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C2-domain CAR proteins mediate membrane interaction of PYR/PYL/RCAR ABA receptors and regulate abscisic acid sensitivity in *Arabidopsis*

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Abstract

Membrane-delimited ABA signal transduction plays a critical role for early ABA signaling but molecular mechanisms linking core signaling components to plasma membrane are unknown. We show that transient calcium-dependent interactions of PYR/PYL receptors with membranes can be mediated through a new 10-member family of C2-domain ABA-related (CAR) proteins. Specifically, we found that PYL4 interacted in an ABA-independent manner with CAR1 both in 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 co-immunoprecipitation assays showed that PYL4-CAR1 as well as other PYR/PYL-CAR pairs interacted in plant cells. Crystal structure of CAR1? and CAR4 was solved and it revealed, in addition to a classical calcium-dependent lipid-binding C2-domain, a specific CAR signature likely responsible of the interaction with PYR/PYL receptors and their recruiting to phospholipid vesicles. This interaction is relevant for PYR/PYL function and ABA signaling since different *car* triple mutants affected in *CAR1, CAR4, CAR5* and *CAR9* genes showed reduced sensitivity to ABA in seed establishment and root growth assays. In summary, we have identified PYR/PYL-interacting partners that mediate a transient Ca^{2+}-dependent interaction with phospholipid vesicles, which affects PYR/PYL sub-cellular localization and positively regulates ABA signaling.

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 (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). 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/OST1/E, which are key players to regulate transcriptional response to ABA and stomatal aperture (Cutler et al., 2010). 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 or 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; 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 and 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 on PYR/PYL function indicates they play a major role in quantitative regulation of ABA response, 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., 2011a; 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 et al., 2005; Scott and Pawson, 2009). PYR/PYL proteins are intracellular ABA receptors localized both at the cytosol and nucleus; however, a detailed knowledge on their sub-cellular 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 et al., 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 to control ion transporters and membrane-associated enzymes that generate second messengers involved in ABA signaling (Geiger *et al.,* 2009; Lee *et al.,* 2009; Sato et al., 2009; Sirichandra et al., 2009; Geiger et al., 2011; Cutler et al., 2010); 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 ABI1 and PYL9 the association of the signaling and transport complex CPK21/SLAH3 within plasma membrane domains 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, 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 is a representative member of the PYR/PYL family, it 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 of new interacting partners of PYL4 resulted in the discovery of a novel 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, 1998). 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, as CAR proteins, that lack additional catalytic domains have also been identified in plants (Kim et al., 2003, Wang et al., 2009; Yokotani 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).

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 novel PYL4-interacting protein, At5g37740, whose binding to PYL4 was not ABA-dependent (Figure 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 (Supplemental Figure 1). Other PYL receptors, such as PYL1, PYL6 or PYL8 were also able to interact in Y2H assays with this small C2-domain protein (Figure 1A). Deletion of the N-terminal region of PYL4, PYL6 and PYL8 severely impaired the interaction with At5g37740 (Figure 1B). A similar N-terminal deletion did not affect the binding of PYR/PYL receptors to PP2Cs since this region is not involved in the formation of the receptor-ABA-phosphatase complex (Santiago et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Dupeux et al., 2011b). We named At5g37740 as CAR1 and BLAST search at TAIR revealed a CAR gene family composed by 10 members (CAR1 to CAR10) in *Arabidopsis* (Supplemental Figure 2). The CAR family was also found in other plant species such as tomato and rice (Supplemental Figure 3). *Arabidopsis* CAR proteins range between 165 to 185 amino acid residues and estimated molecular mass of 18-20 kDa (Supplemental Figure 2).

C2 domains are usually found as regulatory modules of different polypeptides that include also a catalytic domain, such as the typical PKC-C2, phosphatidylinositol 3-kinase-C2 or phospholipase A2-C2 combinations found in mammals (Zhang and Aravind, 2010). Thus, C2 domains are able to translocate to membrane compartments the associated catalytic activity in response to Ca^{2+} peaks. In *Arabidopsis*, we found combinations with different catalytic domains, such as phospholipase D, lysine decarboxilase, phosphoribosylanthranilate transferase, endonucleases, inositol 1,4,5-trisphosphate phosphatases or phospholipase C (Supplemental Figure 1). However, the *Arabidopsis* