supporting the current model for PYR/PYL-mediated signaling able to perceive changes in ABA levels (Cutler et al., 2010).

Another question related to the ABA signaling core, which until now remain controversial, is the possible existence of quaternary PYL-ABA-PP2C-SnRK2 complexes and whether this complex is stable in plant cells. Umezawa et al. (2009) have observed no significant changes in PP2C-SnRK2 interactions with or without ABA after *Agrobacterium* –infiltration of *Nicotiana benthamiana* leaves and Nishimura et al. (2010) have seen that SnRK2.2, SnRK2.3 and SnRK2.6 could be coimmunoprecipitated with ABI1 in *Arabidopsis* protein extracts with and without exogenous ABA (Umezawa et al., 2009, Nishimura et al., 2010). Since direct interaction between SnRK2s and PYR/PYL/RCAR has not been described, the only explanation for these results could be the constitutive interactions between PP2Cs and SnRK2s *in vivo.* Moreover, *in vitro* experiments have suggested the possible existence of quaternary PYL-ABA-PP2C-SnRK2 complexes (Soon et al., 2012). Therefore, based on these results, some groups have proposed that C-terminal ABA box of SnRK2.2/ SnRK2.3/ SnRK2.6 could serve to maintain in close proximity these kinases and PP2Cs in the presence of ABA, so that ABA phosphatases could inactive them very rapidly (Figure 5.1A). In contrast, Fujii et al. (2009) showed in Yeast Three-Hybrid (Y3H) analyses that the interactions of ABI1/2 and HAB1 with SnRK2.6 could be disrupted in response to ABA by PYL5/8 receptors (Fujii et al., 2009). Moreover, in the presence of ABA, SnRK2-PP2C constitutive interactions are not consistent with the structure based mechanism of molecular mimicry between ABA receptors, PP2Cs and SnRK2s, which postulates that ABA-PYR/PYL/RCAR receptors act as a competitive inhibitors of the phosphatase at the substrate-binding site (Ng et al., 2011; Dupeux et al., 2011a). In fact, quaternary PYL-ABA-PP2C-SnRK2 complexes have not been recovered from plant extracts yet. In our TAP experiments, we also did not recover any ABA-activated SnRK2 forming part of complexes with PYL8 receptor. Therefore, our TAP results support the idea that all components may not be part of the same signalosome simultaneously and PP2C-SnRK2 and PP2C-ABA-PYR/PYL/RCAR complexes are mutually exclusive (Figure 5.1B). Thus, the ABI1-interacting proteins identified by MS analyses by Nishimura et al., (2010) might simply reflect the recovery of independent PYL-ABA-ABI1 and ABI1-SnRK2 complexes.

However, we should note that TAP is more stringent technique than single step GFP affinity purification, which might result in loss of the SnRK2 ABA box-PP2C interaction or weaker interactions than ternary ABA complexes.

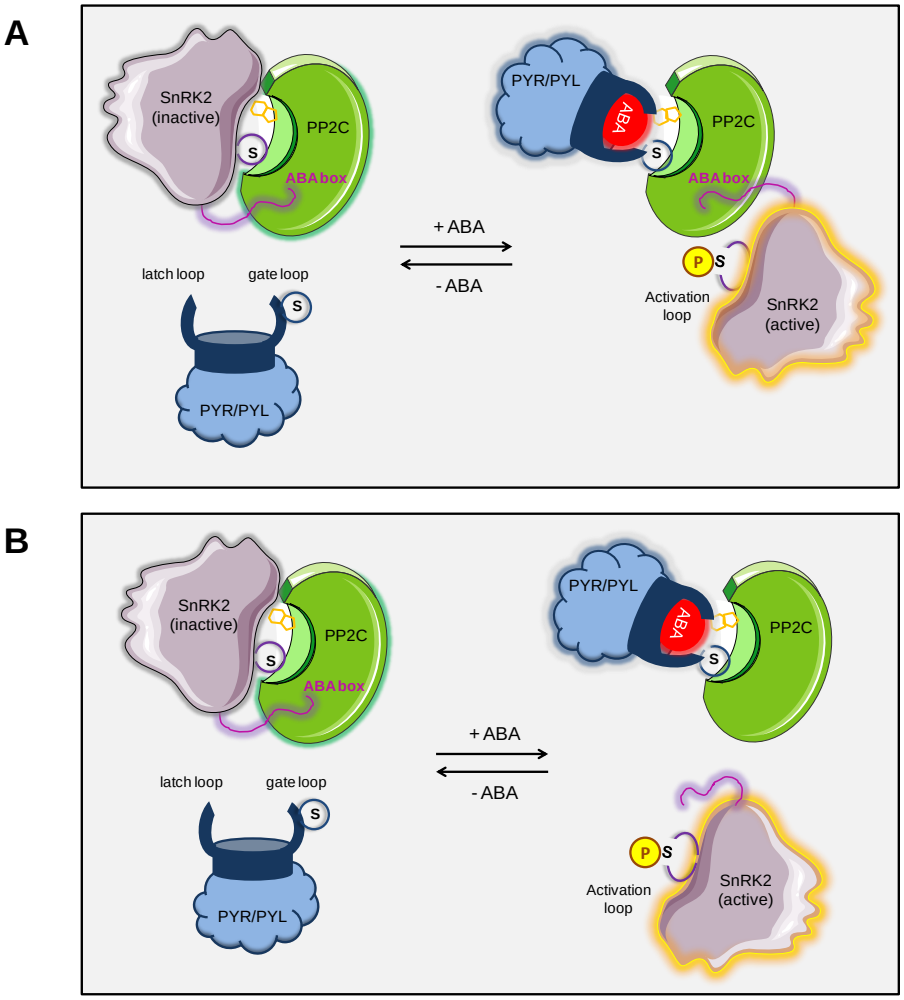

Figure 5.1 Cartoon presentation of the two alternative models of SnRK2–PP2C interactions within ABA sgnaling core. In the absence of ABA, PYR/PYL receptors are in an ‘‘open’’ conformation with the ‘‘gate’’ loop facing away from the ligand-binding pocket and the ‘‘latch’’ loop. PP2Cs are active and interact with SnRK2s trough two binding sites: kinase domain and C-terminal ABA box. Thus, while the conserved ABA-sensing tryptophan of PP2Cs inserts into the kinase catalytic cleft, the kinase activation loop of SnRK2s docks into the active site of PP2Cs, causing dephosphorylation of the serine (S) residue and further inactivation of SnRK2s by allosteric inhibition. This SnRK2-PP2C complex in the absence of ABA, mimics a PYR/PYL-ABA-PP2C interaction. For instance, in the presence of ABA, ABA-binding induces a conformation change in the receptors, causing swing of the gate loop to a closed position and promoting binding of the ABA receptor to the catalytic site of the PP2Cs as those involved in SnRK2s interaction. While the conserved tryptophan of PP2Cs establishes water-mediated hydrogen bond with ABA, increasing stability of PYR/PYL-ABA-PP2C ternary complex, the conserved serine residue (S) of the receptor “gate” loop blocks kinase seine entrance to the catalytic domain of PP2Cs and inhibit phosphatase activity. Complex formation therefore inhibits the activity of the PP2C in an ABA-dependent manner, allowing autoactivation of SnRK2s. Thus, PYR/PYL-ABA act as competitive inhibitor of PP2C and makes SnRK2-PP2C and PYR/PYL-ABA-PP2C interactions mutually exclusive. A, Model proposed by several groups, in which, in the presence of ABA, SnRK2s remain interacting with PP2C through C-terminal ABA box and this interaction is not affected by binding of ABA-PYR/PYL. Thus, in the presence of ABA, it is possible formation of quaternary PYR/PYL-ABA-PP2C-SnRK complex. B, Model suggested by results obtained in PYL8 TAP experiments in this work, in which formation of the ternary PYR/PYL-ABA-PP2Cs complex completely disrupts physical interaction between PP2Cs and SnRK2s.

Interestingly, although PP2Cs are the mayor interacting partners of PYR/PYL/RCARs in presence of ABA, in the last two years a number of new interacting partners of ABA receptors have been identified in several studies. It has been seen that regulation of the PYR/PYL turnover could occur at the plasma membrane or other cellular locations through interaction with different E3 ubiquitin ligases, promoting targeted degradation of PYL ABA receptors. For instance, Irigoyen et al. (2014) have seen that multi-subunit E3 ligase formed by the CRL4 complex through substrate adapter DDA1 binds to PYL8, as well as PYL4 and PYL9, in nucleus *in vivo* and Bueso et al. (2014) have been seen interactions between RING FINGER OF SEED LONGEVITY1 (RSL1) E3 ligase, targeted at the plasma membrane, with PYR1 and PYL4 ABA receptors (Irigoyen et al., 2014; Bueso et al., 2014). Together these works represent a new regulatory mechanism to modulate ABA responses based on the control of ABA receptor stability. PYL8, independently of the core ABA signaling pathway, has also been shown to promote lateral root growth by direct interaction with the transcription factor MYB77 and its paralogs, MYB44 and MYB73, leading to transcription factor enhanced activities and augmented auxin signaling (Zhao et al., 2014). Finally, it has been identified *Arabidopsis* single C2-domain superfamily, C2-domain ABA-related (CAR) proteins as new interacting partners of PYR/PYLs ABA receptors, which mediate their approach to the plasma membrane in a Ca^{2+}-dependent manner (Results, chapter II). However, in our TAP experiments we did not recover any new possible interaction partners of PYL8. One possible explanation could be the fact that, although TAP is more stringent technique than single step affinity purification, maintenance and identification of low abundance transient interactions, so important for rapid hormone signaling, remains a challenge. It is thought that such dynamic interactions can be lost during the longer purification times necessary for tandem purifications. Additionally, it has been reported that there are some interactors, which are involved in ubiquitin-proteasome degradation pathway and might affect the stability of ABA receptors (Wang and Huang, 2008). Some steps of the TAP protocol, such as TEV cleavage step, which require incubation at 16ºC for 1 hour, actually have some alternatives that not affect protein stability and are available to deal with those weak point (Dedecker et al., 2015). On the other hand, in our TAP experiments, the purification protocol started from total soluble protein extract from Arabidopsis suspension cell cultures, which often leads to dilution of possible interacting partners, depending on its subcellular localization specificity, e.g. chloroplast, mitochondrial, nuclei and membrane associated proteins. To make easier recruitment of such interacting proteins, the extraction protocol need to be optimized by the fractionation step enrichment. Thus, protein stability and subcellular localization are factors to be taken into account for next generation of TAP approach.

Identification and characterization of C2-domain CAR proteins as new plasma membrane interacting partners of PYR/PYL/RCAR ABA receptors, which positively regulate abscisic acid sensitivity

In this work we describe a family of C2-domain calcium binding proteins that interact with PYR/PYL ABA receptors and mediate their approach to the plasma membrane in a Ca^{2+}-dependent manner. We suggest that the high local calcium concentration found at cellular membranes in response to different stimuli (ABA, abiotic stress, pathogen attack) might allow CAR proteins to translocate to cell membranes in response to calcium oscillations as we have demonstrated using in vitro assays. Therefore, CAR-interacting proteins, such as the PYR/PYL ABA receptors, are membrane-recruited in a Ca^{2+}-dependent manner (Figures 4.10 and 4.11). We have demonstrated that PYL4 and CAR1 interact in the plasma membrane of plant cells; however, we cannot exclude interaction in other membrane compartments for the full set of 10 CAR and 14 PYR/PYL proteins. Depending on the membrane system targeted by CAR proteins, we suggest it might affect the activity, half-life, trafficking or targeting of the interacting PYR/PYLs by changing their sub-cellular localization, which would in turn affect receptor-mediated regulation of clade A PP2Cs. Genetic evidence obtained with combined *car* mutants supports the notion that CAR proteins regulate at least a subset of ABA responses, so CAR-dependent transient interactions of ABA receptors with plasma membrane affect ABA signaling.

Different abiotic stresses induce Ca^{2+} fluctuations, which serve as a second messenger to elicit plant responses to changing environment (McAinsh and Pittman, 2009; Bari and Jones, 2009; Dodd et al., 2010). For instance, both osmotic stress and cold require Ca^{2+} signaling in order to regulate gene expression and to cope with cellular damage, such as repair of plasma membrane (Yamazaki et al., 2008; Schapire et al., 2008; Dodd et al., 2010). Members of the *CAR* family are transcriptionally regulated by different abiotic stresses (Kilian et al., 2007; Figure 5.2) and an homologous rice gene of the *CAR* family, Os-*SMCP1*, was previously found to confer tolerance to both abiotic and biotic stresses in transgenic *Arabidopsis* (Yokotani et al., 2009). Therefore, it is possible that CAR proteins, either through regulation of ABA signaling or additional downstream targets regulated by PP2Cs, are also involved in responses to abiotic stress involving calcium fluctuations. ABA signaling involves increases in intracellular [Ca^{2+}] levels, which has primarily been studied in guard cells (Kim et al., 2010). However, it is likely that other plant tissues responsive to ABA, such as root, also utilize Ca^{2+} as a second messenger of ABA signaling or some interplay occurs between Ca^{2+} signaling induced by abiotic stress and ABA. For instance, it is well known that the root response to abiotic stress involves increases in cytoplasmic free calcium (Kiegle et al., 2000). Osmotic and salt stress cause increases in Ca^{2+}

levels in the endodermis, and this tissue is the target cell layer for ABA-dependent regulation of lateral root growth in response to osmotic stress (Kiegle et al., 2000; Duan et al., 2013). Therefore, a Ca^{2+}-mediated connection between osmotic/salt stress and ABA signaling is envisaged in the root response to environmental stress. CAR proteins might mediate such cross-talk since *car* mutants showed reduced sensitivity to ABA-mediated inhibition of primary root growth and they were also less sensitive to salt-mediated inhibition of lateral root growth (Figures 4.16 and 4.17).

It has been also reported that ABA can prime the sensitivity of Ca^{2+}-dependent processes (Young et al., 2006). Although a molecular explanation for this priming mechanism has not been reported yet, it is possible that ABA affects the function of several Ca^{2+} associated proteins such as CDPKs/CPKs, calmodulins (CaMs), calcineurin B-like (CBLs) and CBL-

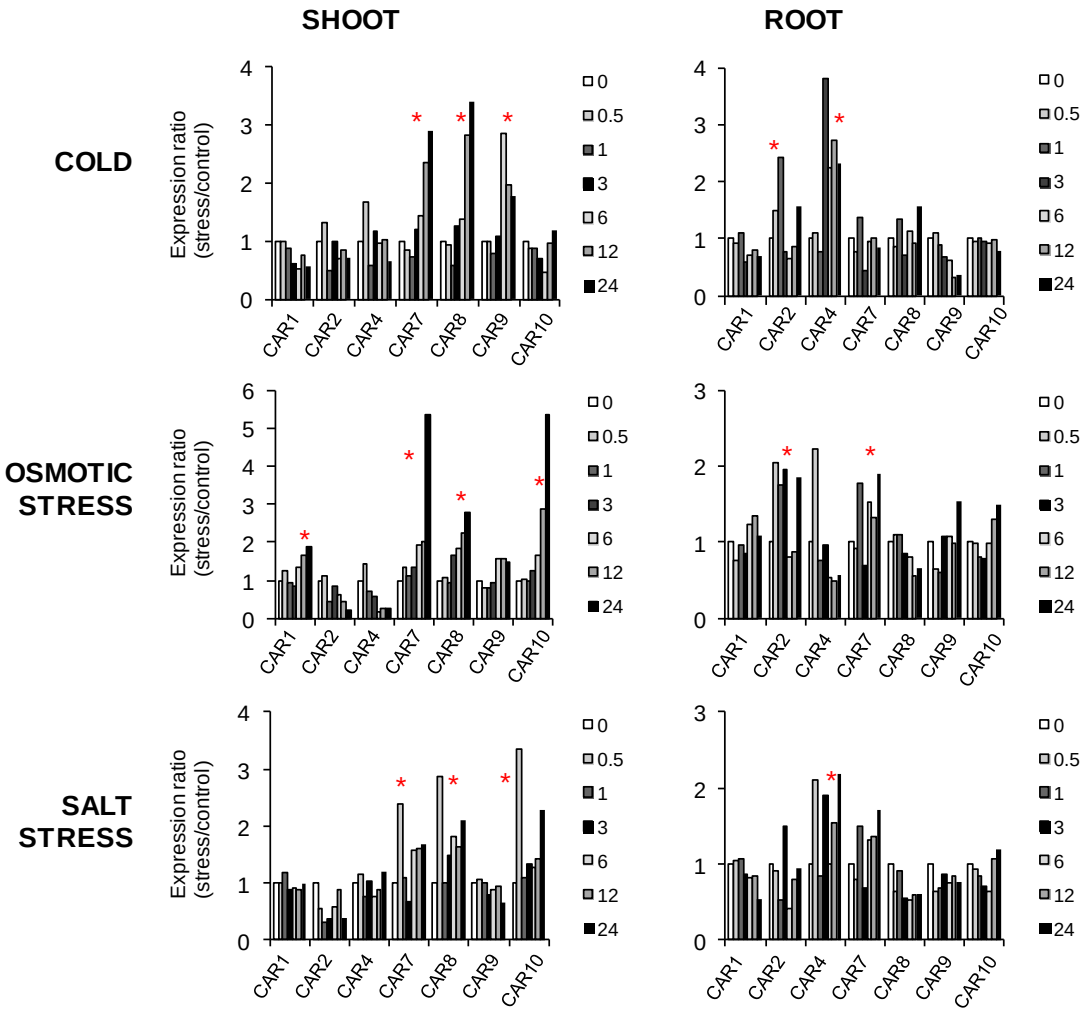

Figure 5.2 Induction of CAR genes by cold, osmotic and salt stress in shoot or root tissues. Data were obtained from the AtGenExpress data set for abiotic stress-induced gene expression (Kilian et al., 2007). Those *CAR* genes represented in the AtGenExpress data set are shown. Stress treatments were initiated at 18 d after sowing and root and shoot samples were taken at 0, 0.5, 1, 3, 6, 12 and 24 h after the onset of the treatment. Cold stress was applied by transferring the boxes containing the plants to 4ºC, whereas osmotic and salt stress were applied by transferring rafts containing the plants to MS medium supplemented with 300 mM mannitol or 150 mM NaCl, respectively. Mock-treated plants were used to normalize gene expression data. Red asterisks mark those *CAR* genes that showed at least 2-fold upregulation by stress treatment in any sample.

interacting protein kinases (CIPKs), which mediate plant responses to environmental stress (Hubbard et al., 2010). Indeed, some of these components are involved in the interplay between abiotic stress and ABA signaling (reviewed in Dodd et al., 2010). In this work we provide an additional point of crosstalk for Ca^{2+} and ABA signaling, since we describe a family of Ca^{2+}-binding proteins that are able to modify the subcellular localization of ABA receptors, which presumably affects their ability to regulate downstream targets, i.e., PP2Cs and SnRK2s. A lipid nanodomain plasma membrane localization of core ABA signaling components, PYL9 and ABI1, has been reported to be required to regulate the activity of CPK21 and SLAH3 and ABA signaling (Demir et al., 2013), and other PYL-PP2C targets are localized to the plasma membrane (Cherel et al., 2002; Lee et al., 2007; 2009; Geiger et al., 2009; Brandt et al., 2012; Pizzio et al., 2013).

CAR1 and CAR4 proteins also localize into the nucleus and interact there with PYR/PYL receptors, although the role of nuclear CAR-PYR/PYL interactions as well as the putative role of Ca^{2+} in this interaction remains to be investigated. Interestingly, other small C2 domain proteins described in plants also show a dual localization at the plasma membrane and nucleus (Wang et al., 2009) and several CBLs show both cytosolic and nuclear localization, further confirming that Ca^{2+} fluctuations induced by abiotic stress can be sensed in the nucleus (Batistic et al., 2010). Ca^{2+} signals are not exclusively from the cytosol, since they also exist in noncytosolic locations, such as mitochondria, chloroplast and nucleus (McAinsh and Pittman, 2009; Dodd et al., 2010). In the latter case, Ca^{2+} may permeate from the cytosol into the nucleus or it can be released by different transporters from Ca^{2+} stores in the lumen of the nuclear envelope contiguous with the endoplasmic reticulum. Many mechanisms account for nuclear calcium signaling and calcium-regulated transcription in plants (Galon et al., 2010; Charpentier and Oldroyd, 2013). Recently, a nuclear calcium-sensing pathway required for the salt stress response has been reported in *Arabidopsis* and it is well known that calcium/CaM binding transcription activators (CAMTAs) mediate abiotic stress responses (Galon et al., 2010; Guan et al., 2013; Antoni et al., 2013). CAMTAs include a C-terminal CaM-binding domain and an N-terminal domain that mediates binding to DNA cis-elements, such as abscisic acid-responsive elements (ABREs), and ABREs confer transcriptional regulation to stress-dependent calcium-responsive genes, linking nuclear calcium signaling to transcription (Kaplan et al., 2006; Dodd et al., 2010). Calcium-mediated transcriptional regulation can be achieved through phosphorylation-dephosphorylation events. For instance, nuclear CDPKs can regulate ABRE binding factors by phosphorylation (Galon et al., 2010). Nuclear CBLs and their interacting CIPKs also regulate nuclear targets and interact with the ABA signaling components ABI1, ABI2 and ABI5 (Lyzenga et al., 2013). It is tempting to speculate that PYR/PYLs, either through regulation of PP2C activity or downstream kinase targets, might link calcium nuclear signals perceived through CAR proteins to transcriptional regulation.

The analyses of our structural data and the available structural information on the interaction of various C2 domains with membranes (Medkova and Cho, 1998; Davletov et al., 1998a; Verdaguer et al., 1999; Kohout et al., 2003; Frazier et al., 2003) allowed us to simulate the CAR4 interaction with a phospholipid bilayer (Figure 5.3). C2 domains use a combined mechanism for membrane binding based on phospholipid headgroup binding, electrostatic interaction and membrane insertion of hydrophobic residues (Lemmon et al., 2008). In this model, the calcium atoms bridge CAR4 with the phosphate moiety of phospholipids, and the hydrophobic tip of loop L3 (Met88 Phe89) is inserted into the membrane as has been described for other C2 domains (Cho and Stahelin, 2006; Ausili et al., 2011). In this situation, the characteristic α1βAβB CAR-signature domain is fully solvent accessible as it is placed opposite to the calcium binding site. This suggests a role for the α1βAβB CAR-signature domain in the recruitment of the PYR/PYL receptor to an area near the membrane. Interestingly, the protein face opposite to Ca^{2+}-binding loops has previously been reported to be involved in C2-mediated protein-protein interactions (Law et al., 2010). For instance, membrane binding by lymphocyte perforin relies on the Ca^{2+} binding loops of a C2-domain, which in the opposite face is linked to a membrane attack complex/perforin like (MACPF) fold (Law et al., 2010). We have also shown that the N-terminal helix of ABA receptors is involved in CAR binding using limited deletion analysis (Figure 4.1B). This receptor area is opposite to the ABA and PP2C phosphatase binding sites and is not involved in the receptor-phosphatase interaction or receptor dimer formation (Figure 5.2). This suggests that CAR proteins might bind a functional ABA receptor or facilitate the formation of receptor-ABA-PP2C ternary complexes at cell membranes. Since early events of ABA signaling are linked to membrane proteins regulated by PP2Cs and SnRK2s, the reported interaction might facilitate the connection of ABA perception to downstream regulatory event.

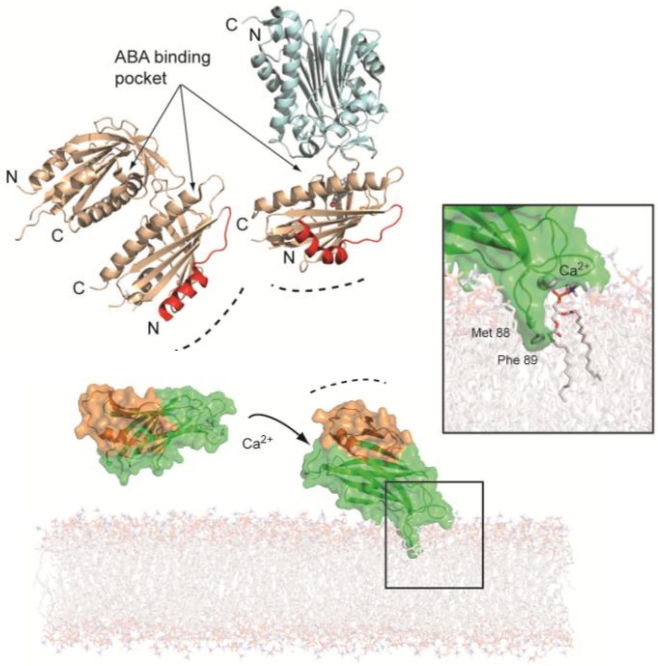

Figure 5.3 A working model for the calcium-dependent CAR membrane binding and its interaction with the PYR/PYL ABA receptors. CAR4, represented as a semitransparent surface, has been docked into a phosphatidyl choline model membrane. The dimeric structure of the apo PYL1 receptor (PDB code 3KAY) and the ternary complex of PYL1, ABA and ABI1 phosphatase (PDB code 3JRQ) are displayed as wheat and pale cyan ribbons respectively. The CAR4 a1b1b2 extra-domain and the N-terminal receptor interacting areas are highlighted in orange and red respectively. The inset represents a close up of the modeled CAR4 calcium-phospholipid complex. Membrane insertion of CAR4 exposes the CAR-signature extra-domain to the cytosol.

6. CONCLUSIONS

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CONCLUSIONS CHAPTER I

1. The combined data obtained from biochemical, proteomic and genetic analysis reveal that PYL8 ABA receptor establishes multiple interactions with PP2Cs, which leads to high sensitivity and combinatorial possibilities to modulate ABA signaling in root. This work demonstrated for the first time that a single ABA-receptor is able to interact *in vivo* with different PP2Cs.
2. TAP experiments indicate that at least five clade A PP2Cs (HAB1, HAB2, ABI1, ABI2, PP2CA) interact *in vivo* with PYL8 in an ABA-dependent manner. In the absence of ABA, PYL8 did not interact significantly with PP2Cs under our experimental conditions.
3. Under our experimental conditions the formation of the ternary PYL8-ABA-PP2C complex disrupts physical interaction between PP2Cs and SnRK2s and prevents the formation of quaternary PYL8-ABA-PP2C-SnRK complexes.

CONCLUSIONS CHAPTER II

1. The search for new interacting partners of PYL4 by Y2H screening led us to the identification of *Arabidopsis* C2-domain ABA-related (CAR) proteins as new interacting partners of ABA receptors.
2. The *Arabidopsis* genome contains 10 members of the CAR family (CAR1 to CAR10), which show high homology at the amino acid sequence level. All members encode small single C2-domain-containing proteins, between 165 to 185 amino acid residues. An additional extradomain responsible of the interaction with ABA receptors was also identified.
3. BiFC assays and coimmunoprecipitation (coIP) experiments confirmed that CAR1 and CAR4 interact with several members of the PYR/PYL/RCAR family, such as PYR1, PYL1, PYL4, PYL6 and PYL8 *in vivo*. Thus, co-localization analysis with OFP-TM23 plasma membrane marker revealed interactions between CARs and PYR/PYL/RCARs in the plasma membrane.
4. *In vitro* phospholipid binding assays revealed that CAR1 and CAR4 proteins are able to bind to phospholipid vesicles in Ca^{2+}-dependent manner as well as to mediate interaction of ABA receptors with phospholipid vesicles in Ca^{2+}-dependent manner.
5. Elucidation of the atomic structure of CAR4 revealed the characteristic β-sandwich C2 fold with two calcium binding sites. In addition, as a unique CAR signature, all members of the CAR family have an extra-domain insertion that connect the two 4-stranded beta-sheets and

folds as an alpha helix followed by a beta hairpin (α1βAβB). This extra-domain present in CAR proteins is required for interaction with PYR/PYL/RCAR ABA receptors.

6. Overexpression of CAR1 led to enhanced ABA sensitivity in seedling establishment and root growth, conversely, *car* triple mutants are less sensitive to ABA-mediated inhibition of seedling establishment and root growth than the wild type, thus indicating that CARs proteins are positive regulators of ABA response and CAR-dependent transient interactions of ABA receptors with the plasma membrane affect ABA signaling.
7. We propose a working model in which, in response to calcium oscillations, CAR proteins dock ABA receptors or facilitate the formation of PYR/PYL/RCAR-ABA-PP2C ternary complexes at cell membranes. Since early events of ABA signaling regulate different membrane proteins through PP2Cs and SnRK2s, the docking of ABA receptors through CAR proteins might facilitate the connection of ABA perception to downstream regulatory events.

7. REFERENCES

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8. APPENDIX I

The single subunit RING-type E3 ubiquitin ligase RSL1 targets PYL4 and PYR1 ABA receptors in plasma membrane to modulate abscisic acid signaling

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Eduardo Bueso*, **Lesia Rodriguez***, Laura Lorenzo-Orts, Miguel Gonzalez-Guzman, Enric Sayas, Jesús Muñoz-Bertomeu, Carla Ibañez, Ramón Serrano and Pedro L. Rodriguez.

*Both authors contributed equally to this work

SUMMARY

Membrane-delimited events play a crucial role for ABA signaling and PYR/PYL/RCAR ABA receptors, clade A PP2Cs and SnRK2/CPK kinases modulate the activity of different plasma membrane components involved in ABA action. Therefore, the turnover of PYR/PYL/RCARs in the proximity of plasma membrane might be a step that affects receptor function and downstream signaling. In this study we describe a single subunit RING-type E3 ubiquitin ligase RSL1 that interacts with the PYL4 and PYR1 ABA receptors at the plasma membrane. Overexpression of RSL1 reduces ABA sensitivity and *rsl1* RNAi lines that impair expression of several members of the RSL1/RFA gene family show enhanced sensitivity to ABA. RSL1 bears a C-terminal transmembrane domain that targets the E3 ligase to plasma membrane. Accordingly, bimolecular fluorescent complementation (BiFC) studies showed the RSL1-PYL4 and RSL1-PYR1 interaction is localized to plasma membrane. RSL1 promoted PYL4 and PYR1 degradation in vivo and mediated in vitro ubiquitylation of the receptors. Taken together, these results suggest ubiquitylation of ABA receptors at plasma membrane is a process that might affect their function via effect on their half-life, protein interactions or trafficking.

INTRODUCTION

Abscisic acid (ABA) is a key regulator of plant adaptation to stress and different aspects of plant growth and development. PYR/PYL/RCAR receptors play a major role for ABA perception and signaling (Cutler et al., 2010; Gonzalez-Guzman et al., 2012). ABA elicits plant responses through binding to soluble PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptors, which constitute a 14-member family. PYR/PYL/RCAR receptors perceive ABA intracellularly and as a result, form ternary complexes with clade A protein phosphatases type-2C (PP2Cs), thereby inhibiting them (Park et al., 2009; Ma et al., 2009). This allows the activation of downstream targets of the PP2Cs, such as the sucrose non-fermenting 1-related subfamily 2 (SnRK2s) protein kinases, i.e. SnRK2.2/D, 2.3/I and 2.6/OST1/E, which are key players to regulate transcriptional response to ABA and stomatal aperture (Umezawa et al., 2009; Fujita et al., 2009; Vlad et al., 2009a; Fujii and Zhu, 2009b). Besides OST1, calcium-dependent protein kinases (CPKs) also regulate stomatal aperture in an ABA- and PYR/PYL/RCAR-PP2C dependent manner (Geiger et al., 2010; Brandt et al., 2012). This process involves regulation of the activities of membrane-localized factors, such as ion channels and proton pumps, which is dependent on both ABA perception by PYR/PYL/RCAR receptors and regulation of clade A PP2C activity in the proximity of plasma membrane (Osakabe et al., 2013; Kollist et al., 2014). The existence of a functional signaling complex in plant membrane nanodomains that is

positively regulated by ABA via RCAR1/PYL9-dependent inhibition of ABI1 has been recently reported (Demir et al., 2013).

Although the biochemical function of PYR/PYL receptors has been well established, little is known about other aspects of the family with biological relevance, such as the detailed sub-cellular localization of receptors in cell compartments, putative post-translational modifications or half-life regulation. A recent advance in this field has been the discovery of PYL8 ubiquitylation as a mechanism to modulate ABA signaling (Irigoyen et al., 2014), adding another example to the ubiquitous role played by the ubiquitin (Ub)-proteasome pathway in plant hormone signaling (Vierstra et al., 2009; Santner and Estelle, 2009). Approximately 5% of the *Arabidopsis thaliana* genome encodes components that can be connected to the Ub/26S proteasome pathway, which is one of the most elaborate and prevalent regulatory mechanisms in plants (Smalle and Vierstra, 2004; Vierstra et al., 2009). Ubiquitylation, in addition to commit proteins to degradation by the 26S proteasome, also directs nonproteolytic outcomes, for instance regulation of protein trafficking (Kim et al., 2013). Recent proteomic studies have provided a glimpse into the plant ubiquitylome (Manzano et al., 2008; Saracco et al., 2009; Kim et al., 2013) and for instance, Kim et al., (2013) have identified 950 ubiquitylation substrates in *Arabidopsis* seedlings. Through an ATP-dependent conjugating cascade, free Ubs are attached to intracellular targets via E1, E2 and E3 Ub ligase enzymes. In particular, E3 ubiquitin ligases, as the last component of the Ub conjugation cascade, are responsible for specific recognition of the many cellular proteins that are ubiquitylated. More than 1500 genes encode putative E3 subunits, being a major part represented by the almost 700 F-box proteins from the multi-subunit E3 ligases and in second place by the more than 500 single subunit RING/U-box E3 ligases. Among the multi-subunit E3 ligases, the cullin-based E3 ligases are the best characterized to date (Hua and Vierstra, 2011). In *Arabidopsis*, five cullins have been identified as components of the cullin-based E3 ligases. Recent findings have revealed that the multisubunit CULLIN4-based E3 ubiquitin ligase complex (CRL4) formed by CUL4, the RING-BOX RBX1, COP10-DET1-DDB1 (CDD) and the substrate adapter DDB1-ASSOCIATED1 (DDA1) binds PYL4, PYL8 and PYL9 through DDA1 (Irigoyen et al., 2014). This interaction occurs in the nucleus of plant cells and, in the case of PYL8, it has been demonstrated that PYL8 is ubiquitylated and DDA1 overexpression promotes PYL8 degradation, which reduces ABA sensitivity in seedling establishment and root growth assays (Irigoyen et al., 2014). Therefore, this work represents an example of a multi-subunit E3 ubiquitin ligase complex that promotes targeted degradation of PYL ABA receptors.

On the other hand, many proteins containing RING domains show in vitro ubiquitin ligase activity and are defined as single-subunit E3 ubiquitin ligases (Smalle and Vierstra, 2004; Lyzenga and Stone, 2011). Indeed the *Arabidopsis* genome encodes approximately 544 putative single-subunit RING/U-box E3 ligases (Smalle and Vierstra, 2004). The RING/U-box serves as

an Ub-E2 docking site that activates transfer of the Ub to substrate lysines (Smalle and Vierstra, 2004). Recently, we identified a RING-type gene family encoding single subunit E3 ubiquitin ligases, which are structurally characterized by the presence of three putative RING domains in tandem, i.e. a canonical RING domain containing a C6HC zinc finger flanked by two RING finger-like structures represented by a RING-HC variant domain and a C5HC2 octet (Bueso et al., 2014). We have analyzed in more detail one member of the family, RING FINGER OF SEED LONGEVITY1 (RSL1, At2g26130), which encodes a functional E3 ubiquitin ligase and whose overexpression altered hormonal responses in seed and vegetative tissues (Bueso et al., 2014). In this study, we found that *35S:RSL1* plants showed reduced sensitivity to ABA-mediated inhibition of growth compared to wt plants and silencing of members of the RSL1 family by RNA interference (RNAi) conferred enhanced response to ABA, which suggests that RSL1 and other RSL1-like genes encode negative regulators of ABA signaling. RSL1 and other members of the family bear a C-terminal transmembrane domain and we have shown RSL1 is targeted to plasma membrane, where interacts with PYR1 and PYL4 ABA receptors.

RESULTS

PYR1 and PYL4 ABA receptors are ubiquitylated and degraded by the proteasome

Recent results from Irigoyen et al., (2014) have shown that PYL8 is ubiquitylated and degraded by the 26S proteasome. To further investigate the stability of other PYR/PYL proteins in vivo, we generated transgenic lines that express HA-tagged versions of PYR1 and PYL4 ABA receptors, which play relevant roles for ABA signaling (Gonzalez-Guzman et al., 2012; Pizzio et al., 2013). Both PYR1 and PYL4 show high expression levels in different tissues and their inactivation is required to generate strongly ABA-insensitive combined *pyr/pyl* mutants (Gonzalez-Guzman et al., 2012). We treated 8-d-old seedlings that express either 3HA-PYR1 or 3HA-PYL4 protein with cycloheximide (CHX) to block protein synthesis and to investigate the half-life of these proteins. We found that both PYR1 and PYL4 protein levels diminished after CHX treatment, which suggests they are subjected to proteolytic degradation (Figure 1A). In agreement with this notion, upon treatment with the proteasome inhibitor MG132, we found a 2-fold and 3-4 fold increase of PYR1 and PYL4 levels, respectively (Figure 1A). We also found the 3HA-PYL8 protein accumulated in MG132-treated plants (Figure 1A), which confirms previous results from Irigoyen et al., (2014). Interestingly, we also found that ABA treatment affected differentially PYR/PYL levels, increasing PYL8 levels, whereas it had no significant effect on PYR1 and PYL4 accumulation (Figure 1A). This result suggests the drought-induced increase of ABA levels might reinforce PYL8 action over other receptors (via sustained protein accumulation), which could explain, for instance, the singular role of PYL8 in root ABA signaling (Antoni et al., 2013).

PYR1 and PYL4 levels increased upon MG132 treatment, which suggests engagement of the 26S proteasome in the regulation of ABA receptor levels via protein ubiquitylation. To investigate this possibility, we performed affinity purification of ubiquitylated proteins in *Arabidopsis* lines that overexpress HA-tagged versions of PYR1, PYL4 or PYL8 (Figure 1B). Ub-conjugated proteins were recovered using commercially available p62 resin, which exploits the Ub-interacting motif of the mammalian p62 protein. Immunoblot analysis using anti-HA antibodies of the pull-down recovered with the p62 resin showed the presence of high-molecular mass bands for 3HA-PYR1, 3HA-PYL4 and 3HA-PYL8 proteins, which likely correspond to ubiquitylated forms of the ABA receptors (Figure 1B), as it has been previously demonstrated for 3HA-PYL8 (Irigoyen et al., 2014).

The RSL1 single-subunit RING-type E3 ubiquitin ligase negatively regulates ABA sensitivity

In a recent study (Bueso et al., 2014), we found that lines over-expressing (OE) the single-subunit RING E3 ubiquitin ligase RSL1 showed enhanced paclobutrazol (PAC) resistance in vegetative tissue, which might be attributed to either enhanced gibberellin response or diminished ABA signaling. To further investigate it, characterization of ABA sensitivity was performed in *35S:RSL1* lines. As a result, we found they were resistant to ABA-mediated inhibition of vegetative growth, which indicated reduced sensitivity to ABA as observed in plants that over-express the negative regulator of ABA signaling HAB1 (Figure 2A). In order to analyze the sensitivity of *35S:RSL1* lines to ABA-mediated inhibition of root growth, we transferred 5-d-old seedlings grown in MS medium to plates lacking or supplemented with 20 □M ABA. Root growth was measured 15-d after transfer and we found that *35S:RSL1* plants showed reduced sensitivity to ABA-mediated inhibition of root growth compared to wt, which was also observed in the *pyr/pyl* mutant lacking PYR1, PYL4 and PYL8 ABA receptors or *35S:HAB1* lines (Figure 2B). Thus, overexpression of RSL1 leads to diminished sensitivity to ABA-mediated inhibition of vegetative growth as observed in plants impaired in ABA perception or showing enhanced PP2C activity. Finally we measured expression of three ABA-responsive genes, i.e. *RAB18*, *RD29B* and *RD29A*, in *35S:RSL1* plants and we found a reduced induction by ABA of these genes compared to wt (Figure 2C). Taken together, these results suggest that RSL1 is a negative regulator of ABA signaling in vegetative tissue, affecting growth sensitivity to ABA and regulation of ABA-responsive gene expression; however, ABA-mediated inhibition of seedling establishment was similar in OE RSL1 lines and wt (Figure 2D).

RNAi lines that silence at least three members of the RSL1-like gene family show enhanced sensitivity to ABA

RSL1 encodes a protein of 398 amino acid residues and contains three Zinc finger (Znf) domains as well as a C-terminal transmembrane (TM) domain located at amino acid residues 372-394 (Figure S1). A cysteine-rich C6HC zinc finger domain is located between amino acid residues 246-292, which is also known as IBR domain because it occurs "in between ring fingers" (Van der Reidjen et al., 1999; Capili et al., 2004). Thus, the IBR domain of RSL1 was flanked by two Znf domains, which were located between residues 157-208 and 321-349 (Figure S1). The first Znf corresponds to a RING-HC variant domain and the second Znf is an octet C5HC2 that does not belong to canonical RING domains (Kosarev et al., 2002). BLAST search of the *Arabidopsis* genome using RSL1 as a query identified many members of the *Arabidopsis* RING/U-box family and by establishing an arbitrary cutoff at p value $<10^{-60}$, we could identify 13 predicted proteins that showed sequence and structural similarity to RSL1 (a conserved IBR domain flanked by two Znf). However, three of them were proteins over 600 amino acid residues, containing additional regions without any homology to RSL1-like proteins. Thus, in addition to RSL1, we have included in the family other 9 members, which we have named RFA1 to RFA9 for RING finger ABA-related 1-9 (Figure S2). Five members have the C-terminal TM domain, i.e. RSL1 and RFA6-9, which might act as a membrane anchor of the E3 ligase (Figure S1 and S2). The other members of the family, i.e. RFA1-5, lack the C-terminal TM domain and two of them display a long very acidic C-terminal domain (At2g21420/RFA4 and At5g37560/RFA5) (Figure S2).

Genes that belong to multigene families usually display functional redundancy when single loss-of-function mutants are tested and require combined inactivation of several members of the family to reveal phenotype (Gonzalez-Guzman et al., 2012). Therefore we decided to generate RNAi lines that might target several members of the RSL1-like gene family, e.g. At2g26130, At2g26135, At3g43750 and At3g45580. We designed the RNAi lines to putatively interfere with the expression of several branches of the RSL1-like family (Figure S2). Silencing of the genes was verified using semi-quantitative RT-PCR analysis of independent RNAi lines using as an internal control the At5g55840 gene, which is a reference gene for transcript normalization in *Arabidopsis* (Czechowski et al., 2005). As a result we found at least three genes, At2g26130, At3g43750, At3g45580, whose expression was impaired in *rsl1* RNAi lines compared to wt (Figure 3A). Unexpectedly, expression of a close relative, At2g26135, was not affected in the RNAi lines (Figure 3A). Overexpression of RSL1 led to reduced sensitivity to ABA-mediated inhibition of root growth (Figure 2B). Conversely, partial loss-of-function of several members of the RSL1-like family led to enhanced sensitivity to ABA-mediated inhibition of root growth (Figure 3B). We also examined ABA-sensitivity of RNAi lines in germination and seedling establishment assays. As a result, we found enhanced sensitivity to

ABA-mediated inhibition of seed germination and early seedling growth in *rsl1* RNAi lines compared to wt (Figure 3C). We also found enhanced PAC-mediated inhibition of seed germination in *rsl1* RNAi lines, OE PYL8 line and the double *hab1-1abi1-2* mutant compared to wt (Figure 3D). This result reflects a higher requirement for GAs to germinate, which is in agreement with the antagonistic role of GAs and ABA during germination and the ABA-hypersensitive response observed in these genetic backgrounds. Finally, seedling establishment was markedly inhibited by 0.5 µM ABA or 0.5 µM PAC in *rsl1* RNAi lines compared to wt (Figure 3E). Interestingly, it was previously described that *35S:RSL1* lines showed enhanced resistance to PAC-mediated inhibition of hypocotyl growth during skotomorphogenesis (Bueso et al., 2014).

RSL1 is localized to plasma membrane

Five members of the RSL1-like gene family contain a TM domain at the C-terminus of the protein, i.e. At2g26130, At2g26135, At3g45460, At3g45480 and At3g4550 (Figure S1). This domain might act as a membrane anchor of the putative E3 ubiquitin ligases, targeting protein ubiquitylation to membrane compartments of the cell. Ubiquitylation of proteins in membrane compartments plays critical roles, for instance, in the endoplasmic reticulum associated degradation (ERAD) quality control system to ensure that only properly folded proteins are released to their appropriate destinations or to regulate the activity of rate-limiting enzymes (Hirsch et al., 2009; Pollier et al., 2013). Additionally, ubiquitylation of plasma membrane proteins is an internalization signal for the endocytic route and also to redirect Golgi-localized membrane proteins into the vacuolar degradation pathway via the endosomal sorting complex required for transport (ESCRT)-machinery (Teis et al., 2009; Viotti et al., 2010; Richardson et al., 2011; Scheuring et al., 2012; MacGurn et al., 2012).

In order to investigate the sub-cellular localization of RSL1, we generated a 35S:GFP-RSL1 construct and delivered it into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration (Voinnet et al., 2003). Whereas the expression of 35S:GFP led to a fluorescent signal both in the cytosol and the nucleus, the expression of 35S:GFP-RSL1 appeared to be mostly localized to plasma membrane (Figure 4). As a marker of plasma membrane localization, we used the red fluorescence emitted by orange/red fluorescent protein OFP-TM23, a modified version of OFP containing a TM domain that results in plasma membrane targeting (Batistic et al., 2012). We co-expressed GFP-RSL1 and OFP-TM23 into tobacco leaf cells and we found that Pearson-Spearman correlation coefficients (French et al., 2008) indicated co-localization of OFP-TM23 and GFP-RSL1 proteins (Figure 4). Interestingly video recording of the GFP-RSL1 fluorescent signal indicated trafficking of the fusion protein between cell membrane compartments and plasma membrane.

Plasmolysis of cells is an additional test that serves to distinguish plasma membrane from cell wall localization. Tobacco leaf cells were plasmolyzed using a 500 mM NaCl treatment for 30 min. Again, both GFP-RSL1 and OFP-TM23 co-localized at plasma membrane and localization of GFP-RSL1 in Hechtian strands, which connect the plasmolyzed plasma membrane to the cell wall (Popper et al., 2011), could be visualized (Figure 4). Staining with the plasma membrane FM4-64 dye of cells that had been plasmolysed by osmotic-stress treatment further confirmed location of GFP-RSL1 at the plasma membrane (yellow color results from merging GFP-RSL1 and FM4-64 staining, Figure S3). In contrast, the cytosolic location of single GFP clearly diverged from the membrane pattern of FM4-64 staining (Figure S3).

To get further insight on the traffic of GFP-RSL1 to reach plasma membrane and its possible recycling via the endocytic pathway, we used a marker of the trans-Golgi network/early endosome, named VTI12/WAVE13 as well as Brefeldin A (BFA) treatment (Figure 4C and 4D) (Robinson et al., 2008; Geldner et al., 2009). Interestingly, when we obtained a CLSM 3D projection through a full z-serie, we could find that both GFP-RSL1 and VTI12/WAVE13 colocalized in trans-Golgi/endosome compartments. It suggests that GFP-RSL1 can recycle from the plasma membrane via the endocytic pathway. Finally, we used BFA treatment to interfere with Golgi-based secretion processes and therefore to affect the traffic of proteins to plasma membrane (Batistic et al., 2008; Robinson et al., 2008). This drug has been extensively used to probe vesicle trafficking pathways in eukaryotic cells because it inhibits the function of certain BFA-sensitive ARF GTPases by interacting with their associated GEFs (Peyroche et al., 1999). Its use in plant cells generates membrane compartments (termed BFA-compartments) formed by Golgi bodies that cluster around aggregates of vesicles (Robinson et al., 2008). Figure 4C shows that BFA treatment generated such vesicles in tobacco cells, which were decorated with the fluorescent signal of GFP-RSL1. The effect of BFA in plants is complicated because at least some plant species (including *Arabidopsis* and tobacco) contain an unusual ARF-GEF, named GNOM, which localizes to elements of the endocytic pathway and therefore BFA can also affect endocytic processes, as observed in the subcellular trafficking of PIN auxin transporters (Friml, 2010). Therefore, the presence of GFP-RSL1 in the BFA-compartments might also reflect a blockade of the endocytic pathway.

RSL1 interacts with PYR1 and PYL4 ABA receptors

The overexpression of RSL1 led to reduced sensitivity to ABA-mediated growth inhibition; whereas RNAi lines that silenced several members of the RSL1-like gene family showed enhanced response to ABA. Taken together, these results suggest that RSL1 and RFA genes act as negative regulators of ABA signaling. Taking into account they encode E3 ubiquitin ligases, we reasoned they might target a positive regulator of ABA signaling for

ubiquitylation. PYR/PYL/RCAR ABA receptors are key elements of the signaling pathway and at least PYR1, PYL4, and PYL8 have been shown to be ubiquitylated proteins (Irigoyen et al., 2014; Figure 1). In order to test whether PYR1 and PYL4 receptors might be interacting partners of RSL1, we performed BiFC assays. To this end, either PYR1 or PYL4 were translationally fused to the YFP^C protein in the pYFP43 vector (Belda-Palazon et al., 2012), whereas RSL1 was fused to YFP^N in the pSPYNE vector. The corresponding constructs were co-delivered into tobacco epidermal leaf cells by Agroinfiltration. As a result, we found interaction of RSL1 and either PYR1 or PYL4 in tobacco leaf cells (Figure 5A). We co-expressed either RSL1-YFP^N/YFP^C-PYR1 or RSL1-YFP^N/YFP^C-PYL4 interacting proteins together with the plasma membrane marker OFP-TM23 and we found that Pearson-Spearman correlation coefficients indicated co-localization of OFP-TM23 and reconstituted YFP proteins; therefore a significant amount of the RSL1-PYR1 or RSL1-PYL4 interactions was localized to plasma membrane (Figure 5A and B). We also conducted coIP experiments where we precipitated RSL1-myc-YFP^N and tested the simultaneous presence of either YFP^C-PYR1 or YFP^C-PYL4 using α-GFP^C. As a result, we could detect coIP of either PYR1 or PYL4 and RSL1, but only when MG132 was included during the protocol for extraction and coIP (Figure 5C).

Finally, we conducted additional studies on the interaction between RSL1 and PYL4. We co-expressed RSL1-YFP^N/YFP^C-PYL4 in tobacco leaf cells and video recording revealed the presence of fluorescent dots in or at the proximity of the plasma membrane and some trafficking of the fluorescent signal between membrane compartments of the cell. We found that a short BFA treatment (2 h) generated small vesicles where the fluorescent signal generated by the RSL1-PYL4 interaction was localized (Figure 5D), which confirms that it occurs in membrane compartments. This finding was confirmed through protein fractionation and biochemical studies. Thus, we submitted the non-nuclear fraction to a 100000g centrifugation to separate the soluble cytosolic fraction from the pelleted microsomal fraction. Most of the RSL1 protein was localized to the microsomal fraction, as well as a significant amount of YFP^C-PYL4 (Figure 5). Therefore, when PYL4 was co-expressed with RSL1, a significant fraction of the total PYL4 pool was localized to the microsomal fraction.

RSL1 promotes degradation of PYR1 and PYL4 in vivo

By using Agroinfiltration in tobacco, we tested whether RSL1 promoted in vivo degradation of PYR1 and PYL4 according to the protocols described by Liu et al., (2010) and Zhao et al., (2013). First, co-infiltration experiments were performed using agrobacteria encoding constructs to express either PYR1 or PYL4 and using increasing amounts of the Agrobacterium that promotes RSL1 expression. Samples were then collected for detection of both protein and RNA levels of transfected constructs (Figure 6A). As the amount of RSL1

increased, the protein level of either PYL4 or PYR1 decreased (Figure 6A). In this experiment, GFP abundance was detected as an internal control, which was not affected by increasing the amount of RSL1. Additionally, both ACTIN8 and PYR1/PYL4 mRNAs were analyzed by RT-PCR to ensure equal amounts of PYR1/PYL4 were expressed in different co-infiltrations.

We also analyzed whether RSL1 promoted PYL4/PYR1 degradation during a time-course using a semi-in vivo degradation assay (Liu et al., 2010). We expressed RSL1, PYR1 and PYL4 separately via different Agroinfiltrations. Then, we mixed together PYL4 and RSL1 or PYR1 and RSL1 and a time course was followed to determine the status of the PYL4 and PYR1 proteins (Figure 6B). The amount of both PYL4 and PYR1 was reduced along the time course in those samples lacking the proteasome inhibitor MG132, whereas it remained stable when MG132 was present. The level of PYL4 and PYR1 remained stable in samples that were mixed with mock control extract.

RSL1 interacts with PYL4 in vitro and promotes the ubiquitylation of PYL4

In order to obtain purified recombinant RSL1, we generated a MBP-RSL1 fusion protein lacking the C-terminal TM domain (MBP-RSL1□TM), since this domain interfered with bacterial expression and purification of recombinant protein. Using a pull-down assay we found MBP-RSL1ΔTM was able to interact with PYL4 and PYR1 in vitro, whereas MBP was not (Figure 7A). In order to test whether RSL1 catalyzes the transfer of ubiquitin to lysine groups of PYL4/PYR1, we performed an enzymatic reaction in column using immunoprecipitated HA-tagged PYL4/PYR1 from Arabidopsis transgenic lines (Pizzio et al., 2013; this work) (Figure 7B). Enzymatic reactions with the immunoprecipitated proteins can be carried out while the protein remains bound to the column, which avoids free diffusion of the substrate and allows the washing of the reaction components once the reaction is finished (those not bound to the column or the substrate). Thus, we used anti-HA paramagnetic microbeads to immunoprecipitate either HA-PYL4 or HA-PYR1 and incubated each substrate in the presence of biotinylated ubiquitin, ATP, human activating enzyme E1, *Arabidopsis* conjugating enzyme UBC8 E2 and MBP-RSL1ΔTM or controls lacking E1+E2 or E3. The incorporporation of biotinylated Ub into the substrate was monitored using Streptavidin-HRP. RSL1 was able to efficiently catalyze the transfer of ubiquitin to both PYL4 and PYR1 (Figure 7B). Immunoblot analysis using anti-HA antibody confirmed that the high-molecular weight species generated upon ubiquitylation corresponded to HA-tagged PYL4/PYR1. In the case of PYR1, some background was observed in the control reaction (E1+E2) lacking RSL1, but the addition of RSL1 (together with E1+E2) greatly enhanced the efficiency of the ubiquitylation reaction.

DISCUSSION

A recent article by Irigoyen et al., (2014) has reported that nuclear PYL8 ABA receptor is ubiquitylated and targeted to degradation by a multi-subunit CRL4-type E3 ubiquitin ligase complex where the substrate adapter DDA1 carries out the recognition of the target protein. In the present study we show that in addition to PYL8, both PYR1 and PYL4 are also ubiquitylated, indicating that ubiquitylation of PYR/PYL ABA receptors appears to be a general mechanism that might regulate their half-life, protein interactions or trafficking processes. Data mining in previous proteomic studies aimed to obtain the *Arabidopsis* ubiquitylome confirmed the ubiquitylation of PYR1, which increased after treating seedlings with MG132 (Kim et al., 2013). In this study we also identify a single subunit E3 ligase, RSL1, able to interact with PYR1/PYL4 ABA receptors at the plasma membrane and ubiquitylate them in vitro. This finding suggests that ubiquitylation of PYR/PYL receptors by RSL1 might be connected to trafficking towards endosome compartments, since proteins ubiquitylated at the plasma membrane can be degraded in the vacuole after endosomal and multivesicular body trafficking (MacGurn et al., 2012). The abundance of integral membrane proteins (nutrient transporters, ion channels, signaling receptors) is constantly adjusted to adapt functional responses to environmental cues and ubiquitylation initiates sorting of plasma membrane proteins into the endosome/vacuolar degradation pathway (Geldner and Jurgens, 2006; Viotti et al., 2010; MacGurn et al., 2012; Scheuring et al., 2012). This has been demonstrated in plants for some plasma membrane proteins such as IRON-REGULATED TRANSPORTER 1 PINFORMED1 (PIN1), REQUIRES HIGH BORON (BOR1) transporter and BRASSINOSTEROID–INSENSITIVE 1 receptor (BRI1) (Barberon et al., 2011; Spitzer et al., 2009; Kasai et al., 2011; Viotti et al., 2010). PYR/PYL ABA receptors are not integral membrane proteins but according to the function of PYR/PYLs and their associated clade A PP2Cs as regulators of different plasma membrane proteins (ion transporters, membrane-bound enzymes), they could be considered as peripheral membrane proteins (at least for a fraction of the total pool). It is possible that auxiliary proteins are involved in approaching transiently PYR/PYL ABA receptors (including the PP2C/SnRK2 ABA signalosome) to plasma membrane in order to regulate ion transporters and membrane associated enzymes involved in ABA signaling (Geiger et al., 2009; Lee et al., 2009; Sato et al., 2009; Sirichandra et al., 2009; Geiger et al., 2011). For instance, recently it was shown that ABI1 and PYL9 modulate the association of the CPK21/SLAH3 complex within plasma membrane domains, implying that PYL9 functions in the proximity of lipid nanodomains reminiscent of animal lipid rafts (Demir et al., 2013). In agreement with this notion, BiFC assays show that RSL1 protein is able to interact with PYR1 and PYL4 in plasma membrane and biochemical fractionation experiments show that PYL4 is partially localized to microsomes when co-expressed with RSL1 (Figure 5D).

Ub-mediated membrane trafficking is a well studied mechanism to regulate the turnover of different proteins that exert their function in the plasma membrane. In eukaryotic cells, ubiquitylation is required to target molecules for ESCRT-mediated internalization of membrane proteins (Teis et al., 2009; Spitzer et al., 2009; Richardson et al., 2011). Taking into account that RSL1 targets PYL4 at the plasma membrane and it is able to ubiquitylate it in vitro, we suggest that this event might initiate the recycling of a certain fraction of the receptor pool to endosomal compartments via the ESCRT machinery (Figure 8, pathway A). Proteins that mediate plasma membrane interaction of PYR/PYLs have been recently identified following protein-protein interaction studies (Rodriguez et al., manuscript under preparation) and might vehiculate PYR/PYL trafficking through the endosomal system. Additionally, ABA signaling proteins show extensive connection with transporters and other membrane proteins in the recently identified membrane-linked interactome database of *Arabidopsis* (Jones et al., 2014). Alternatively, ubiquitylated transiently membrane-bound PYR/PYLs might follow a cytosolic pathway to be degraded by the 26S proteasome (Figure 8, pathway B). We also note that several members of the RSL1/RFA family lack C-terminal transmembrane domains and are candidates to target PYR/PYL receptors to the 26S proteasome. Ultimately, both the 26S proteasome and endosomal-vacuolar pathways could lead to degradation of PYR/PYL receptors to attenuate ABA signaling. This notion is coherent with genetic evidence obtained in RNAi lines, which suggests that RSL1 and RFA genes play a negative role in ABA signaling (Figure 2 and 3). In summary, we suggest that ubiquitylation of PYR/PYL receptors through members of the RSL1/RFA family regulates their half-life and protein turnover (Figure 8). The regulation of the PYR/PYL turnover could occur at the plasma membrane or other cellular locations through interaction with different E3 ubiquitin ligases, for instance members of the RFA family lacking the TM domain or the previously described multi-subunit E3 ligase formed by the CRL4 complex and the substrate adapter DDA1.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions.

Arabidopsis thaliana plants were routinely grown under greenhouse conditions (40-50% relative humidity) in pots containing a 1:3 vermiculite-soil mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4ºC for 3 days. Then, seeds were sowed on Murashige-Skoog (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% sucrose and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and

incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 μE m-2 sec-1.

Transient protein expression assay in N. benthamiana

Agrobacterium infiltration of tobacco leaves was performed basically as described by Voinnet et al., (2003). Constructs to investigate the subcellular localization of RSL1 was done in pMDC43 (Curtis and Grossniklaus, 2003). The construct encoding the plasma membrane marker OFP-TM23 was reported in Batistic et al., (2012). To investigate the interaction of RSL1 and PYR/PYL proteins in planta, we used the pSPYNE-35S and pYFP^{C}43 vectors. The coding sequence of RSL1 was obtained by RT-PCR and cloned into pCR8/GW/TOPO, it was excised using a double digestion *Bam*HI-*Eco*RV and cloned into *Bam*HI-*Sma*I pSPYNE-35S. The coding sequences of PYR1 and PYL4 were recombined by LR reaction from pCR8 entry vector to pYFP^{C}43 destination vector. The different binary vectors described above where introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) by electroporation and transformed cells were selected in LB plates supplemented with kanamycin (50 mg/ml). Then, they were grown in liquid LB medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM morpholinoethanesulphonic (MES) acid-KOH pH 5.6 containing 10 mM MgCl2 and 150 mM acetosyringone to an OD600 nm of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1 (pCH32 35S:p19) expressing the silencing suppressor p19 of tomato bushy stunt virus (Voinnet et al., 2003) so that the final density of *Agrobacterium* solution was about 1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old *Nicotiana benthamiana* plants. Leaves were examined 48-72 h after infiltration using confocal laser scanning microscopy.

Biochemical fractionation, protein extraction, analysis and immunoprecipitation

Constructs to express GFP-RSL1 or HA-tagged PYR1/PYL4/PYL8 were done in pMDC43 or pALLIGATOR2 vectors, respectively. Generation of PYL4 and PYL8 constructs in pALLIGATOR2 has been described previously (Antoni et al., 2013; Pizzio et al., 2013) and a similar construct was done for PYR1. The different binary vectors described above where introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) and used for infiltration of tobacco leaves. Protein extracts for immunodetection experiments were prepared from tobacco leaves 48-72 h after infiltration. Plant material (~200 mg) for direct Western blot analysis was extracted in 2X Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.001% bromophenol blue), proteins were run in a 10% SDS-PAGE gel and analyzed by immunoblotting.

Cytosolic and microsomal fractionation of YFP-tagged proteins was performed as described previously (Antoni et al., 2012). Proteins from the non-nuclear fraction were immunoprecipitated using super-paramagnetic micro MACS beads coupled to monoclonal anti-myc antibody according to the manufacturer´s instructions (Miltenyi Biotec). Purified immunocomplexes were eluted in Laemmli buffer, boiled and run in a 10% SDS-PAGE gel. Proteins immunoprecipitated with anti-GFP antibody were transferred onto Immobilon-P membranes (Millipore) and probed with anti-HA-HRP to detect coIP of HA-tagged receptors. Immunodetection of green fluorescent protein (GFP) fusion proteins was performed with an anti-GFP monoclonal antibody (clone JL-8, Clontech) as primary antibody and ECL anti-mouse-HRP (GE Healthcare) as secondary antibody. Detection was performed using the ECL advance western blotting chemiluminiscent detection kit (GE Healthcare). Image capture was done using the image analyzer LAS3000 and quantification of the protein signal was done using Image Guache V4.0 software.

Protein stability assays and affinity purification of ubiquitylated proteins using p62-agarose

Surface sterilized seeds of transgenic lines overexpressing HA-tagged PYR1, PYL4 and PYL8 were sown in MS plates and grown vertically for 14 days. Seedlings were transferred to 6-well plates containing 3 mL of liquid MS medium and they were either mock- or 100 µM cycloheximide-, 50 µM MG132- or 50 µM ABA-treated. A time course at 0, 4 7 and 9 hours was followed and plant material was collected and frozen in liquid nitrogen at the indicated times. Plant material (0.2 g) was extracted in 3 volumes of PBS supplemented with 1 mM EDTA, 0.05% Triton X-100, and protease inhibitor cocktail (Roche). After protein quantification of each plant extract, 10 µg of total protein was loaded on a 12% SDS-PAGE gel. Proteins were transferred onto Immobilon-P membranes (Millipore) and probed with anti-HA horseradish peroxidase (HRP) antibody (Roche). Detection was performed using the ECL select Western blotting chemiluminiscent detection kit (GE Healthcare). Image capture was done using a cooled CCD camera system and the image analyzer LAS3000 and quantification of the protein signal was done using Image Guache V4.0 software. The signal intensities of the digitalized images were quantified using Image-Gauge version 4.0

Isolation of ubiquitylated proteins was performed in protein extracts prepared from HA-tagged PYR1, PYL4 and PYL8 lines that were 50 µM MG132-treated for 6h. The protocol used was basically as described by Manzano et al., (2008) with small modifications introduced by Irigoyen et al., (2014). Immunoblotting was performed for detection of ubiquitylated proteins using anti-Ub antibody (Ub P4D1:sc-8017, Santa Cruz Biotechnology) or anti-HA-HRP for detection of HA-tagged PYR/PYL proteins

Seed germination and seedling establishment assays

After surface sterilization of the seeds, stratification was conducted in the dark at 4 ºC for 3d. Next, approximately 100 seeds of each genotype were sown on Murashige and Skoog plates supplemented with 1% sucrose lacking or supplemented with the indicated concentrations of ABA. To score seed germination, radical emergence was analyzed at 24, 48 and 72 h after sowing. Seedling establishment was scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves at 7-9 days.

Root and shoot growth assays

Seedlings were grown on vertically oriented Murashige and Skoog plates for 4-5 days. Afterwards, 20 plants were transferred to new plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10 days to produce image files suitable for quantitative analysis of root growth using the NIH Image software ImageJ versión 1.37. As indicator of shoot growth, fresh weight was measured after 14 days.

RNA analyses

Seedling total RNA was extracted using a NucleoSpin RNA plant kit and cDNA synthesis and qRT-PCR analyses of ABA-inducible genes was performed as described by Saez et al. (2006). RNA isolation for gene expression analyses of At2g26130, At2g26135, At3g43750, At3g45580 and At5g55840 in *rsl1* RNAi lines was performed using 3 days stratified seeds (Oñate-Sánchez and Vicente-Carbajosa, 2008). The sequences of the primers used for PCR amplifications are listed in Table S1.

Generation of overexpressing and knock-down RNAi lines

Transgenic lines overexpressing RSL1 coding sequence (*35S:RSL1*) are described in Bueso et al., (2014). HA-tagged PYR1/PYL4/PYL8 constructs were done in pALLIGATOR2 vector and transgenic lines were generated as described by Pizzio et al., (2013). *rsl1* RNAi lines were designed to knock-down several genes of the RSL1-like gene family: At2g26130, At2g26135, At3g43750, At3g45580 and At3g45540. To this end, we used the microRNA designer webpage (http://wmd3.weigelworld.org/cgibin/webapp.cgi?page= Designer;project=stdwmd). The amiRNAs were obtained according to the protocol described in (http://wmd3.weigelworld.org/downloads/ Cloning_of_artificial_microRNAs.pdf). The sequences of the primers used for PCR amplifications for amiRNAs are listed in Table S1. PCR products were cloned in the pCR8/GW/TOPO plasmid (Invitrogen) and then where recombined in the plasmid pMDC32 (Curtis and Grossniklaus, 2003) using the Gateway technology and LR Clonase reaction (Invitrogen). All PCR-derived constructs were verified by DNA sequencing.

Confocal Laser Scanning Microscopy (CLSM)

Confocal imaging was performed using a Zeiss LSM 780 AxioObserver.Z1 laser scanning microscope with C-Apochromat 40x/1.20 W corrective water immersion objective. The following fluorophores, which were excited and fluorescence emission detected by frame switching in the single or multi-tracking mode at the indicated wavelengths, were used in tobacco leaf infiltration experiments: GFP (488 nm/500-530 nm), YFP (488 nm/529-550 nm), OFP (561 nm/575-600 nm) and mCherryFP (561 nm/605-630 nm). Pinholes were adjusted to 1 Air Unit for each wavelength. Post-acquisition image processing was performed using ZEN (ZEISS Efficient Navigation) Lite 2012 imaging software and ImageJ (http://rsb.info.gov/ij/).

Epifluorescence confocal images of epidermal tobacco leaves co-infiltrated with the constructs described in the text were merged to quantitatively estimate co-localization of fluorescent markers (French et al., 2008). Statistical analyses for fluorescence colocalization were performed through determination of the linear Pearson's and nonlinear Spearman's correlation coefficients between fluorescent signals. Pearson's and Spearman's correlation coefficients can range from [+1] to [-1], depending on the percentage of co-localization observed for the fluorescent signals. Values in the range +0.4-1 indicate partial co-localization, whereas lower values or negative values indicate lack of co-localization.

***In vivo* and semi-*in vivo* protein degradation assays**

Protein degradation assays were performed as described by Liu et al., (2010) with small modifications**.** For *in vivo* protein degradation experiments, *A. tumefaciens* cultures containing constructs that express GFP-RSL1 E3 ligase, 3HA-PYR1 or 3HA-PYL4 and the silencing suppressor p19 were co-infiltrated at different ratios in tobacco leaves. Three days after infiltration, samples were collected, ground in liquid nitrogen and immediately placed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail) on ice for protein extraction. Homogenates were cleared by centrifugation at 12000 g, 4ºC for 15 min, and supernatants were used for protein immunoblot analysis. Samples were also collected for Actin and PYR1/PYL4 mRNA analyses to ensure equal amounts of PYR1/PYL4 were expressed in different co-infiltrations.

For semi-*in vivo* protein degradation experiments, we expressed GFP-RSL1, 3HA-PYR1 and 3HA-PYL4 separately via different Agroinfiltrations of tobacco leaves. Samples were separately harvested and proteins were extracted in native extraction buffer (50 mM Tris-HCl pH 8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT and protease inhibitor cocktail). Extracts were clarified by centrifugation as aforementioned and a final concentration of 25 μM ATP was added to preserve the function of the 26S proteasome. Then equal amounts of HA-PYR1 or 3HA-PYL4 extracts were mixed with protein extracts containing either GFP-

RSL1 or GFP and they were incubated at 4ºC with rotation for 4h in presence or absence of 50 µM MG132. Samples were collected at 0, 1, 2, and 4 h in Laemli buffer and subjected to immunoblot analysis.

Pull-down assays

The construction, expression in bacteria and purification of MBP (maltose-binding protein) fused to RSL1 lacking the C-terminal TM domain was described in Bueso et al., (2014). Purification of 6His-PYL4 and 6His-PYR1 was described in Santiago et al., (2009). For pull-down assays, 5 µg of MBP-RSL1ΔTM or MBP and 5 µg of either 6His-PYL4 or 6His-PYR1 were incubated 1 h at 4 ºC with constant rocking in 0.5 ml binding buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5% Tween-20). Afterwards, MBP proteins were purified using amylose affinity chromatography, eluted and analysed by SDS-PAGE, followed by western blotting and immunodetection using anti HIS-tag monoclonal antibodies (Novagen).

***In vitro* ubiquitylation assay**

Enzymatic reactions were performed on the column by using immunoprecipitated substrate, either 3HA-PYL4 or 3HA-PYR1, bound to super-paramagnetic micro MACS beads coupled to monoclonal anti-HA antibody. Immunoprecipitation of 3HA-PYL4 or 3HA-PYR1 was performed according to the manufacturer´s instructions (Miltenyi Biotec) using Arabidopsis extracts of HA-tagged PYR/PYL lines containing 600 µg of total protein in 50 mM Tris-HCl pH 8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, 10 µM MG132 and protease inhibitor cocktail. The immobilized substrate (100 ng) was incubated at 30º for 2 h in Ubi buffer (50 mM Tris pH 7.5, 10 mM MgCl2, 0.2 mM DTT, 50 µM ZnCl2 and 5 mM ATP) containing 100 ng human E1 (BostonBiochem), 250 ng 6His-AtUBC8 (E2), 2 µg MBP-RSL1ΔTM and 1 µg biotinylated ubiquitin (Enzo Life Sciences). Reaction was stopped by washing the column with Ubi buffer followed by elution with Laemmli buffer. The eluted substrate was then subjected to SDS-PAGE/blotting followed by detection using either streptavidin-HRP (St-HRP) or antiHA-HRP to detect either ubiquitylated or 3HA-tagged proteins, respectively.

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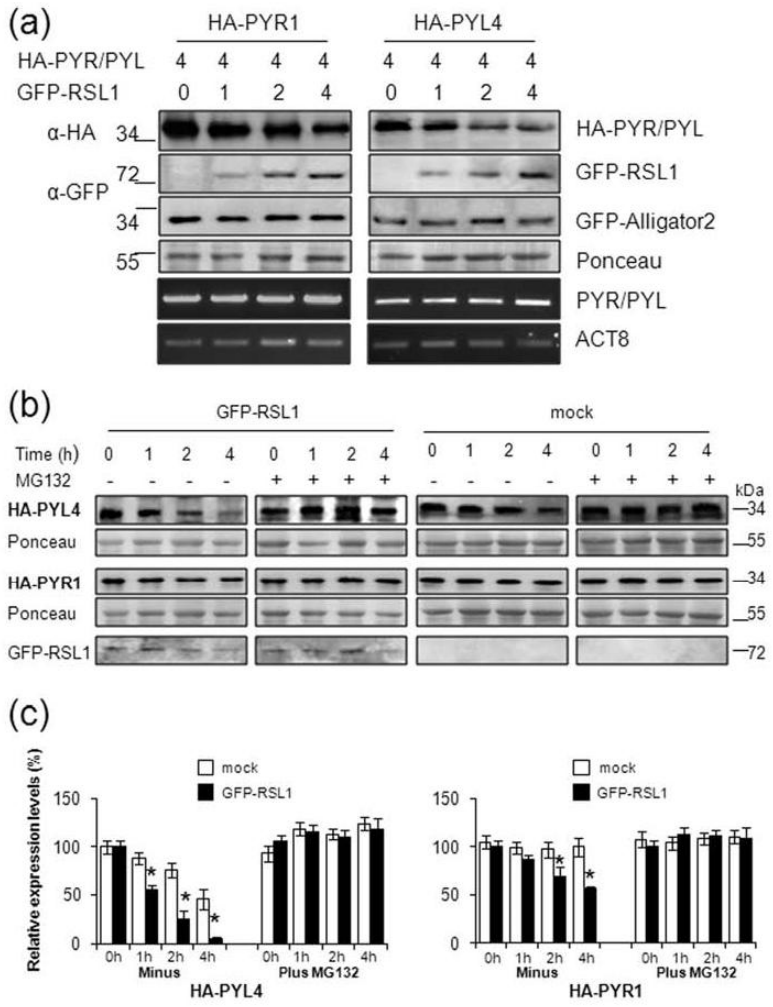

Figure 1. PYR1, PYL4 and PYL8 proteins are ubiquitylated. (A) Effect of CHX, MG132 and ABA treatment on PYR1, PYL4 and PYL8 protein levels. 10-d-old seedlings expressing HA-tagged PYR/PYL proteins were either mock- or chemically-treated with 100 µM CHX, 50 µM MG132 or 50 µM ABA for the indicated time period. Immunoblot analysis using anti-HA was performed to quantify protein levels. Three-independent experiments were performed and the histogram represents the average relative protein level with respect to time 0 (error bars indicate standard error). The right panel shows the specificity of the anti-HA antibody to recognize HA-tagged PYR/PYL proteins. (B) Pull-down (PD) using p62-agarose (+p62) or agarose alone (-p62) of protein extracts containing HA-tagged PYR/PYLs reveals PYR1, PYL4 and PYL8 are ubiquitylated. Immunoblot analysis using anti-Ub reveals the p62 domain binds ubiquitylated proteins from *Arabidopsis* plants.

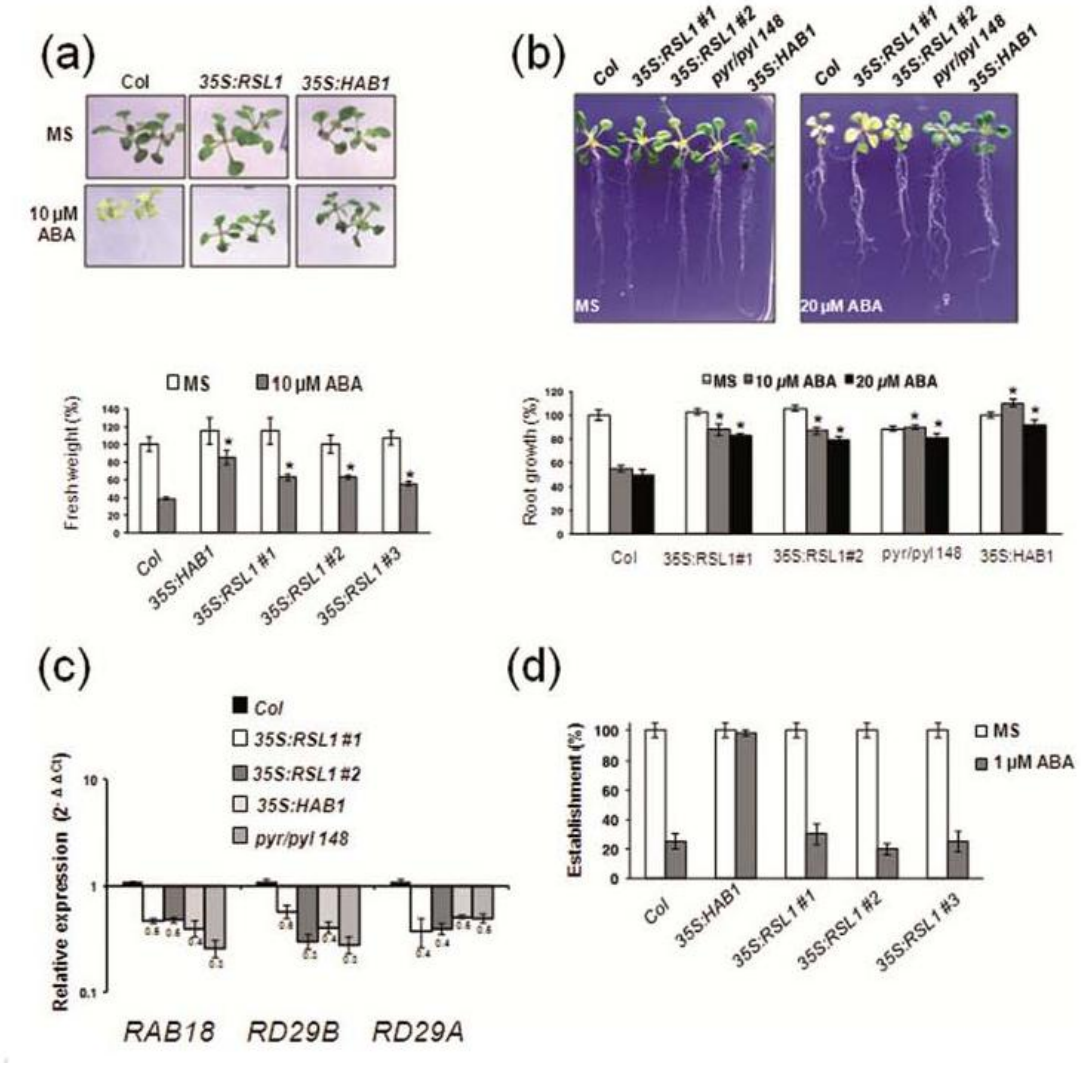

Figure 2. *35S:RSL1* lines show reduced sensitivity to ABA-mediated inhibition of vegetative growth. (A) The photograph shows representative seedlings after 10 d in medium lacking or supplemented with ABA. Seeds were germinated in medium lacking ABA and transferred to medium supplemented with 10 µM ABA. The histograms represent average values of fresh weight ±SE after 10 d. * indicates $p<0.05$ (Student´s t test) when comparing data of the indicated genetic background to Col plants in the same assay conditions. (B) Reduced sensitivity to ABA-mediated inhibition of root growth in plants OE either RSL1 or the negative regulator HAB1, and plants lacking three PYR/PYL ABA receptors (pyr/pyl 148). The histograms represent the quantification of root growth in seedlings grown for 14 d in medium lacking or supplemented with 20 µM ABA. (C) Expression of ABA-inducible genes is reduced in *35S:RSL1* plants compared to wt. Relative expression of *RAB18, RD29B* and *RD29A* genes in the indicated genotypes after 50 µM ABA-treatment for 3 h compared with the wt (value 1) as determined by qRT-PCR. Expression of the genes was upregulated 75-, 530- and 63-fold by ABA in the wt, respectively. (D) ABA-mediated inhibition of seedling establishment is similar in *35S:RSL1* plants and Col wt. *Arabidopsis* plants.

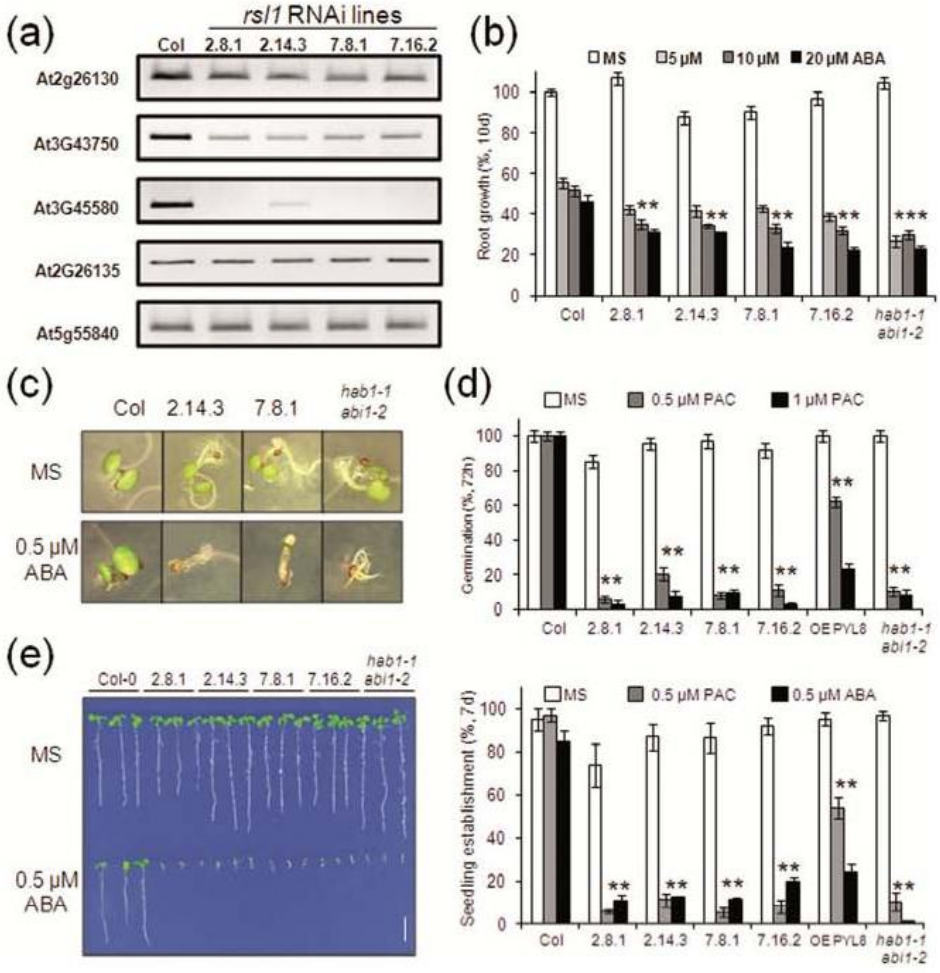

Figure 3. *rsl1* RNAi lines show enhanced sensitivity to ABA as compared to wt. (A) Semiquantitative RT-PCR analysis of wt and 4 independent *rsl1* RNAi lines using primers specific for the indicated genes reveals partial silencing of *RSL1* and two *RSL1*-like genes. *At5g55840* is a reference gene for transcript normalization. (B) Enhanced sensitivity to ABA-mediated inhibition of root growth in *rsl1* RNAi plants and the double *hab1-1abi1-2* mutant. The histograms represent the quantification of root growth in seedlings grown for 10 d in medium lacking or supplemented with 5, 10 or 20 µM ABA. (C) Enhanced sensitivity to ABA-mediated inhibition of seed germination and early seedling growth. The photographs show representative seeds/seedlings after germination of approximately 100 seeds in MS medium lacking or supplemented with 0.5 µM for 4 days. (D) Enhanced sensitivity to PAC-mediated inhibition of seed germination in *rsl1* RNAi lines, OE PYL8 line and *hab1-1abi1-2*. Percentage of seeds germinated (radicle emergence at 72 h) in the presence of the indicated concentration of PAC. (E) Enhanced sensitivity to ABA- and PAC-mediated inhibition of seedling establishment in *rsl1* RNAi plants and *hab1-1abi1-2*. Approximately 100 seeds of each genotype were sowed on each plate and seedling establishment was scored after 7 d. * indicates $p<0.05$ (Student´s t test) when comparing data of the indicated genetic background to Col plants in the same assay conditions.

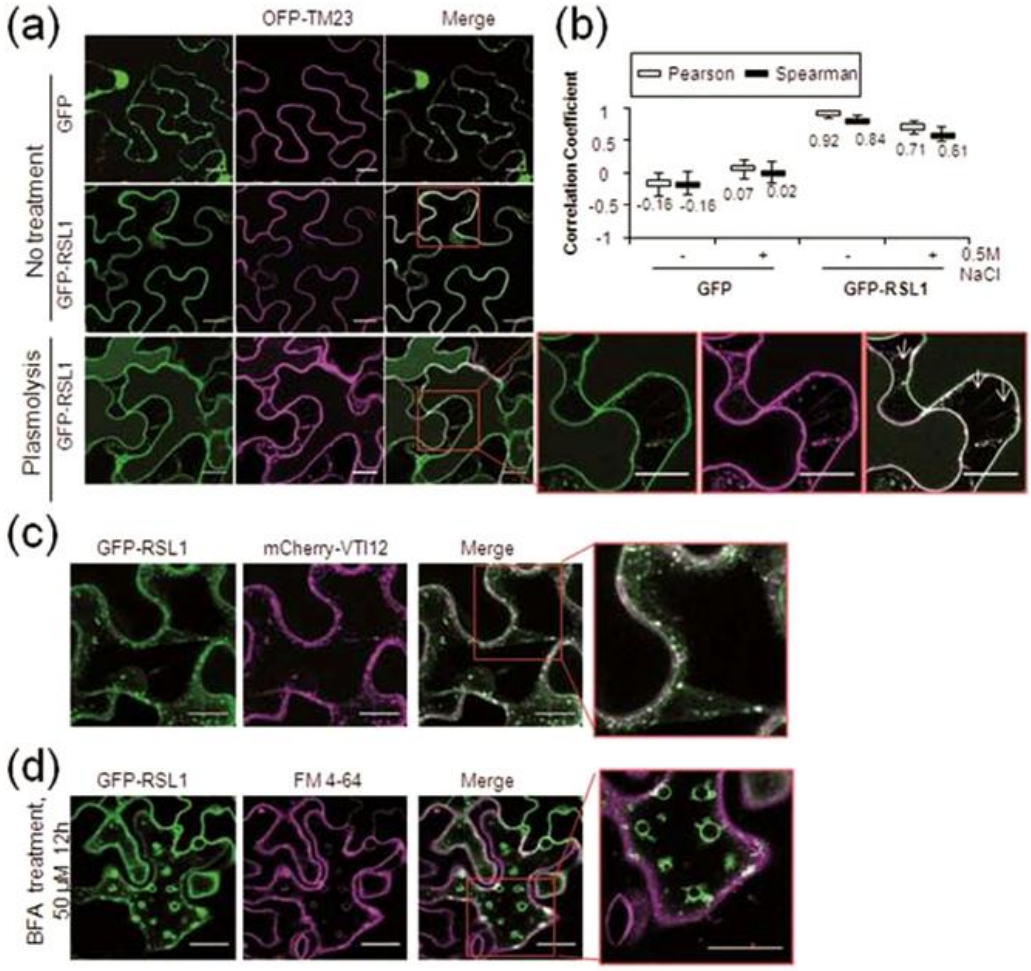

Figure 4. GFP-RSL1 fusion protein localizes to plasma membrane upon transient expression in *Nicotiana benthamiana*. (A) Confocal images of transiently transformed tobacco epidermal cells co-expressing GFP or GFP-RSL1 and the plasma membrane marker OFP-TM23. In the case of GFP-RSL1, images were also obtained after cell plasmolysis by 0.5 M NaCl treatment for 30 min. The degree of colocalization between the two fluorescent signals was analyzed using merged images and Zeiss software (Zen Lite 2012). The red box highlights the merging of the green and magenta signals, which generates white labelling of the plasma membrane. The white arrows label the Hechtian strands, which connect the plasmolyzed plasma membrane to the cell wall. (B) Pearson-Spearman correlation coefficients indicate co-localization of GFP-RSL1 and the plasma membrane marker. Epifluorescence confocal images of epidermal leaves co-infiltrated with the indicated constructs were merged to quantitatively estimate co-localization of GFP and OFP fluorescence (French et al., 2008). At least 10 single-scanned cell images per experiment were collected and analyzed using the same conditions of laser intensity, pinhole size and gain levels. (C) CLSM 3D projection through a full z-serie shows partial colocalization of GFP-RSL1 and the VTI12/WAVE13 marker of the trans-Golgi network/early endosome. (D) GFP-RSL1 decorates BFA-compartments.

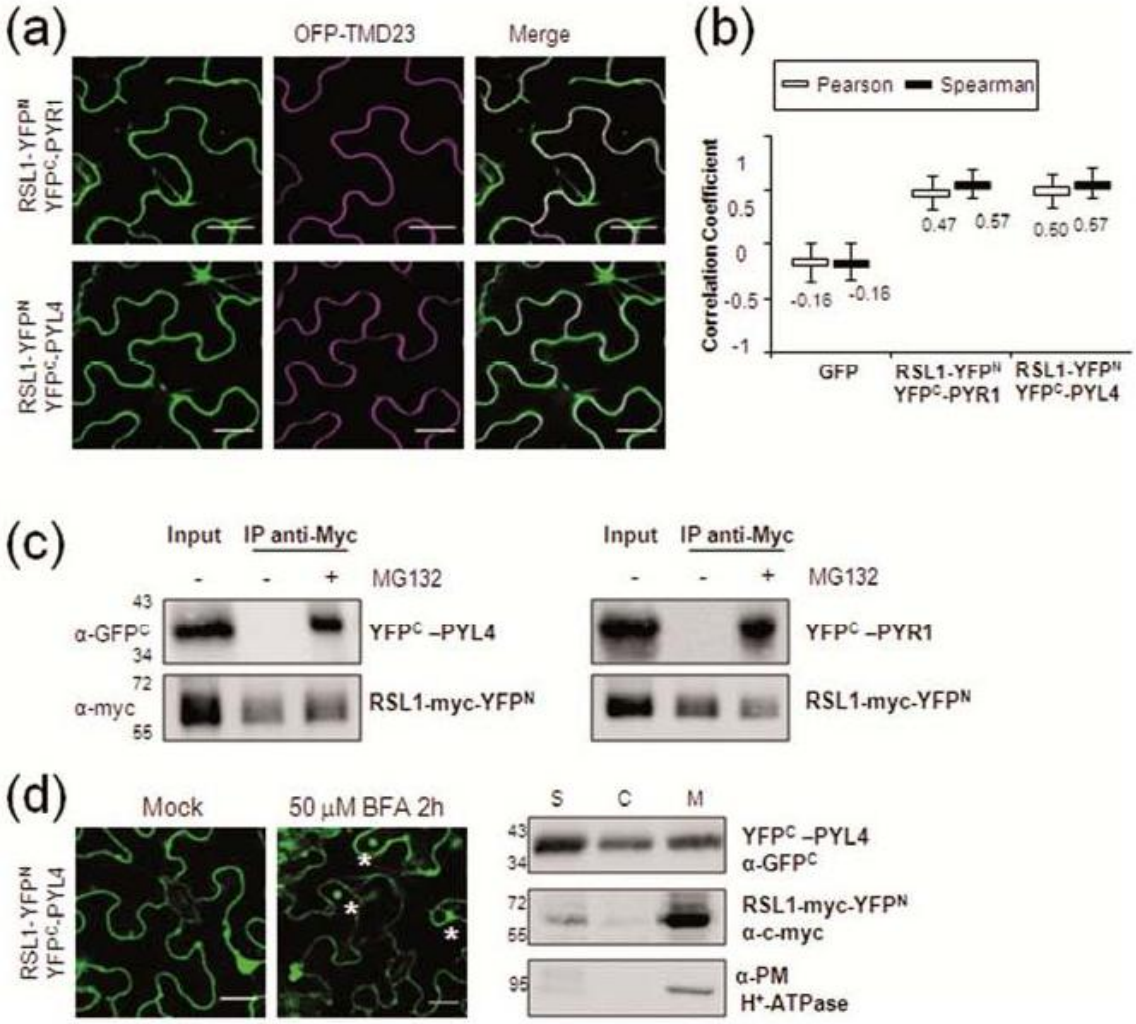

Figure 5. BiFC and coimmunoprecipitation (coIP) assays show interaction of RSL1 and PYR1/PYL4 in tobacco epidermal cells. (A) RSL1 and PYR1/PYL4 interact in the plasma membrane of tobacco cells. Confocal images of transiently transformed tobacco epidermal cells co-expressing RSL1-YFP^N and YFP^C-PYR1/PYL4 interacting proteins and the plasma membrane marker OFP-TM23. BiFC of RSL1-YFP^N and YFP^C-PYR1 or YFP^C-PYL4 was observed and this interaction co-localizes with the plasma membrane marker OFP-TM23. (B) Statistical analysis of colocalization for RSL1-PYR1 or RSL1-PYL4 interacting proteins and OFP-TM23 using Pearson´s and Spearman´s correlation factors. Epifluorescence confocal images of epidermal tobacco leaves infiltrated with the indicated constructs were merged to quantitatively estimate co-localization of YFP and OFP fluorescence (French et al., 2008). The degree of colocalization between the two fluorescent signals was analyzed using Zeiss software. (C) CoIP assays demonstrate the interaction of RSL1 and PYR1/PYL4. Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harbouring the indicated constructs were analyzed using anti-GFP^C or anti-myc antibodies. Input levels of epitope-tagged proteins in crude protein extracts (20 µg of total protein) were analyzed by immunoblotting. Immunoprecipitated (IP) RSL1-myc-YFP^N protein was probed with anti-GFP^C antibodies to detect coIP. Protein extracts were prepared in the absence or presence of the proteasome inhibitor MG132. (D) The RSL1-YFP^N/YFP^C-PYL4 interaction decorates membrane vesicles generated by BFA treatment. Protein biochemical fractionation shows that most RSL1 protein is localized to the microsomal fraction as well as a significant fraction of PYL4. Immunoblotting using anti-plasma membrane (PM) proton-ATPase antibody validated the fractionation procedure.

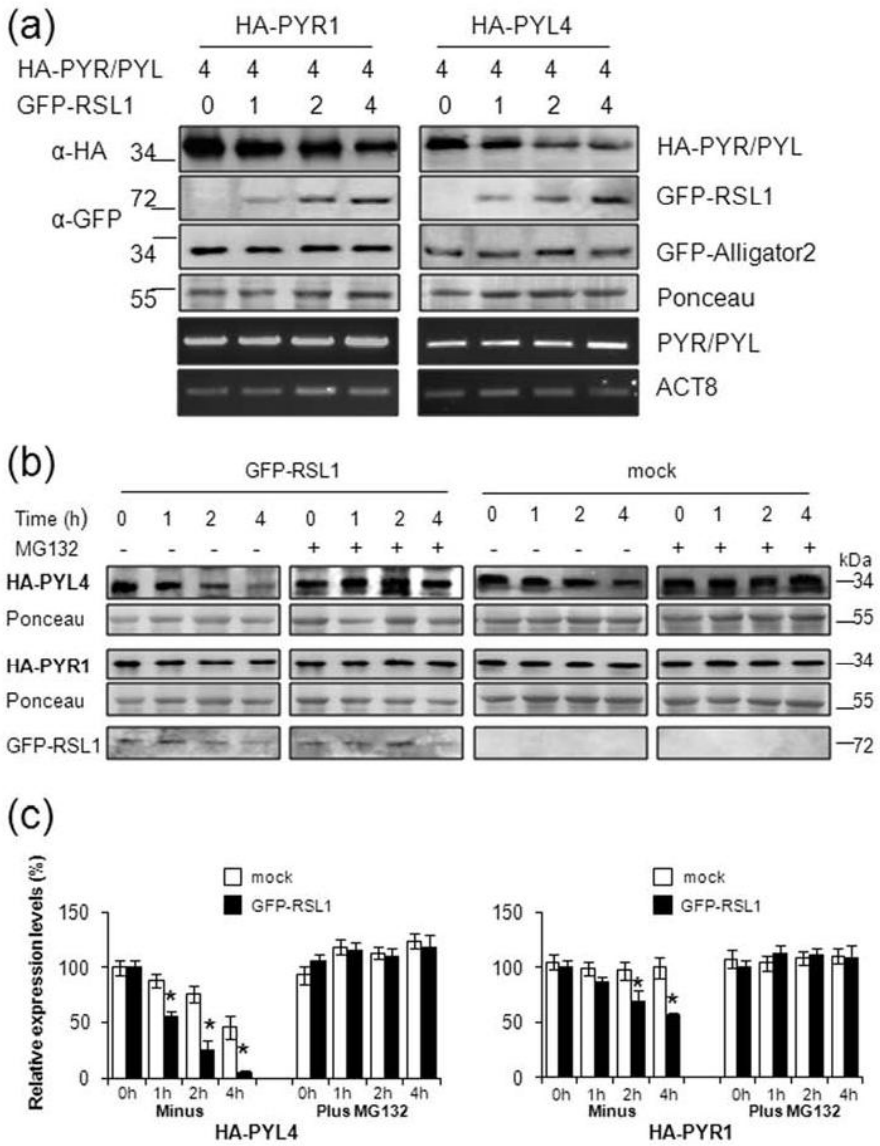

Figure 6. Analysis of RSL1-promoted degradation of PYR1 and PYL4 by in vivo and semi-in vivo assays. (A) In vivo degradation of either PYR1 or PYL4 was observed in co-infiltration experiments with increasing amounts of RSL1. The ratio of the relative concentration of agrobacteria used in the different co-infiltrations is indicated by numbers (top). Cell extracts were analyzed using a-HA to detect HA-tagged PYR1/PYL4 and a-GFP to detect both GFP-RSL1 and the internal control of GFP. mRNA expression levels of the target PYR1 and PYL4 genes and ACTIN8 (ACT8) were analyzed by RT-PCR. Molecular masses of marker proteins are indicated in kilodaltons. (B) Time-course of HA-tagged PYR1/PYL4 degradation promoted by RSL1. Degradation of either HA-PYL4 or HA-PYR1 was performed by mixing tobacco cell extracts from separated agroinfiltrations. HA-PYL4 or HA-PYR1 extract was mixed with GFP-RSL1 extract or mock control extract and incubated at 4ºC during a time-course either in the absence or the presence of MG132. Proteins were detected by immunoblot as described above. Ponceau staining of the rubisco protein is shown as a loading control. (C) Quantification of the experiment described in B.

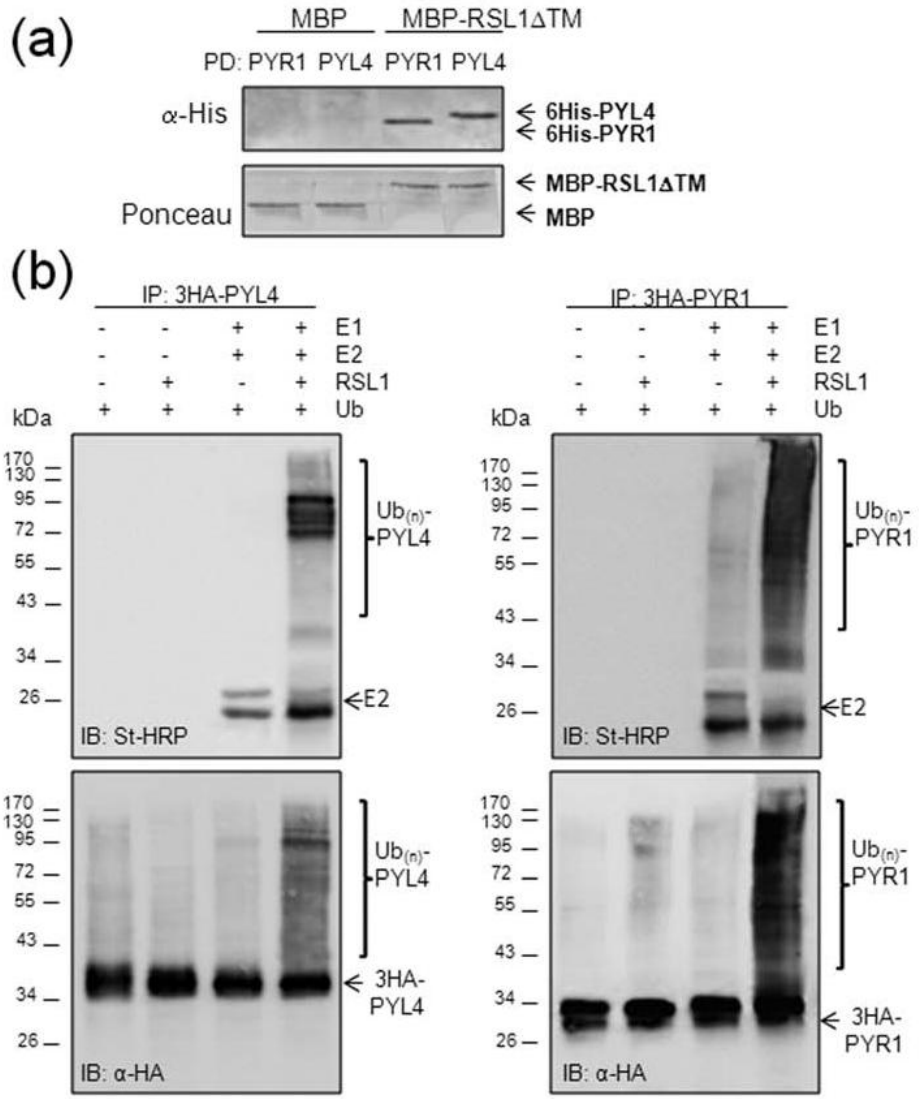

Figure 7. MBP-RSL1DTM interacts in vitro with 6His-PYR1 and 6His-PYL4. RSL1 has E3 ubiquitin ligase activity and promotes ubiquitylation of PYL4 and PYR1. (A) Interaction between PYR1 or PYL4 and RSL1 in a pull-down (PD) assay. Purified MBP or MBP-RSL1DTM (5 μg each) and 5 μg of 6His-PYR1 or 6His-PYL4 proteins were incubated 1 h at 4 ºC with constant rocking in 0.5 ml binding buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5% Tween-20). Next, MBP or MBP-RSL1 proteins were purified using amylose affinity chromatography, eluted and analysed by immunoblotting using anti-HIS and Ponceau staining. (B) MBP-RSL1DTM (2 μg) was assayed for E3 ligase activity in the presence of 100 ng human E1, 250 ng of purified 6His-AtUBC8 (E2), 3HA-PYL4 or 3HA-PYR1 immunoprecipitated from the corresponding HA-PYR/PYL tagged transgenic line and 1 μg of biotinylated ubiquitin. After incubation at 30ºC for 2h, the mixture was subjected to SDS-PAGE/blotting followed by detection using either streptavidin-HRP (St-HRP) or antiHA-HRP to detect either ubiquitylated or 3HA-tagged proteins, respectively.

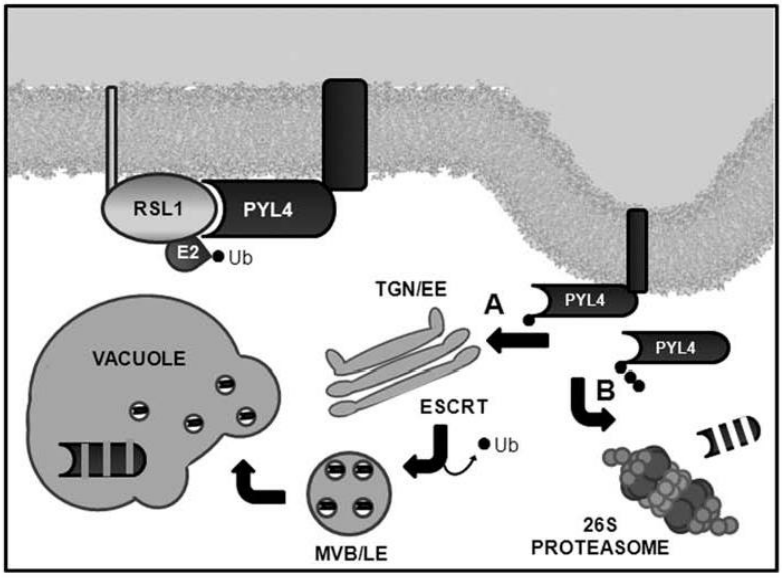

Figure 8. A working model depicting the interaction of PYL4/PYR1 and RSL1 at the plasma membrane is shown. We suggest that ubiquitylation might trigger endocytosis of the receptor to endosomal compartments, i.e. trans-Golgi network/early endosomes (TGN/EE) and multivesicular body/late endosomes (MVB/LE). The presence of ABA receptors as peripheral membrane proteins might be mediated through auxiliary proteins or interaction with plasma membrane components regulated by PYR/PYLs and clade A PP2Cs, according to the presence of ABA signaling proteins in the membrane-linked interactome of Arabidopsis (Jones et al., 2014). Alternatively, ubiquitylated transiently membrane-bound or cytosolic PYR/PYLs might be degraded by the 26S proteasome.

Supplemental material

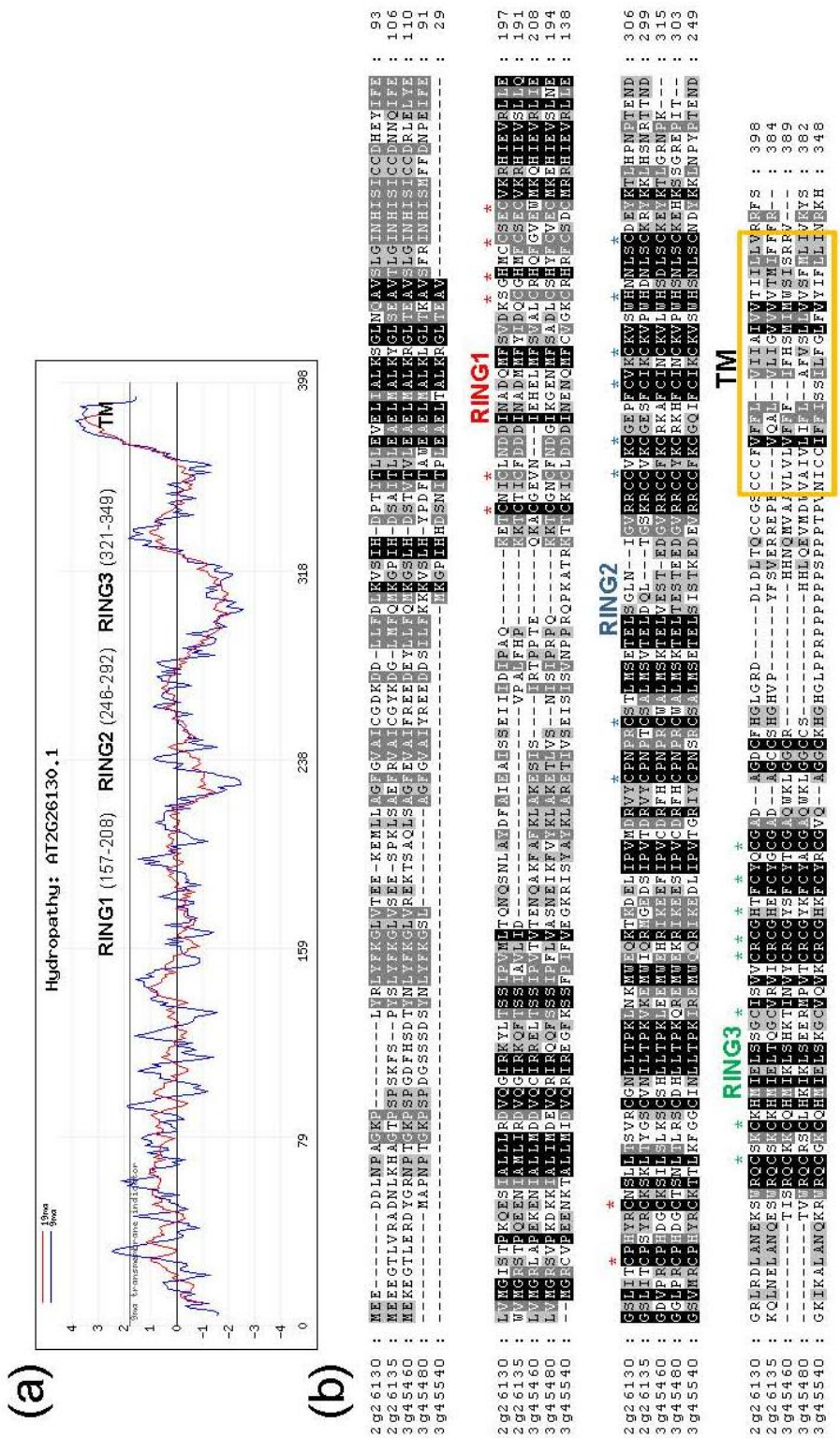

Figure S1. RSL1 and RFA6-9 contain a C-terminal TM domain. (a) The hydropathy plot of RSL1 predicts a C-terminal TM domain according to Kyte-Doolittle hydrophobicity scales. The bioinformatic prediction for protein subcellular location was generated in the SubCellular Proteomic Database (http://suba3.plantenergy.uwa.edu.au). (b) Amino acid sequence alignment of RSL1 and RFA6-9 E3 ubiquitin ligases that bear a C-terminal TM domain: At3g45480/RFA6, At3g45460/RFA7, At3g45540/RFA8 and At2g26135/RFA9. The position of the RING fingers and TM domains are indicated.

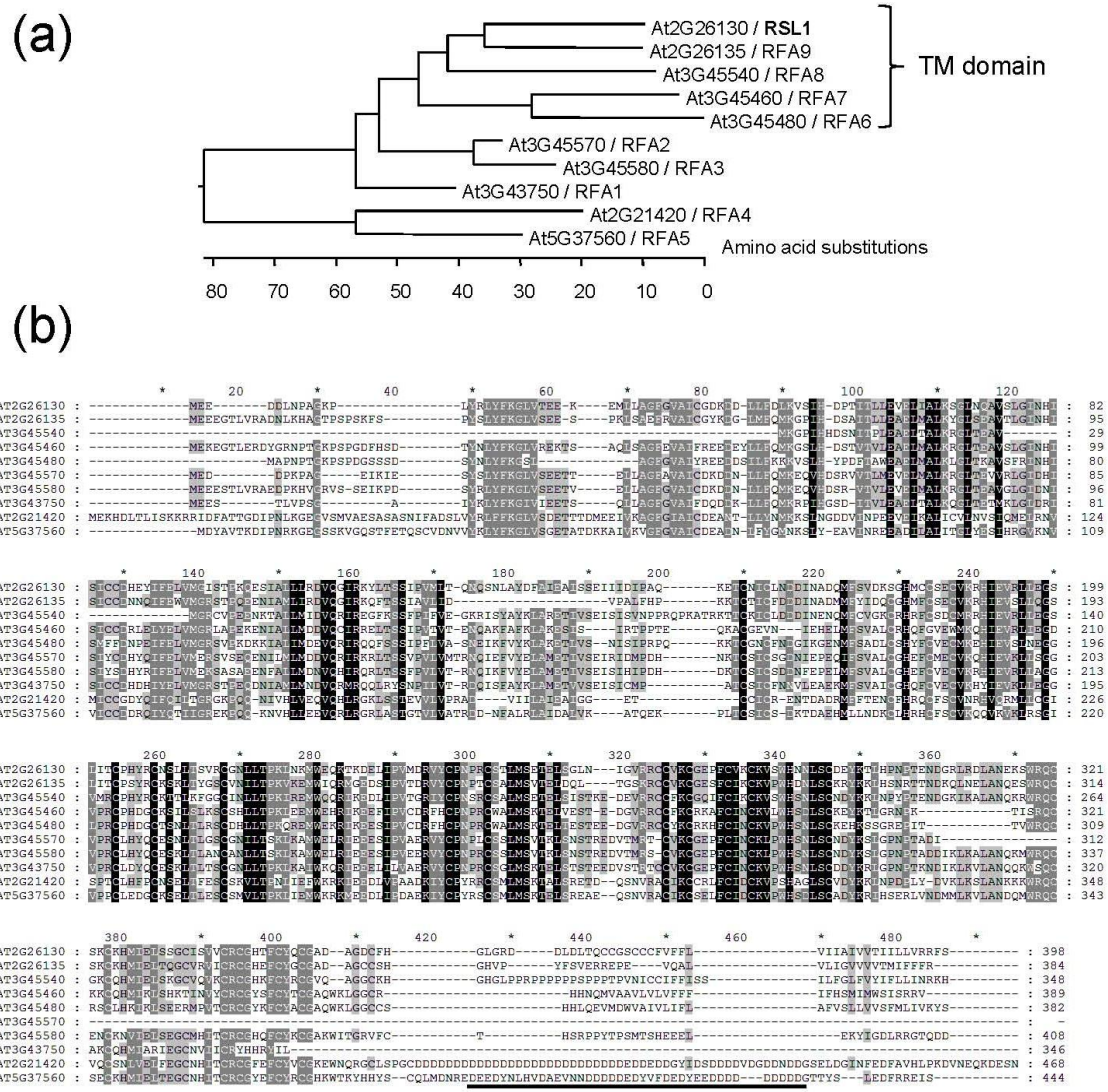

Figure S2. Cladogram and amino acid sequence alignment of RSL1 and RFA1-9 proteins. (a) Cladogram of the RSL1/RFA gene family. A family composed by 10 members was revealed by BLAST search at the TAIR database and visual inspection of the hits showing p value $<10^{-60}$. The nomenclature RFA (RING finger ABA-related) is described in the text. (b) Amino acid sequence alignment of RSL1 and RFA putative E3 ubiquitin ligases. The alignment was generated using Clustal W program and Genedoc software. The acidic C-terminal domain present in At2g21420/RFA4 and At5g37560/RFA5 is underlined.

Figure S3. Location of GFP-RSL1 in plasma membrane is revealed by FM4-64 staining. Confocal images showing either GFP or GFP-RSL1 in transiently transformed tobacco epidermal leaf cells that were plasmolyzed using 0.5 M NaCl for 2 h. FM4-64 staining of the same cell shows red fluorescence at the plasma membrane. Merging of the GFP-RSL1 and FM4-64 fluorescent signals reveals location of GFP-RSL1 at plasma membrane (yellow color resulting from merging GFP-RSL1 and FM4-64 fluorescence). In contrast, the cytosolic location of single GFP clearly diverges from red FM4-64 fluorescent signal.

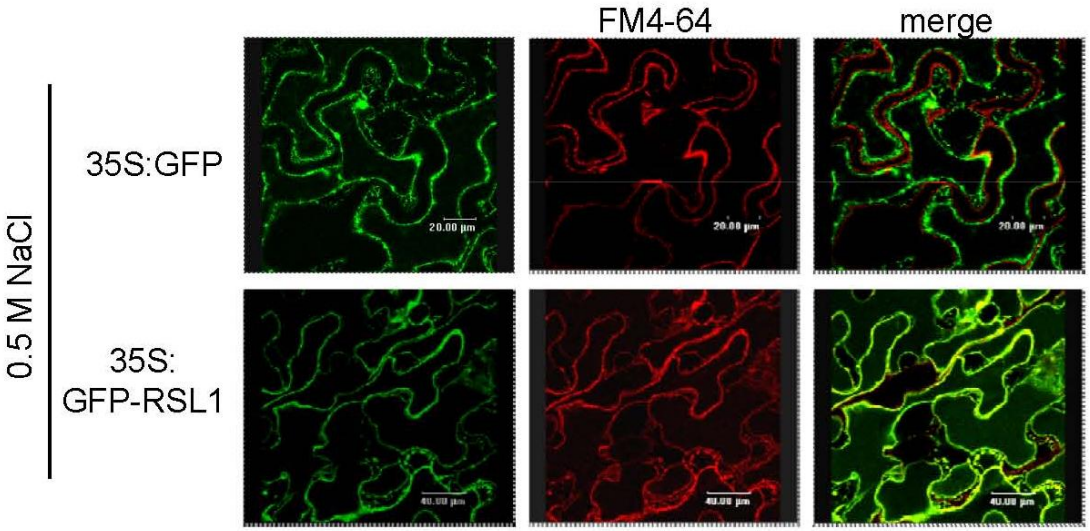

Table S1. List of oligonucleotides used in this work.

RSL1 (At2g26130)	
Forward	5´-TCCAGCATCGGCTCCACATTGG
Reverse	5´-CGAGAACCGTCTTACGAGTAATA
Reverse	5´-TTACGAGAACCGTCTTACGAGTA
RAB18 (At5g66400)	
Forward	5′-ATGGCGTCTTACCAGAACCGT
Reverse	5′-CCAGATCCGGAGCGGTGAAGC
RD29B (At5g52300)	
Forward	5′-ATGGAGTCACAGTTGACACGTCC
Reverse	5′-GAGATAGTCATCTTCACCACCAGG
RD29A (At5g52310)	
Forward	5′-GGAAGTGAAAGGAGGAGGAGGAA
Reverse	5′-CACCACCAAACCAGCCAGATG
β-actin-8 (At1g49420)	
Forward	5′-AGTGGTCGTACAACCGGTATTGT
Reverse	5′-GAGGATAGCATGTGGAAGTGAGAA
At2g26130	
Forward	5′- TTGGCAGAGATGATCTGGATTT
Reverse	5′-CAGTCTTACGAGAACCGTCTTAC
At2g26135	
Forward	5′-CTCTCATGGTCATGTACCGTATTT R
Reverse	5′-CCTGCTCAAAGCAAGTCCTTAT
At3g43750	
Forward	5′-CATGCGATGATTACAAGAGATTGG
Reverse	5′-TACGTTGCATCCTTCGATACG
At3g45580	
Forward	5′-AACGTAATCGAACTATCGGAAGG
Reverse	5′-TGCGATGTCATTGAAGGAGTAT
At5g55840	
Forward	5′-GATGATATTGCAGTTTGTCACCGT
Reverse	5′-CACTGTCTTGCTTGTCTTGTTCTG

amiRNA2:	
I:microRNA	GATCAATATGTCGTTTCACACATCTCTCTTTTGTATTCCA
II:microRNA	AGATGTGTGAAACGACATATTGATCAAAGAGAATCAATGA
III:microRNA	AGATATGTGAAACGAGATATTGTTCACAGGTCGTGATATG
IV: microRNA	GAACAATATCTCGTTTCACATATCTACATATATATTCCTA
amiRNA7	
I:microRNA	GATCTATATGTCGTTTTACGCACCTCTCTTTTGTATTCCA
II:microRNA	AGGTGCGTAAAACGACATATAGATCAAAGAGAATCAATGA
III:microRNA	AGGTACGTAAAACGAGATATAGTTCACAGGTCGTGATATG
IV: microRNA	GAACTATATCTCGTTTTACGTACCTACATATATATTCCTA

9. APPENDIX II

Tomato PYR/PYL/RCAR ABA receptors show high expression in root, differential sensitivity to the ABA-agonist quinabactin and capability to enhance plant drought resistance

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Miguel González-Guzmán*, **Lesia Rodríguez***, Laura Lorenzo-Orts, Clara Pons, Alejandro Sarrión-Perdigones, Maria A. Fernández, Marta Peirats-Llobet, Javier Forment, Maria Moreno-Alvero, Sean R. Cutler, Armando Albert, Antonio Granell and Pedro L. Rodríguez.

* These authors contributed equally to this work.

ABSTRACT

ABA plays a crucial role for plant response to both biotic and abiotic stress. Sustainable production of food faces several key challenges, particularly the generation of new varieties with improved water use efficiency and drought tolerance. Different studies have shown the potential applications of *Arabidopsis* PYR/PYL/RCAR ABA receptors to enhance plant drought resistance. Consequently the functional characterization of orthologous genes in crops holds promise for agriculture. We have identified the full set of tomato (*Solanum lycopersicum*) PYR/PYL/RCAR ABA receptors. From the 15 putative tomato ABA receptors, 14 of them could be grouped in three subfamilies that correlated well with corresponding *Arabidopsis* subfamilies. High expression of *PYR/PYL/RCAR* genes was found in tomato root and some genes showed predominant expression in leaf and fruit tissues. Functional characterization of tomato receptors was performed through interaction assays with *Arabidopsis* and tomato clade A PP2Cs as well as phosphatase inhibition studies. Tomato receptors were able to differentially inhibit in an ABA-dependent manner the activity of clade A PP2Cs, and at least three receptors were sensitive to the ABA-agonist quinabactin, which inhibited tomato seed germination. Indeed, the chemical activation of ABA signalling induced by quinabactin was able to activate stress-responsive genes. Both dimeric and monomeric tomato receptors were functional in *Arabidopsis* plant cells, but only overexpression of monomeric-type receptors conferred enhanced drought resistance. In summary, gene expression analyses, chemical and transgenic approaches revealed distinct properties of tomato PYR/PYL/RCAR ABA receptors that might have biotechnological implications.

Keywords: ABA, tomato ABA receptor, tomato clade A PP2C, drought resistance

Abbreviations: ABA, abscisic acid; QB, quinabactin; PP2C, protein phosphatase type 2C; PYR/PYL/RCAR, PYRABACTIN RESISTANCE1 /PYR1-LIKE/ REGULATORY COMPONENTS OF ABA RECEPTORS

INTRODUCTION

Drought, high salinity and cold have adverse effects on plant growth and seed production. ABA-induced changes play a capital role among the various biochemical and physiological processes required to acquire abiotic stress tolerance (Verslues et al., 2006). Thus, in order to maintain water, ABA promotes stomatal closure through the control of membrane transport systems (Osakabe et al., 2013). On the other hand, shoot growth is inhibited whereas root growth rate is maintained to gain access to water (Sharp et al., 2004; Des Marais et al., 2012). Gene expression is widely regulated by ABA and as a result genes encoding proteins

involved in protection and damage repair are up-regulated, such as LEA/dehydrins, ROS scavengers or osmolite biosynthetic enzymes (Verslues et al., 2006). A wide knowledge on ABA perception and signal transduction has emerged in recent years in *Arabidopsis thaliana* (Cutler et al., 2010). PYR/PYL/RCAR receptors perceive ABA intracellularly and as a result, form ternary complexes with clade A PP2Cs, thereby inactivating them (Park et al., 2009; Ma et al., 2009). This allows the activation of downstream targets of the PP2Cs, such as the sucrose non-fermenting 1-related subfamily 2 (SnRK2s) protein kinases, i.e. SnRK2.2/D, 2.3/I and 2.6/OST1/E, which are key players to regulate transcriptional response to ABA and stomatal aperture ((Umezawa et al., 2009; Fujita et al., 2009; Fujii and Zhu, 2009a; Vlad et al., 2009g). PP2Cs also dephosphorylate other classes of kinases or kinase regulated-proteins (Finkelstein et al., 2013; Rodrigues et al., 2013). According to the oligomeric nature of the apo receptors, they can be classified in two major classes, i.e. dimeric (PYR1 and PYL1-PYL3) or monomeric (PYL4-PYL10, except the untested PYL7) (Hao et al., 2011; Dupeux et al., 2011a). Upon ligand binding, dimeric receptors dissociate to make available the PP2C interaction surface (Dupeux et al., 2011a). In particular, PYL3 suffers a severe cis- to trans-dimer transition by a protomer rotation of 135º to facilitate subsequent dissociation (Zhang et al., 2012). Monomeric receptors are able to interact with PP2Cs in the absence of ABA; however the presence of ABA is required for a major inhibition of PP2Cs when protection of phosphorylated protein substrates by ABA receptors is evaluated (Antoni et al., 2012; Pizzio et al., 2013).

Several biochemical and structural studies of apo/ABA-bound PYLs and ternary receptor-ABA-phosphatase complexes have provided the molecular details of the ABA perception and signalling mechanism (Melcher et al., 2009; Miyazono et al., 2009; Santiago et al., 2009b; Nishimura et al., 2009; Yin et al., 2009). Two loops located between β3-β4 and β5-β6 sheets control the access of the ABA molecule to the ABA binding pocket. These loops generate an open conformation of the ligand-binding pocket in the apo receptors. In response to ABA, conformational changes occur in these loops that serve as gate and latch to stabilize the closed conformation of ABA-bound receptor. A conserved Ser residue flips out of the β3-β4 loop and inserts into the phosphatase catalytic site, blocking access of potential substrates. The mechanism of PP2C inhibition is further explained by the structure of the ternary receptor-ABA-phosphatase complex. In particular, a conserved Trp residue located in a β-hairpin of PP2C establishes contact with the gate and latch loops and indirectly with the ABA´s ketone group through a hydrogen bond mediated by a critical water molecule (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009; Dupeux et al., 2011b). As a result of this gate-latch-lock mechanism and PP2C interaction, the ternary complex shows high affinity for ABA binding (Kd between 20-40 nM) (Ma et al., 2009; Santiago et al., 2009a).

Different works have shown the potential applications of *Arabidopsis* PYR/PYL receptors to enhance plant drought resistance, either through genetic engineering or chemical approaches (Santiago et al., 2009a; Saavedra et al., 2010; Mosquna et al., 2011; Okamoto et al., 2013; Cao et al., 2013; Pizzio et al., 2013). Thus, either through overexpression of wild-type, constitutively active receptors or mutated versions that enhance ABA-dependent inhibition of PP2Cs, enhanced drought resistance could be conferred to *Arabidopsis* plants (Santiago et al., 2009a; Saavedra et al., 2010; Mosquna et al., 2011; Pizzio et al., 2013). On the other hand, chemicals acting as ABA agonists have proved to be effective for similar purposes (Okamoto et al., 2013; Cao et al., 2013). Given the physiological and practical implications of the ABA signalling pathway in agriculture, it is expected that similar approaches could be implemented in crops. Identification of core components of the ABA signalling pathway taking advantage of the knowledge generated in *Arabidopsis* is now possible (Ben-Ari, 2012). Thus, from the discovery of the PYR/PYL/RCAR ABA receptor family in *Arabidopsis*, several reports have described orthologous genes in commercial crops, such as tomato (Sun et al., 2011), strawberry (Chai et al., 2011), rice (Kim et al., 2012), grape (Boneh et al., 2012), sweet orange (Romero et al., 2012) and soybean (Bai et al., 2013). Recently, overexpression of *OsPYL5* in the monocot rice was shown to confer enhanced drought tolerance (Kim et al., 2014). In this work we identified a comprehensive list of putative PYR/PYL/RCAR receptors in the dicot tomato and different studies were performed to validate their function and to show that they encode functional ABA receptors in plant cells. As a result, we found they inhibited tomato clade A PP2Cs in an ABA-dependent manner and some of them could be activated by the ABA-agonist quinabactin, which induced tomato stress-responsive genes. The overexpression in *Arabidopsis* of two tomato receptors from the monomeric sub-groups AtPY4-6 and AtPYL7-10 conferred enhanced drought resistance, whereas overexpression of a tomato dimeric receptor from the sub-group AtPYL1 failed to confer this phenotype.

RESULTS

The tomato genome encodes 15 putative PYR/PYL/RCAR ABA receptors

A partial analysis of the tomato PYR/PYL/RCAR family was published by Sun et al., (2011), leading to the discovery of eight receptors. We have extended the analysis to the complete tomato genome (Tomato Genome Consortium, 2012) and, as a result, we have identified 15 receptors (Fig. 1A). With the exception of 2g076770, they were distributed in three subfamilies, which matched the corresponding groups from *Arabidopsis* PYR/PYL/RCAR receptors. Since it is possible that biochemical or physiological features already known in *Arabidopsis* receptors might be translated to crop receptors, we have tried to correlate tomato receptors with the corresponding groups in *Arabidopsis* (Fig. 1A). On this basis, we propose a

new nomenclature. Thus we found in subfamily I two tomato receptors, 8g076960 and 6g061180, closely related to *Arabidopsis* PYL1/PYR1 (Supplementary Fig. S1) and two other receptors, 12g095970 and 8g065410, more closely related to AtPYL2/PYL3. In the subfamily II we found 6 tomato receptors related to AtPYL4/PYL5/PYL6 and in the subfamily III we found 4 tomato receptors related to AtPYL7/PYL8/PYL9/PYL10. No close relative for the *Arabidopsis* group AtPYL11/12/13 was found in tomato. Finally, the putative tomato receptor 2g076770 was ungrouped, likely because it lacked key conserved residues of the PYR/PYL/RCAR family, such as the conserved Leu of the β3-β4 and β5-β6 loops and the Asn residue before α4-helix (Fig. 1B). Additionally, 2g076770 gene expression could not be detected by RNA-Sequencing (RNA-Seq) analysis in vegetative or fruit tissue (see below Fig. 2), therefore it remains to be established whether 2g076770 is a functional ABA receptor. In contrast, the key residues of both gate-like and latch-like loops were conserved in the remaining 14 tomato receptors (Fig. 1B).

Differential gene expression of tomato ABA receptors in leaf, root and fruit

Relative expression of all tomato genes has been recently reported using Illumina RNA-Sequencing (RNA-Seq) technology, providing gene expression data for the transcriptome of the inbred tomato cultivar "Heinz 1706" (Tomato Genome Consortium 2012). We performed data mining in the RNAseq transcriptome for the 15 tomato receptor genes in root, leaf and six stages of fruit and additionally we performed our own microarray analysis in other tomato accessions (Fig. 2). The expression pattern of ABA receptors in root and leaf indicates that some members show clearly a higher expression level compared to others (Fig. 2A). Two members from subfamily II, i.e. 10g085310 and 3g095780, showed the highest transcription level in root, whereas a member from subfamily III, i.e. 1g095700, was the most transcribed receptor in leaf. In contrast, expression of some receptors was absent in these tissues. For instance, significant expression of 2g076770 and 12g095970 was not detected both in leaf and root (Fig. 2A). On the other hand, it was interesting to detect a high expression of several tomato ABA receptors in root tissue since ABA signalling is required at low water potentials to maintain primary root elongation, to increase root versus shoot biomass partitioning, to regulate root system architecture and to promote root hydrotropism (Sharp et al., 2004; Des Marais et al., 2012; Duan et al., 2013). RNA-seq data were also compiled from a tomato fruit development series composed of six fruit stages, i.e. 1 cm, 2 cm, 3 cm, mature green, breaker (when colour becomes noticeable) and breaker + 10d. In general, members from subfamily III, for instance 12g055990 and 8g082180, showed high expression levels along fruit development, which suggests a role in this process (Fig. 2B). Additionally, recent studies suggest that ABA might be involved in regulating the onset of fruit ripening through triggering of ethylene biosynthesis and, consequently, ABA content peaks at breaker stages compared to mature green stage (Zhang

et al., 2009; Sun et al., 2011). Some tomato ABA receptors whose expression peaked during breaker stages were identified in subfamily II and III, such as 6g050500 and 12g055990; therefore they are candidate genes to regulate fruit ripening (Fig. 2B).

In order to obtain further data on fruit expression of tomato *PYR/PYL* genes, we performed new studies through microarray expression analysis in fruit pericarp at the breaker stage for cv. Moneymaker and *S. pimpinellifolium* accession TO-937 (Fig. 2C). Both 6g050500 and 12g055990 were among the top three most expressed genes at the breaker stage, although depending on the cultivar considered, other tomato receptors also appeared highly expressed (for instance 3g007310 in Moneymaker and 10g085310 in TO-937). In any case, the comparison between Heinz, Moneymaker and TO-937 indicates that 6g050500 was highly expressed in all three genetic backgrounds at breaker stage, which suggests it could be relevant in the regulation of tomato ripening. ABA receptors of the subfamily III may be also important but in an accession specific manner.

Fruit epidermis might be a putative target of ABA action to minimize fruit water loss, for instance through regulation of cuticle thickness or cuticle-dependent sensing of osmotic stress (Wang et al., 2011), and peel fruit also represents the point of entry for pathogen attack. Different genetic resources in the tomato Microtom background are available, including transgenic lines that modify peel fruit features (Shi et al., 2013). Therefore, we were interested in obtaining gene expression data for ABA receptors in peel fruit. Microarray expression data pointed out different receptors from Subfamily I, II and III that were expressed over the average in peel fruit and are, therefore, candidates to regulate ABA action at fruit epidermis, e.g. 6g050500 and 8g082180 (Fig. 2D).

Tomato ABA receptors interact with Arabidopsis and tomato clade A PP2Cs

A key aspect of receptor function is its ability to interact and inhibit clade A PP2Cs. Both ABA-independent and ABA-dependent Y2H interactions among ABA receptors and PP2Cs have been reported in *Arabidopsis*, which likely reflect the monomeric/dimeric nature of the receptor as well as different Kds in yeast for particular receptor-phosphatase interactions (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009a). ABA-independent interactions can be detected in Y2H assays; however, major inhibition of PP2C activity relies on the presence of ABA (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009a). First of all, we tested whether tomato PYR/PYLs were able to interact with *Arabidopsis* clade A PP2Cs. We selected tomato members from the three subfamilies and we found both ABA-independent and ABA-dependent interactions with *Arabidopsis* PP2Cs (Fig. 3A). Interestingly, both 8g076960 and 6g061180, which are supposed to be dimeric receptors according to their similarity to AtPYL1, showed ABA-dependent interactions with *Arabidopsis* PP2Cs (Fig. 3A). Dimeric receptors occlude their surface of interaction with PP2Cs and require ABA-induced dissociation to form ternary

receptor monomer-ABA-phosphatase complexes (Dupeux et al., 2011a). We assayed three members from subfamily II and we found that two of them showed ABA-independent interactions with some PP2Cs. The third member, 9g015380, is a close relative of AtPYL4 and it showed ABA-dependent interactions with PP2Cs as it was reported previously for AtPYL4 (Lackman et al., 2011). Finally, we assayed two members from subfamily III and found ABA-independent interactions with AtHAB2 and AtABI2. The *Arabidopsis* PP2C AHG1 is resistant to inhibition by ABA receptors because it lacks the conserved Trp residue required for formation of ternary complexes (Dupeux et al., 2011b). Accordingly, this phosphatase did not interact with any tomato receptor, suggesting a similar Trp-dependent mechanism for formation of ternary complexes with tomato receptors. Finally, the PP2C HAI1, which shows a more restrictive pattern of interaction with *Arabidopsis* receptors, only interacted with one tomato receptor (Bhaskara et al., 2012).

Next we cloned the cDNA of two tomato clade A PP2Cs, 5g052980 and 12g096020, which are close relatives of *Arabidopsis* PP2CA and HAB1, respectively (Supplementary Fig. S2). We used the N-terminal deleted version (ΔN) of 12g096020 for both Y2H and activity assays, since the N-terminus is dispensable for interaction with ABA receptors (Santiago et al., 2009a). 5g052980 and ΔN 12g096020 showed both ABA-dependent and ABA-independent interactions with *Arabidopsis* as well as tomato receptors (Fig. 3B and C). Therefore, a similar mechanism to that described in *Arabidopsis* for receptor-phosphatase interaction seems to operate in tomato. Finally, according to Y2H analyses and sequence similarity, we predicted that tomato relatives of AtPYL1, such as 8g076960 and 6g061180, might be dimeric receptors whereas members of subfamilies II and III might be monomeric receptors. To test this prediction, we performed size-exclusion chromatography (SEC) analysis from 4 tomato receptors in the absence of ABA (Fig. 3D). As a result we found that 8g076960 and 6g061180 migrated with an estimated molecular mass of 48 kDa, which corresponds to a dimeric receptor, and 12g055990 migrated with an estimated molecular mass of 27 kDa, which corresponds to a monomeric receptor (predicted molecular masses are 25.6, 23.7 and 25.8 kDa, respectively). However, 6g050500 displays a two peaks profile that corresponds to a distribution between monomeric and dimeric species (predicted molecular mass is 24.5 kDa). Recent results with the monomeric receptor AtPYL9 indicate that it can form dimers during crystal packing and a minor dimeric form also appears after SEC (Zhang et al., 2013).

Tomato ABA receptors inhibit both Arabidopsis and tomato clade A PP2Cs in an ABA-dependent manner

To investigate whether tomato PYR/PYL proteins are actually functional receptors, able to perceive ABA and to inhibit PP2Cs, we measured phosphatase activity using the RRA(phosphoT)VA phosphopeptide as substrate (Fig. 4A, B and C). To this end we used the *Arabidopsis* PP2C ABI2, which showed interaction with all tomato receptors assayed in Y2H

assays, and two tomato PP2Cs, 5g052980 and ΔN 12g096020, which are putative orthologous proteins from AtPP2CA and AtHAB1, respectively. We were able to purify recombinant soluble proteins for five tomato receptors representing the three subfamilies. All of them were able to inhibit phosphatase activity in an ABA-dependent manner, although to different extent. ABI2 and ΔN 12g096020 activity was sensitive to all receptors, whereas 5g052980 activity was hardly affected by 6g061180 and only at 10 μM ABA by 8g076960. Since tomato phosphatases belong to different sub-branches of the clade A PP2C family, these results are in agreement with the differential inhibition by ABA receptors described previously for AtPP2CA and AtHAB1 (Hao et al., 2011; Antoni et al., 2012; Pizzio et al., 2013).

The ABA-agonist quinabactin is selectively perceived by tomato ABA receptors and induces abiotic stress responsive genes

Quinabactin (QB) is an ABA-mimicking ligand able to discriminate among the different *Arabidopsis* PYR/PYL receptors, showing preferential activation of dimeric receptors and certain activation of monomeric PYL5 and PYL7 (Okamoto et al., 2013; Cao et al., 2013). QB application in crop plants (soybean, barley and maize) had ABA-like effects (Okamoto et al., 2013); however its mechanism of action using crop ABA receptors and PP2Cs has not been investigated previously. Since QB represents a synthetic ABA agonist eliciting both seed and vegetative ABA-responses, holding potential to enable plant protection against water stress, we tested its effect on tomato PYR/PYLs. Interestingly, QB inhibited efficiently (compared with ABA) ΔN12g096020 phosphatase activity through the dimeric tomato receptors 8g076960 and 6g061180, and also through 6g050500, although in this latter case less efficiently than ABA (Fig. 4C). 6g050500 belongs to the AtPYL4-6 subfamily and therefore shows similarity with the QB-sensitive AtPYL5, which can explain its capacity to perceive QB. In contrast, two tomato receptors that belong to the AtPYL7-10 family, i.e. 3g007310 and 12g055990, were not activated even by 10 μM quinabactin. These results lend support to the selective effect of QB on ABA receptors and provide biochemical evidence that QB can be perceived by dicot crop receptors and inhibit activity of a crop PP2C. In order to test whether QB has *in vivo* effects on tomato, we analyzed tomato seed germination in the presence of the compound (Fig. 4D). QB was able to inhibit germination of tomato seeds, although at higher concentration compared to ABA. These results suggest that tomato receptors not sensitive to QB are required for full regulation of seed germination or that QB-sensitive receptors are not expressed at high levels during this stage. Alternatively, since QB is less water soluble than ABA, bioavailability of QB could be lower than ABA to inhibit seed germination or follow a less efficient transport system.

Finally, in order to assess the biological activity of QB in tomato seedlings, we treated 10-d-old plants with 10 μM QB or ABA for 3 h. The transcriptional levels of ABA- and

drought-responsive tomato genes were analyzed using qRT-PCR (Fig. 4E). To this end we selected three tomato genes that showed strong sequence similarity with either *Arabidopsis RESPONSIVE TO ABA 18 (RAB18)*, *LATE EMBRYOGENESIS ABUNDANT (LEA)* family or the *DELTA 1-PYRROLINE-5-CARBOXYLATE SYNTHASE (P5CS1)* genes, which were represented by the tomato loci *2g084850*, *6g067980* and *6g019170*, respectively. The *Arabidopsis* genes have been shown to be induced in response to drought, cold, salinity and ABA, and therefore are good markers of plant response to these forms of abiotic stress (Saez et al., 2006). The three tomato genes were activated both by ABA- and QB-treatment, which indicated that ABA signalling was efficiently triggered by the ABA-agonist QB in tomato (Figure 4E).

Overexpression of tomato monomeric-type ABA receptors in Arabidopsis confers enhanced response to ABA and plant drought resistance

Overexpression of some monomeric *Arabidopsis* PYR/PYL receptors is known to enhance ABA response and plant drought resistance (Santiago et al., 2009a; Saavedra et al., 2010; Pizzio et al., 2013). In order to investigate whether tomato PYR/PYLs are functional receptors in plant cells, we generated transgenic plants that overexpress HA-tagged versions of either monomeric-type receptors, 6g050500 or 3g007310, or a dimeric receptor, 8g076960. Expression of HA-tagged tomato PYR/PYLs was verified by immunoblot analysis and two independent transgenic lines were selected for further analysis (Fig. 5A). Overexpression of tomato monomeric-type receptors in *Arabidopsis* enhanced ABA-mediated inhibition of seedling establishment and root growth compared with non-transformed plants, a phenotype similar to that obtained by a double inactivation of the ABI1 and HAB1 PP2Cs (Fig. 5B and C) (Saez et al., 2006). Interestingly, overexpression of the tomato dimeric receptor did not enhance ABA-mediated inhibition of seedling establishment but enhanced root growth sensitivity to ABA and it also generated partial complementation of the ABA-insensitive phenotype of the 112458 *pyr/pyl* mutant (Fig. 5B and C; Supplementary Fig. S3).

Next, we performed drought resistance experiments under greenhouse conditions. Plants were grown under normal watering conditions for two weeks and then irrigation was stopped for 20 d (Fig. 5D). After 20 d lacking watering, non-transformed plants wilted and many rosette leaves yellowed, in contrast to transgenic lines that express tomato monomeric-type PYR/PYLs (Fig. 5D). Interestingly, transgenic lines expressing the tomato dimeric receptor showed a phenotype similar to wt (Fig. 5D). Watering was then resumed and survival of the plants was measured after 3 d. A remarkable enhanced survival (40-50%) was found in the drought-resistant *hab1-1abi1-2* double mutant (Saez et al., 2006) and transgenic plants expressing tomato monomeric-type PYR/PYLs compared to non-transformed plants or transgenic lines expressing the dimeric receptor (Fig. 5E). Thus, a clear distinction regarding drought resistance

was found between overexpressing tomato monomeric-type or dimeric receptors. During the drought stress experiment, the RWC of the rosette leaves was measured at 11, 14 and 17 d after water withdrawal. Both the *hab1-1abi1-2* double mutant and transgenic lines expressing monomeric-type PYR/PYLs showed higher RWC compared to non-transformed plants or transgenic lines expressing the dimeric receptor (Fig. 5F). Thus, either knocking out clade A PP2Cs or overexpressing tomato monomeric-type leads to plants that experience lower water-loss compared to non-transformed plants.

DISCUSSION

In this work we reveal distinct properties of tomato PYR/PYL ABA receptors according to gene expression and biochemical analyses, sensitivity to the ABA-agonist quinabactin and capability to enhance plant drought resistance. We demonstrate that both chemical and transgenic approaches can trigger activity of tomato PYR/PYL ABA receptors, leading to inhibition of crop PP2Cs. Thus, our results indicate that chemical treatment with an ABA-agonist is effective to activate the ABA signalling pathway in a dicot crop plant, inducing key genes for drought stress response. Inhibition of PP2C activity by either overexpression of ABA receptors or combined insertional mutagenesis has proved to be an efficient approach to enhance plant drought resistance in *Arabidopsis* (Saez et al., 2006; Santiago et al., 2009). Our results open the way for similar approaches in tomato given the feasibility of transgenic approaches and availability of tomato mutant libraries and TILLING platforms (Okabe et al., 2012). According to results obtained here when tomato receptors were introduced into *Arabidopsis* and their effect on AtABI2 inhibition, it seems that ABA receptors can be functionally exchanged among different plants. Therefore, the generation of constitutively active receptors or mutated versions that enhance ABA-dependent inhibition of PP2Cs might be used as a transversal approach to enhance drought resistance in different plants (Mosquna et al., 2011; Pizzio et al., 2013). Since over-expression of monomeric-type tomato receptors in *Arabidopsis* conferred enhanced survival and higher RWC upon drought stress, it will be interesting to check it in tomato plants, either through constitutive or stress-induced expression. Interestingly, overexpression of a dimeric tomato receptor was not effective to enhance *Arabidopsis* drought resistance, which suggests that monomeric crop PYR/PYL ABA receptors might perform better to achieve such goal. Indeed, in contrast to monomeric *Arabidopsis* PYR/PYL receptors, overexpression of dimeric receptor has not proved to be effective to enhance plant drought resistance, which might reflect structural constraints of dimeric receptors to interact with PP2Cs in the absence of ABA and therefore lack of basal activation of the pathway (Dupeux et al., 2011a). In this case, chemical treatment with ABA-agonists is an

alternative and efficient approach to activate dimeric receptors (Okamoto et al., 2013; Cao et al., 2013).

Tomato is mainly produced in Mediterranean countries, where fresh water availability is a major problem that could make worse in the event of climate change. When produced in soilless greenhouses, the solution recycling is mandatory and thus any reduction in the water used is relevant for producers. Gene transcription data suggest that a high number of tomato receptors operate in root and their transcript levels are higher than those in leaves, which seems to support a relevant role of ABA perception and signalling in root to cope with drought stress and promote hydrotropic growth response (Sharp and LeNoble, 2002; Antoni et al., 2013). These data are in agreement with a recent transcriptional analysis of *Arabidopsis* genes involved in ABA synthesis and perception, which revealed that whereas synthesis-involved genes show higher levels in shoots than in roots, perception-involved genes show an opposite pattern (Boursiac et al., 2013). On the other hand, some reports have revealed a role of ABA in tomato fruit ripening and cell wall catabolism via regulation of ethylene biosynthesis and major catabolic genes (Zhang et al., 2009; Sun et al., 2012). Therefore, ABA signalling in tomato fruit might also be relevant to regulate fruit texture, shelf life and water-loss through fruit epidermis (Sun et al., 2012; this work). In summary, both RNAseq and microarray expression analyses in tomato have pointed out ABA receptors that showed a preferential expression (Sun et al., 2011; Wang et al., 2013; this work). These data together with their functional characterization open the door to a future biotechnological use in order to enhance tomato drought resistance or modify fruit properties regulated through ABA signalling.

In silico identification of the ABA signalling core components has been performed in several crops. For instance, the soybean and rice genome encode 23 and 13 putative ABA receptors, respectively (Bai et al., 2012; Kim et al., 2012). In general, nomenclature of ABA receptors in crops has followed a numerical order that misses correlation with *Arabidopsis* receptors. As a result, current nomenclature in crops makes it difficult to tentatively correlate biochemical and physiological properties of crop ABA receptors with *Arabidopsis* receptors. Since the ABA signalling pathway is universally conserved in land plants (Hauser et al., 2011), it seems sensible to take advantage from *Arabidopsis* knowledge on PYR/PYL receptors. In this work, we propose a nomenclature based on the adscription of crop receptors to *Arabidopsis* subfamilies of PYR/PYL ABA receptors. This approach is supported by phylogenetic studies showing that PYR/PYL receptors can be grouped in three major clades and it allows tentative prediction of some receptor properties (Hauser et al., 2011). Indeed, we were able to take advantage of this knowledge to infer some properties exhibited by tomato receptors regarding their oligomeric nature, inhibition of PP2C activity and sensitivity to quinabactin, for instance. Subfamily I corresponded with *Arabidopsis* dimeric receptors and indeed, SEC analysis of two tomato members (closely related to AtPYL1) confirmed their dimeric nature. In agreement,

Y2H interaction assays showed ABA-dependent interactions for dimeric receptors and PP2Cs, which presumably reflects the requirement of ABA-induced dissociation previous to interaction with the phosphatase. Subfamily II and III corresponded with AtPYL4-6 and AtPYL7-10 groups, respectively. SEC analysis of one tomato representative member of AtPYL7-10 group revealed a similar monomeric nature; whereas the tomato member of AtPYL4-6 group showed an elution profile that might be explained as a monomeric-dimeric mixture. It is possible that the high protein concentration present in the injected sample for SEC analysis might have promoted such equilibrium as it was described recently for the monomeric receptor AtPYL9 (Zhang et al., 2013).

Y2H analyses performed among tomato ABA receptors and either *Arabidopsis* or tomato PP2Cs revealed both ABA-independent and ABA-dependent interactions (Fig. 3). However, major inhibition of PP2C activity by tomato ABA receptors was ABA-dependent (Fig. 4). Therefore, these results suggest that the formation of stable ternary receptor-ABA-phosphatase complexes is required to achieve a major effect on the activation of tomato PP2C-downstream targets. Clade A PP2Cs constitute a hub for regulation of different environmental responses, allowing the integration of stress signalling pathways into a coordinated response (Rodrigues et al., 2013). A fine tuning of their activity can be achieved by the selective or differential inhibition of PP2C activity carried out by ABA receptors, which was confirmed in the two tomato PP2Cs analyzed in this work. Finally, we also found that the ABA-agonist QB was selective for some tomato receptors and promoted both *in vitro* inhibition of a tomato clade A PP2C and *in vivo* inhibition of tomato seed germination. These results, taken together with previous data from Okamoto et al., (2013), indicate the ABA signalling pathway can be activated in crops by chemicals mimicking ABA action. Thus, chemical treatment of tomato seedlings with quinabactin promoted expression of ABA- and stress-responsive genes. For instance, *P5CS1*, which encodes a key enzyme for proline biosynthesis and osmotic adjustment under drought stress, was efficiently induced by QB treatment (Figure 4E). In summary, both chemical and transgenic approaches based on PYR/PYL ABA receptors might be effective to cope with water stress in tomato.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana and *Solanum lycopersicum* (cv. Moneymaker) plants were routinely grown under greenhouse conditions (40-50 % relative humidity) in pots containing a 1:3 vermiculite-soil mixture. For *Arabidopsis* plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by

commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4ºC for 3 days. Then, seeds were sowed on Murashige-Skoog (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% sucrose and 1% agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 μE m^{-2} sec^{-1}. Tomato seeds were surface sterilized by treatment with commercial bleach (2.5 % sodium hypochlorite) containing 0.05 % Tween 20 for 30 min and four washes with sterile distilled water. MS plates for tomato seeds contained 0.5xMS salts.

Microarray analysis

Fruits at breaker stage were harvested from *S. lycopersicum* ('Moneymaker' and 'Microtom') and *S. pimpinellifolium* ('TO-937') plants. Pericarp and epidermis were excised manually with a sterile scalpel, frozen and ground with liquid nitrogen to a fine powder. At least three biologically replicated samples for RNA isolation were prepared from each genotype and tissue from three or more pooled fruits. RNA was extracted from pericarp with the modified CTAB method (Powell et al., 2012) and from epidermis with the Trizol Reagent (Invitrogen). RNA clean up protocol was done with the RNA Plant Mini Kit (Qiagen). The RNA pellet was resuspended in nuclease-free water. Samples of total RNA were checked for integrity and quality using an Agilent Bioanalyzer (Agilent Technologies). The three biologically replicated RNA samples were amplified, labeled and hybridized to the 34K gene EUTOM3 Exon array (http://www.eu-sol.net/science/bioinformatics/data-and-databases/all-databases) according to manufacturer's instructions (Affymetrix) at Unitat Central d'Investigació (Universitat de Valencia, Spain) as described in Powell et al., (2012). Data were pre-processed and analyzed using Partek Genomic Suite software v6.6 (Partek Inc.) with the probes matching only once with the ITAG annotation 2.30. The configuration consisted of a pre-background adjustment for GC content, Robust Multi-array Analysis for background correction, quantile normalization and probe set summarization using median polishing (Irizarry et al., 2003). Library files were eutom3gene_v2_ucprobes.cdf and the annotation file version was eutom3-annotation-per-scaffold-modif.txt which represents 30,000 tomato genes.

Yeast two hybrid (Y2H) assays

The full-length coding sequences of Sl08g076960, Sl06g061180, Sl09g015380, Sl06g050500, Sl03g095780, Sl12g055990 and Sl03g007310 ABA receptors as well as Sl05g052980 PP2C were amplified by PCR from tomato leaf/fruit cDNA and cloned into the pCR8/GW/TOPO entry vector (Invitrogen). An N-terminal deleted version (ΔN) of Sl12g096020 PP2C was amplified from tomato leaf/fruit cDNA using primers that amplify the

catalytic PP2C core (amino acid residues 178 to 509, ΔN Sl12g096020). All the primers used in this work are listed in supplementary Table 1. Appropriated restriction sites were introduced in some primers to allow the subsequent cloning steps and all constructs were verified by DNA sequencing. Tomato ABA receptors were fused by Gateway recombination to the GAL4 DNA-binding domain (GBD) in pGBKT7GW. As preys, we used a set of *Arabidopsis* clade A PP2Cs fused to the GAL4 activation domain (GAD) in pGADT7 vector (Lackman et al., 2011; Antoni et al., 2012). Tomato Sl05g052980 and ΔN Sl12g096020 PP2Cs were fused to the GAD in the pGADT7GW vector. Protocols for Y2H assays were similar to those described previously (Saez et al., 2008).

Purification of recombinant proteins

Sl06g050500, Sl03g095780, Sl12g055990 and Sl03g007310 coding sequences were cloned in pCR8/GW/TOPO, excised using *Nco*I/*Eco*RI double digestion and subcloned into pETM11. Sl09g015380, Sl08g076960 and Sl06g061180 coding sequences have either *Eco*RI or *Nco*I internal restriction sites and were subcloned using a different strategy. Coding sequences of Sl08g076960 and Sl09g015380 were excised using *Nco*I/*Hind*III and *Nco*I/*Bam*HI double digestion, respectively, and subcloned into pETM11, whereas Sl06g061180 was excised using *Eco*RI digestion and subcloned into pET28a. *Escherichia coli* BL21 (DE3) cells transformed with the corresponding pET28a/pETM11 construct were grown in 50 mL of Luria-Bertani medium supplemented with 50 μg mL^{-1} kanamycin to an optical density at 600 nm of 0.6 to 0.8. Then, 1mM isopropylthio-b-galactoside (IPTG) was added, and the cells were harvested 3 h after induction and stored at -80ºC before purification. The pellet was resuspended in 2 mL of buffer HIS (50 mM Tris-HCl, pH 7.6, 250 mM KCl, 10% glycerol, 0.1% Tween 20, and 10 mM mercaptoethanol), and the cells were sonicated in a Branson Sonifier. A cleared lysate was obtained after centrifugation at 14,000g for 15 min, and it was diluted with 2 volumes of buffer HIS. The protein extract was applied to a 0.5 mL nickel-nitrilotriacetic acid (Ni-NTA) agarose column, and the column was washed with 10 mL of buffer HIS supplemented with 20% glycerol and 30 mM imidazol. Bound protein was eluted with buffer HIS supplemented with 20% glycerol and 250 mM imidazol.

In order to obtain enough protein for size exclusion chromatography (SEC) analysis, 8 ml of an overnight culture were sub-cultured into 800 ml fresh 2TY broth (16 g Bacto tryptone, 10 g yeast extract, 5 g NaCl per litre of solution) plus kanamycin (50 μg mL^{-1}). Protein expression was induced with 0.3 mM IPTG and the cells were harvested after overnight incubation at 16ºC. Pellets were resuspended in 25 mM TrisHCl pH 8.0, 200 mM NaCl, 50 mM imidazole, 5 mM β-mercaptoethanol and disrupted by sonication. After centrifugation for 40 min at 40000 g, the clear supernatant was filtered (pore diameter 0.45 μm; Millipore

Corporation, Bedford, MA, USA). The 6His-tagged proteins were purified using Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions. Proteins were eluted with the following elution buffer: 25 mM TrisHCl pH 8.0, 200 mM NaCl, 500 mM imidazole, 5 mM b-mercaptoethanol, and cleaved with TEV protease (1:100). Proteins 8g076960, 6g061180 and 6g050500 were concentrated to 10 mg/ml and 12g055990 was concentrated to 0.7 mg/ml. Finally, each purified protein was subjected to gel filtration using a prep grade Superdex200 10/30 (Amersham Biosciences Limited, UK) previously equilibrated with 25 mM TrisHCl pH 8.0, 200 mM NaCl, 5 mM b-mercaptoethanol. Approximately 1 mg of 8g076960, 6g061180 or 6g050500 was loaded into the column, whereas 12g055990 was difficult to solubilize and only 0.1 mg was loaded.

PP2C activity assays

Phosphatase activity was measured using the RRA(phosphoT)VA peptide as substrate, which has a K_m of 0.5 to 1 μM for eukaryotic PP2Cs (Donella et al., 1990). Assays were performed in a 100 μL reaction volume containing 25 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, 25 μM peptide substrate, and 0.5 μM PP2C. When indicated, PYR/PYL/RCAR recombinant proteins and ABA or Quinabactin (QB, purchased from Life Chemicals) were included in the PP2C activity assay. ABA and QB concentrations were 0.1, 0.5, 1, 5, 10 and 50 μM. After incubation for 60 min at 30ºC, the reaction was stopped by the addition of 30 μL of molybdate dye (Baykov et al., 1988), and the absorbance was read at 630 nm with a 96-well plate reader. Appropriated controls including DMSO and buffer HIS were included.

ABA and QB treatment of tomato seedlings

10-d-old tomato seedlings (cv. Moneymaker) were mock-, 10 μM ABA or QB-treated for 3 h. Total RNA was extracted using a NucleoSpin RNA plant kit. Synthesis of cDNA and qRT-PCR analyses were performed as described (Saez et al., 2006). Amplification of the ABA- and stress-responsive *Sl02g084850*, *Sl06g067980* and *Sl06g019170* genes was done using the primers described in Table S1. Expression was normalized using the values obtained with *Sl06g009970 (SlEF1a)*.

Generation of transgenic lines

Sl06g050500, Sl03g007310 and Sl08g076960 coding sequences in the pCR8/GW/TOPO entry clone were recombined by LR reaction into the gateway compatible ALLIGATOR2 vector (Bensmihen *et al.,* 2004). The ALLIGATOR2 vector drives expression of the recombined gene under control of the 35S CaMV promoter and introduces a triple hemagglutinin (HA) epitope at the N-terminus of the encoded protein. Selection of transgenic

lines is based on the visualization of GFP in seeds, whose expression is driven by the specific seed promoter At2S3. The ALLIGATOR2 constructs were transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere *et al.*, 1985) by electroporation and used to transform Columbia wild type by the floral dip method. T1 transgenic seeds were selected based on GFP visualization and sowed in soil to obtain the T2 generation. Homozygous T3 progeny was used for further studies and expression of HA-tagged protein was verified by immunoblot analysis using antiHA-peroxidase (Roche).

Seed germination assays

After surface sterilization of the tomato seeds, approximately 100 seeds were sowed on 0.5xMS plates lacking (control plates) or supplemented with either 1 or 10 μM ABA or quinabactin. Seeds were germinated in the dark at 23ºC for 3 d. In order to score seed germination, radical emergence was analyzed at 72 h after sowing. Since quinabactin was dissolved in DMSO, control MS plates for quinabactin experiments were supplemented with 0.1% DMSO.

Root growth assays

Arabidopsis seedlings were grown on vertically oriented MS plates for 3 days. Afterwards, 20 plants were transferred to new MS plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10-d to produce image files suitable for quantitative analysis of root growth using the NIH Image software ImageJ v1.37.

Drought stress and water status measurement

Plants grown under greenhouse conditions (10 individuals per experiment, three independent experiments) were grown under normal watering conditions for 15 days and then subjected to drought stress by stopping irrigation during 20 days. Next, watering was resumed and survival rate was calculated after 3 days by counting the percentage of plants that had more than four green leaves. Photographs were taken at the start of the experiment (day 0), after 16 and 20 days of drought, and 3 days after re-watering. The relative water content (RWC) of the plants was measured in rosette leaves at 11d, 14d and 17d. Samples of 10 leaves from 5 plants were collected and their fresh weight (FW) was obtained. Leaves were then floated for 3 h on demineralized water and weighed again in order to obtain their turgid weight (TW). Finally, leaves were dried for 16 h at 70ºC and weighed to obtain the dry weight (DW). The RWC was calculated as (FW-DW/TW-DW) x 100 and each measurement was made in triplicate.

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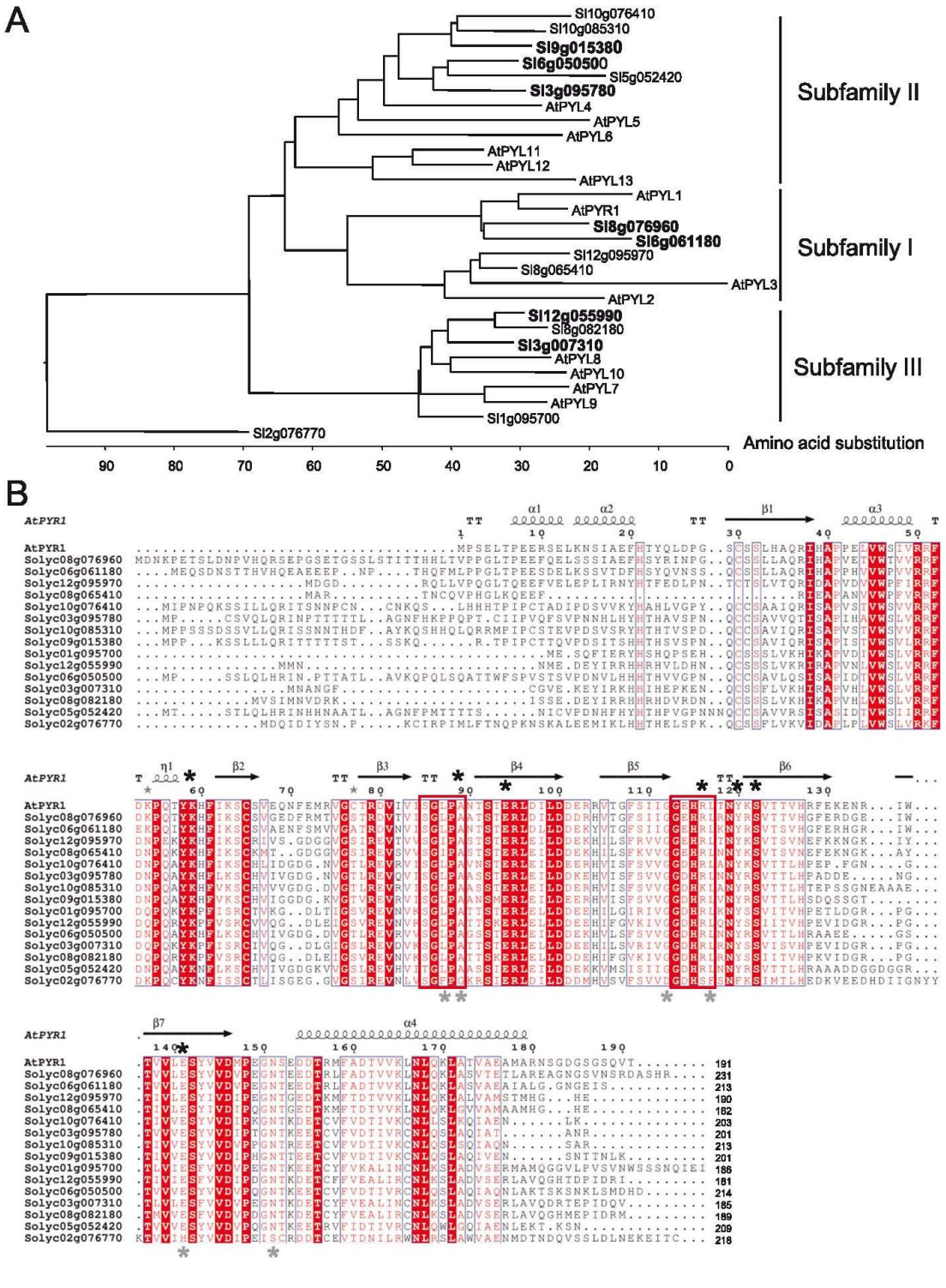

Figure 1. Cladogram and amino acid sequence alignment of tomato PYR/PYL ABA receptors. (A) Cladogram of the multiple sequence alignment of tomato and *Arabidopsis* PYR/PYL receptors, indicating three major subfamilies and the ungrouped 2g076770. In bold face those tomato receptors further described in the text. (B) Sequence and secondary structure alignment of tomato PYR/PYL ABA receptors and *Arabidopsis* PYR1 protein. Predicted secondary structure of the tomato proteins was indicated taking as model the crystallographic structure of PYR1 (Protein DataBank Code 3K90) and using Espript interface (http://espript.ibcp.fr/). Boxes indicate the position of the gate and latch loops. Black asterisks mark residues K59, A89, E94, R116, Y120, S122 and E141 of PYR1 involved in ABA binding. Grey asterisks mark conserved residues of the tomato receptor family that differ in tomato 2g076770 protein.

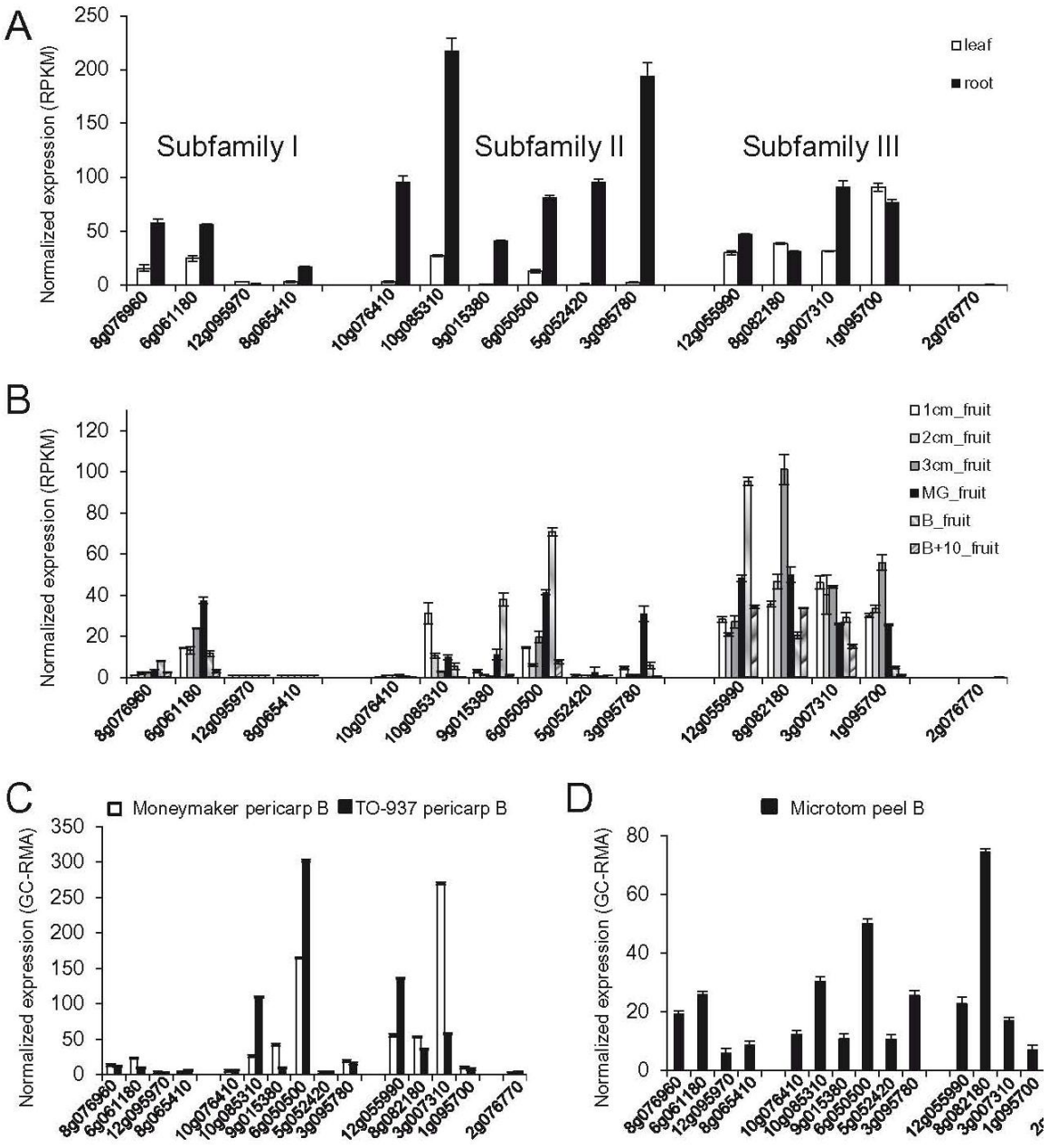

Figure 2. Relative gene expression of tomato ABA receptors in leaf, root and fruit was determined by RNA-Sequencing (RNA-Seq) and microarray analysis. (A, B) The transcriptome of the inbred tomato cultivar Heinz 1706 was analysed using Illumina RNA-Seq technology (Tomato genome consortium, 2012). Data show gene transcription of tomato receptors grouped in three subfamilies and the ungrouped 2g076770 in leaf and root (A) and during fruit development and ripening (B). Significant expression of 2g076770 and 12g095970 was not detected in these tissues. RPKM, reads per kilobase of exon model per million mapped reads; MG, mature green stage; B, breaker stage. (C, D) Relative RNA abundance based on GC-RMA values (GC content-robust multiarray analysis) obtained from Affymetrix exon tomato microarray (EUTOM3) hybridized with fruit pericarp RNA of Moneymaker and TO-937 (C) and fruit epidermis RNA of Microtom background (D) at breaker stage.

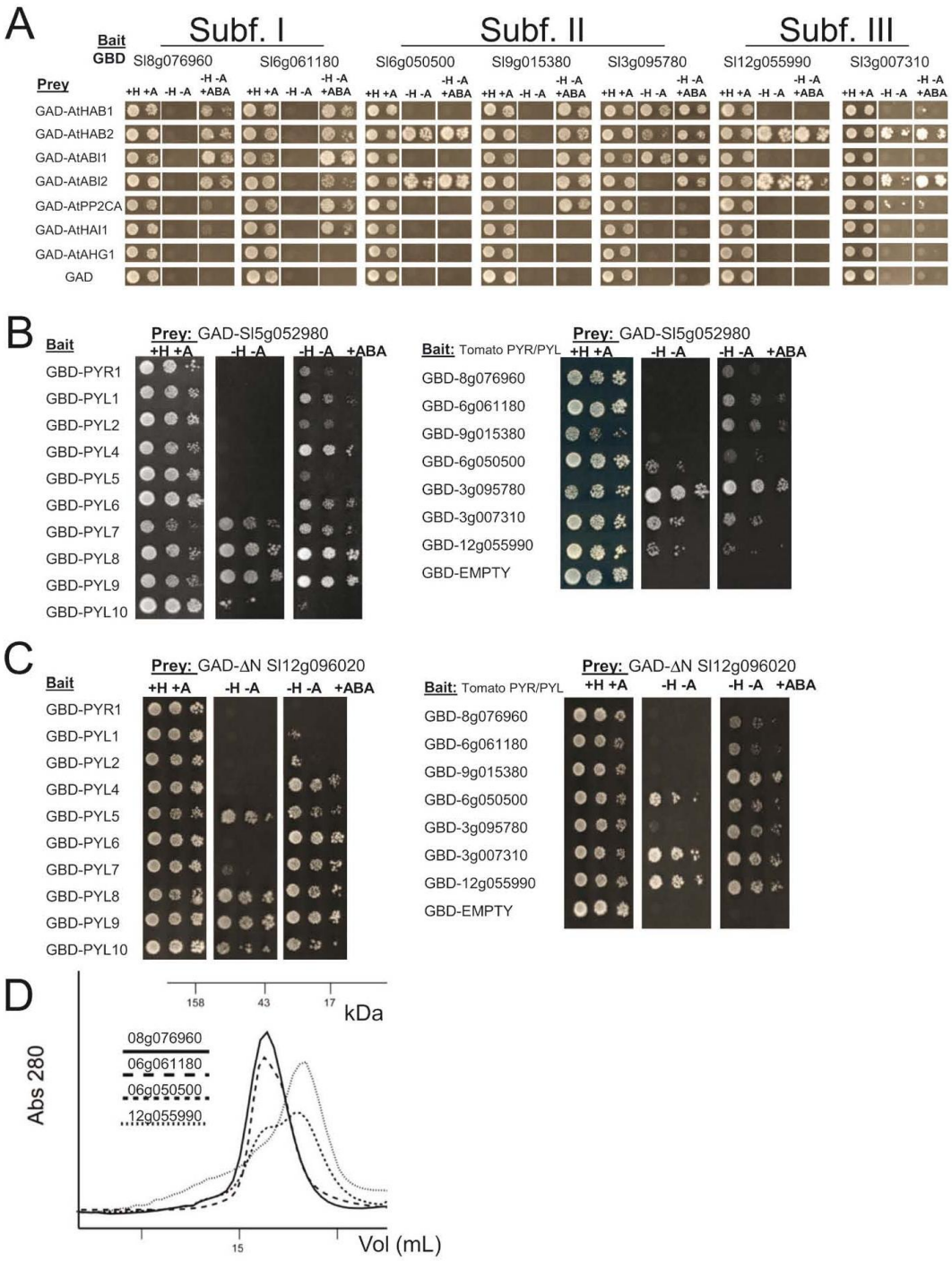

Figure 3. Interactions between PYR/PYL ABA receptors and clade A PP2Cs. Interaction was determined by growth assay on media lacking His and Adenine (-H, -A), which were supplemented or not with 50 mM ABA (+ABA). Dilutions (10^{-1}, 10^{-2} and 10^{-3}) of saturated cultures were spotted onto the plates. (A) Interaction of tomato receptors with *Arabidopsis* PP2Cs. (B) Interaction of a tomato PP2CA-like phosphatase (5g052980) and *Arabidopsis* (left) or tomato (right) PYR/PYL ABA receptors. (C) Interaction of a tomato DN HAB1-like phosphatase (12g096020) and *Arabidopsis* (left) or tomato (right) PYR/PYL ABA receptors. (D) Elution profiles after size-exclusion chromatography of four tomato ABA receptors in absence of ABA. The lines show the absorbance recorded at 280 nm. Molecular mass markers are indicated in kDa.

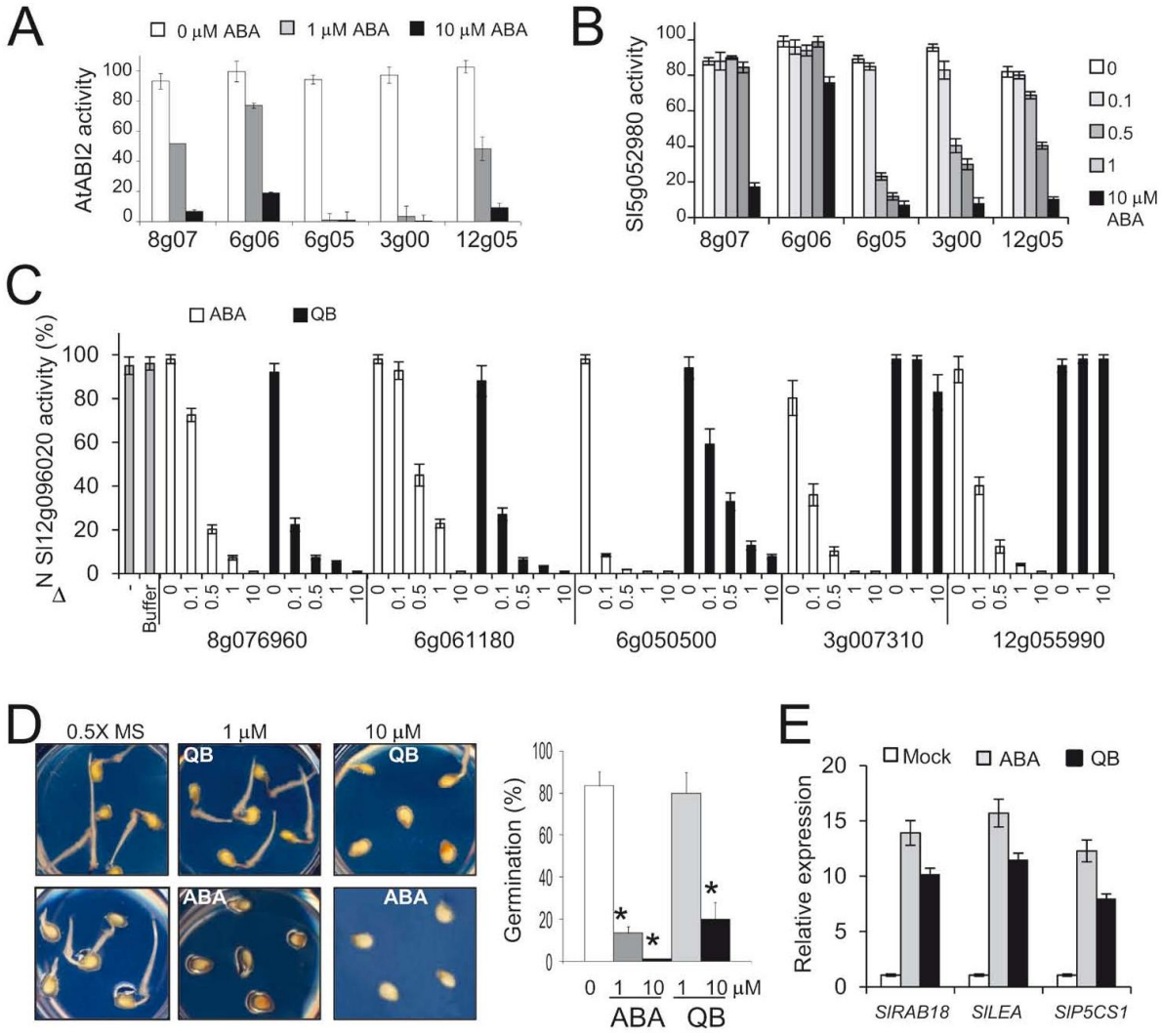

Figure 4. ABA-dependent PP2C inhibition mediated by tomato ABA receptors. PP2C activity was measured *in vitro* using a phosphopeptide substrate in the absence or presence of ABA at a ratio 1:4 phosphatase:receptor (0.5:2 μM stoichiometry). Data are averages ± SD for three independent experiments. (A) ABA-dependent inhibition of AtABI2 by tomato receptors. Values represent percentage activity compared to 100% in the absence of receptor and ABA. (B) Phosphatase activity of tomato 5g052980 in the presence of tomato receptors. (C) Phosphatase activity of tomato DN 12g096020 PP2C in the presence of tomato receptors. PP2C activity was measured in the absence or presence of 0, 0.1, 0.5, 1, or 10 μM ABA or QB. Column labelled as buffer contained an equivalent volume of His elution buffer and 0.5 % DMSO. (D) Inhibition of tomato seed germination is more sensitive to ABA than QB. Seed germination was scored 72 h after sowing. * indicates $p<0.05$ (Student´s t test) when comparing data of plates supplemented with ABA or QB to plates lacking these chemicals. (E) Quinabactin treatment induces expression of ABA- and stress-responsive genes. 10-d-old tomato seedlings were either mock- or 10 μM ABA- or QB-treated for 3 h. The histograms indicate the relative induction by ABA or QB treatment of the indicated tomato genes with respect to mock conditions (value 1).

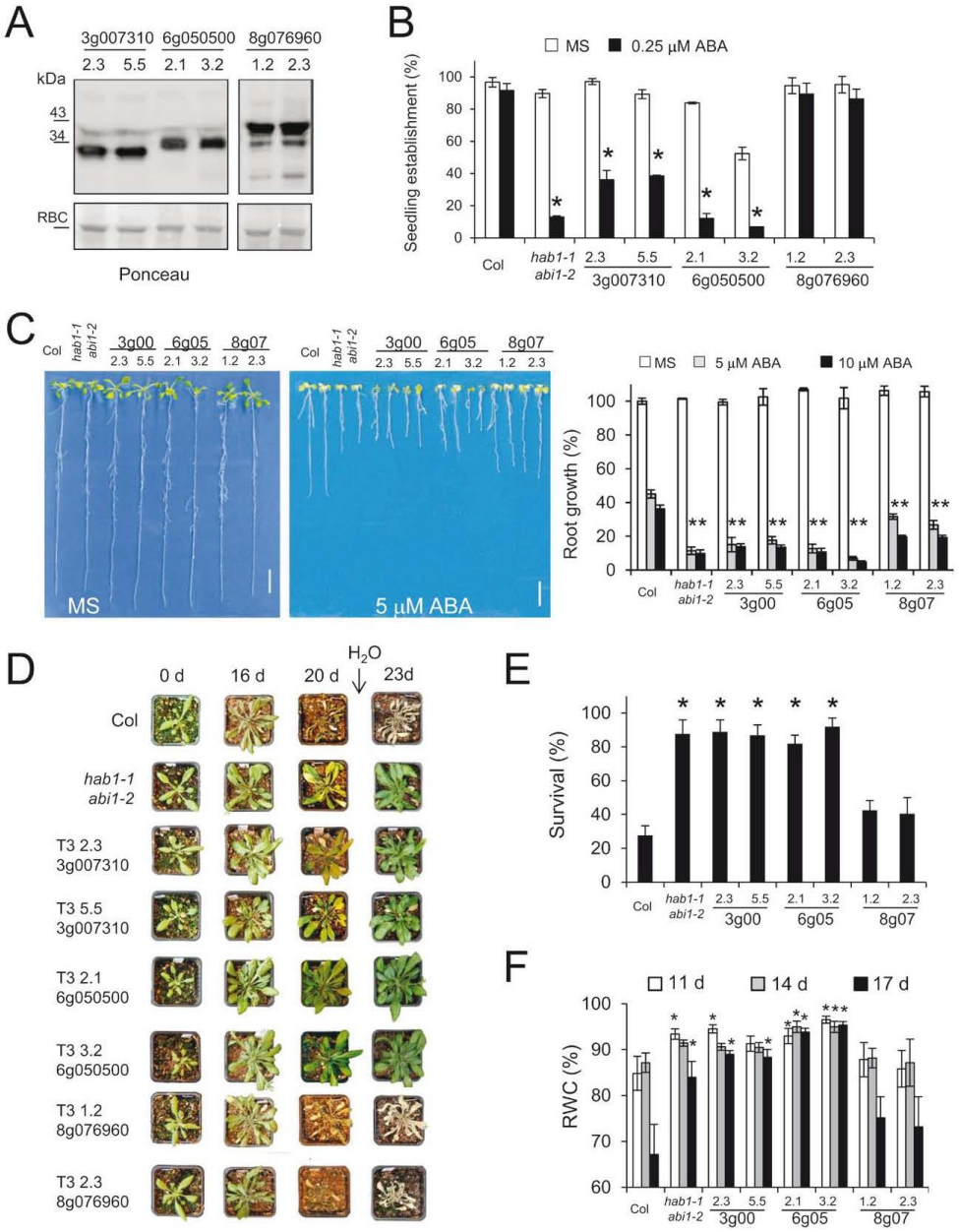

Figure 5. Overexpression of monomeric-type tomato receptors in *Arabidopsis* confers enhanced response to ABA and drought resistance. (A) Immunoblot analysis using antibody against hemagglutinin (HA) tag shows expression of tomato ABA receptors in 21-d-old seedlings (two independent *Arabidopsis* T3 transgenic lines for each tomato receptor). Ponceau staining is shown below. RBC indicates ribulose-1,5-bis-phosphate carboxylase. (B) ABA-mediated inhibition of seedling establishment in transgenic lines compared to non-transformed Col plants and *hab1-1abi1-2* double mutant. * indicates $p<0.05$ (Student´s t test) when comparing data of transgenic lines and *hab1-1abi1-2* mutant to non-transformed Col plants in the same assay conditions. Approximately 100 seeds of each genotype (three independent experiments) were sown on MS plates lacking or supplemented with 0.25 µM ABA. Seedlings were scored for the presence of both green cotyledons and the first pair of true leaves after 8 d. Values are averages ± SE. (C) Enhanced sensitivity to ABA-mediated inhibition of root growth of transgenic lines and *hab1-1abi1-2* mutant compared to non-transformed Col plants. Photographs show representative seedlings 10 d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 5 µM ABA. Right panel, quantification of ABA-mediated root growth inhibition (values are means ±SE; growth of Col wt on MS medium was taken as 100%). (D, E, F) Transgenic lines overexpressing monomeric-type receptors show enhanced drought resistance, survival and higher RWC compared to non-transformed plants. (D) Two-week-old plants were deprived of water for 20 d and then re-watered. Photographs were taken at the start of the experiment (0-d), after 16 and 20 d of drought and 3 d after re-watering. (E) Survival percentage of non-transformed Col, *hab1-1abi1-2* and transgenic lines 3 d after re-watering. (F) RWC of non-transformed Col, *hab1-1abi1-2* and transgenic lines after 11, 14 and 17 d water withdrawal.

Supplemental material

Table S1. List of oligonucleotides used in this work.

Sl12g055990
ACCATGGTGAACAATATGGAAGATGAG FNco12g055990
CTAGATTCTATCAATAGGGTCGGT R12g055990
Sl09g015380
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Sl06g050500
ACCATGGCTTCTTCCCTTCAACTGCATC FNco06g050500
TTAATCATGATCCATAGATAATTTATTAC R06g050500
Sl03g007310
ACCATGGACGCTAATGGATTCTGCGGTG FNco03g007310
TTAGACCTGATCAATGGGTTCTG R03g007310
Sl08g76960
ACCATGGATAATAAACCGGAAACGTC FNco08g76960
AAGCTTTCACCTGTGACTCGCATCACGAC RHindIII08g076960
Sl06g061180
ATGGAGCAATCCGATAACTCAAC F06g061180
TTATGAGATCTCACCGTTACCACC R06g061180
Sl03g095780
ACCATGGCTTGTTCAGTTCAGCTGCAG FNco03g095780
TCAGCGATTTGCAGTTGCGATTTG R03g095780
Sl12g096020
CGCAGTGTTTTTGAAGTGGACTAC FΔN12g096020
TTATGAGGCCAATTGTGTTGAAG R12g096020

Solyc03g096670
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CTATAGACCTCTTTTCAAATCAAC R03g096670
Solyc05g052980
ATGGCTGGAATGTGTTGTGGAGT F05g052980
TTATAGATTTTTTCTCAAATCCACC R05g052980
Solyc07g040990
ACCATGGTTGTGGGTGCTGAAGATT FNcoΔN7g040990
GTCGACTTATGTTTTCTTCTTGAATTTCCT RSalI7g040990
Solyc06g067980 (*SlLEA*)
AGCAGATGTTGGAAAAGGAGC FqPCR06g067980
ATGCCTATGGTGGGGTATGTG RqPCRF06g067980
Solyc02g084850 (*SlRAB18*)
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CACGGGACACCATAACACAC RqPCR02g08450
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AATTATTTCACCCACCTGCCC RqPCR06g019170
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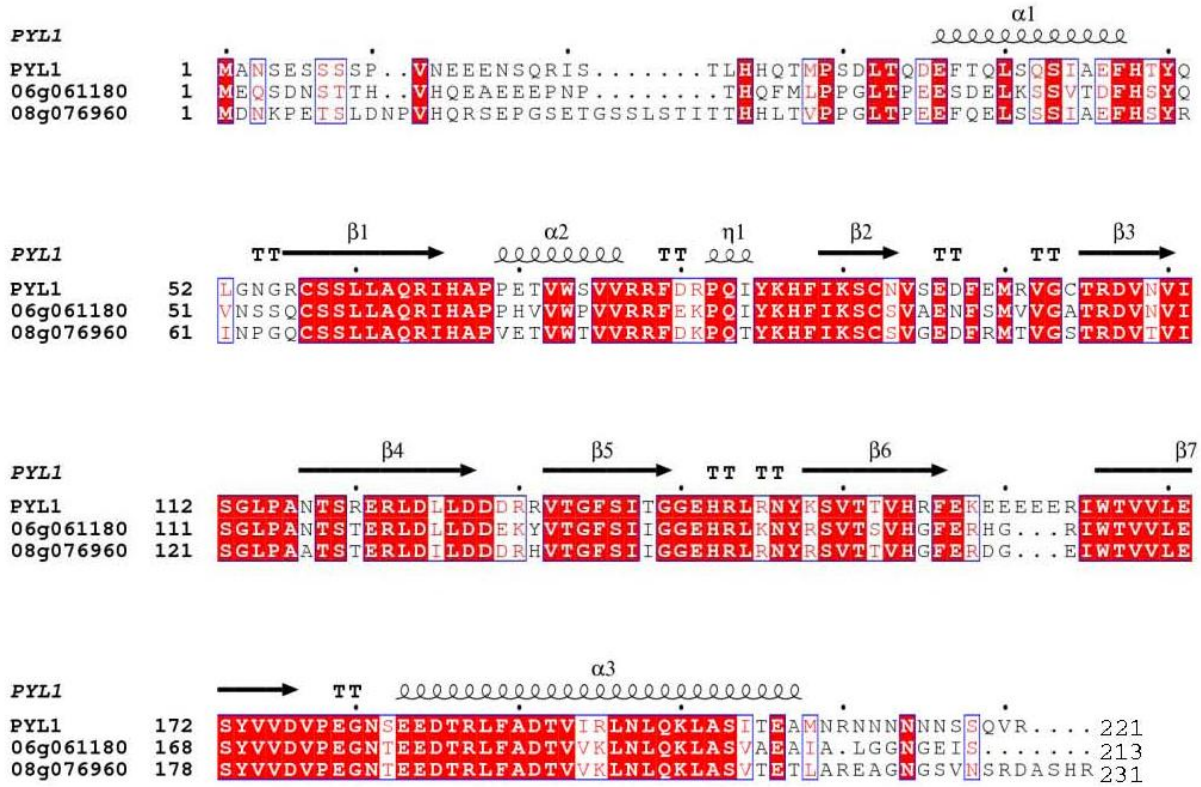

Figure S1. Amino acid sequence alignment of AtPYL1 and putative tomato orthologous ABA receptors. The predicted secondary structure of the tomato proteins was depicted taking as model the crystallographic structure of AtPYL1 (Protein DataBank Code 3KAY) and using Espript interface (http://espript.ibcp.fr/).

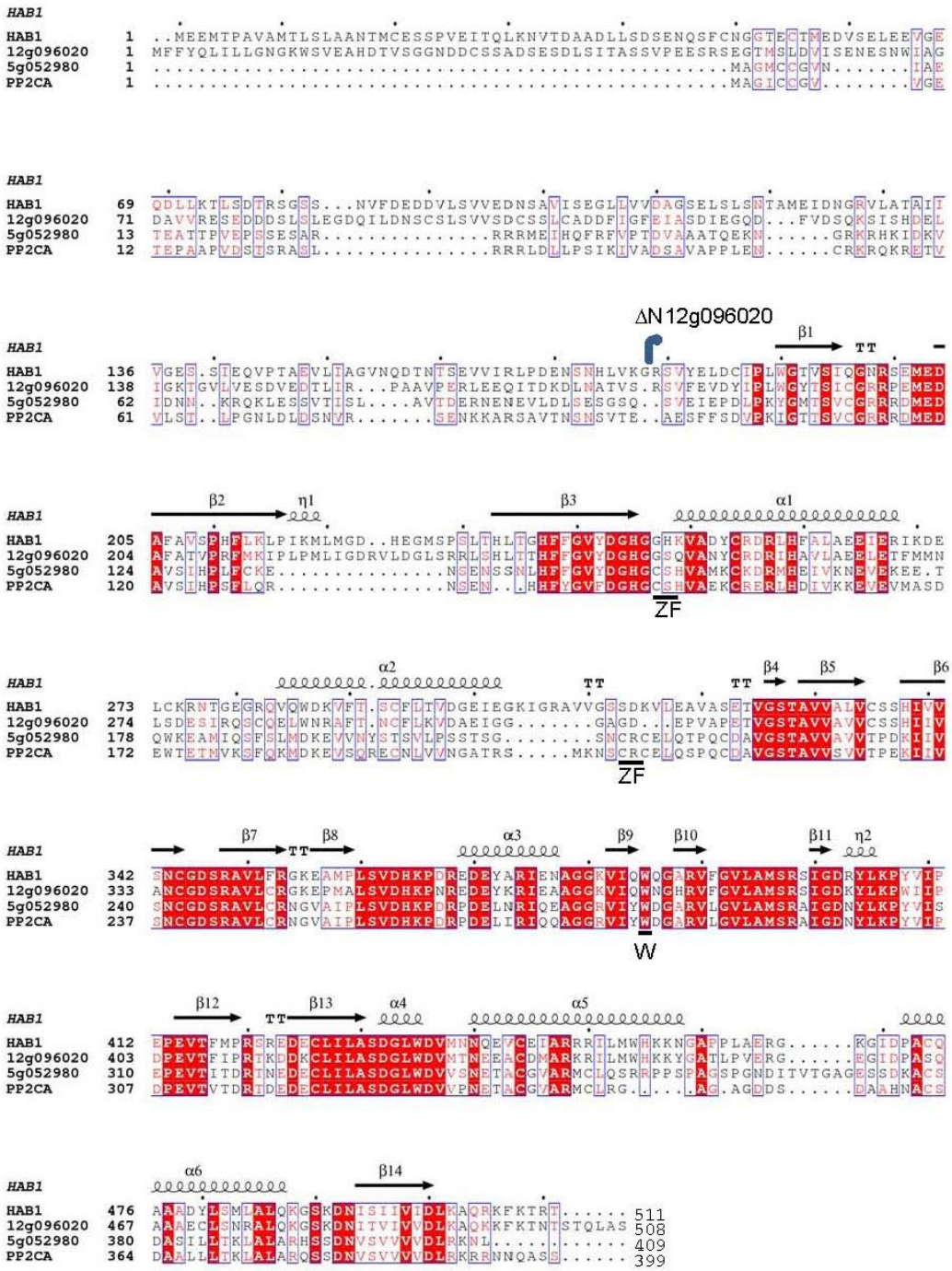

Figure S2. Amino acid sequence alignment of AtHAB1, AtPP2CA and putative tomato orthologous PP2Cs. The predicted secondary structure of the tomato proteins was depicted taking as model the crystallographic structure of AtHAB1 (Protein DataBank Code 3QN1) and using Espript interface (http://espript.ibcp.fr/). The CCCH zing-finger (ZF) motif of AtPP2CA and 5g052980 and the tryptophan (W) conserved residue of clade A PP2Cs are underlined. The start of the ΔN 12g096020 PP2C is indicated.

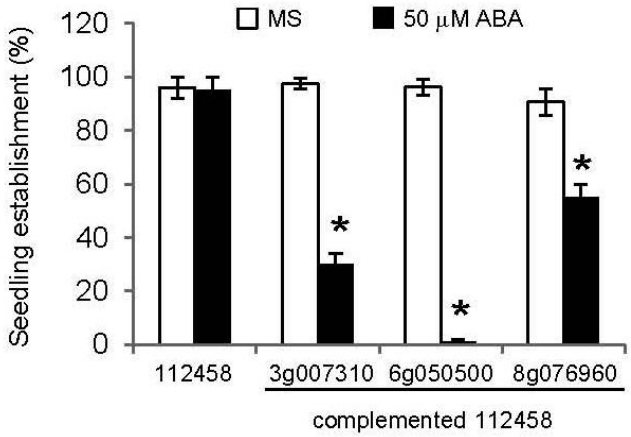

Figure S3. Complementation of the 112458 *pyr/pyl mutant by tomato PYR/PYL ABA* receptors. Expression of HA-tagged 3g007310, 6g050500 or 8g076960 complements (partially in the case of 8g076960) the ABA-insensitive phenotype of 112458 *pyr/pyl mutant, which lacks 6* major *Arabidopsis ABA receptors, i.e. PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8* (Gonzalez-Guzman et al., 2012). Approximately 100 seeds of each genotype (two independent experiments) were sown on MS plates lacking or supplemented with 50 μM ABA. Seedlings were scored for the presence of both green cotyledons and the first pair of true leaves after 8 d. Values are averages ± SE. * indicates $p<0.05$ (Student´s t test) when comparing data of 112458 mutant transformed with the indicated tomato receptor to 112458 in the same assay conditions.

10. APPENDIX III

Curriculum vitae

Name: Lesia Natacha Rodriguez Solovey
Date of birth: 6 December, 1983 (Cuba)
Citizenship: Ucranian
Languages: Spanish (primary), Russian (primary), English, French, Catalan, Ucranian
Contact: Instituto de Biologia Molecular y Celular de Plantas
Consejo Superior de Investigaciones Científicas-Univ. Politécnica
Avd de los Naranjos. Edificio CPI, 8E
ES-46022 Valencia.Spain
E-mail: leirodso@upvnet.upv.es

Education and Training

2009- Present	Ph.D in Biotechnology Instituto de Biología Molecular y Celular de Plantas (IBMCP). Universidad Politécnica de Valencia Supervisor: Dr.Pedro Luís Rodríguea Egea. Co-supervisor: Miguel González Guzman
2008-2009	Graduate Student, Department of Biochemistry and Molecular Biology, Faculty of Biology, Universidad de Valencia Supervisor: Dr. Mª Luisa Salvador Alcober
2007-2008	M.Sc., Master in research in Molecular, Cellular and Genetics Biology Faculty of Biology, Universidad de Valencia
2002-2007	B.Sc, Biochemistry, Universidad de Valencia

Research articles

Castillo MC*, Lozano-Juste J*, Gonzalez-Guzman M, **Rodriguez L**, Rodriguez PL, and León J (2015): Inactivation of PYR/PYL/RCAR ABA receptors by tyrosine nitration may enable rapid inhibition of ABA signaling by nitric oxide in plants. *Science Signaling*, 8 (392), pp.ra89

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* These authors contributed equally to this work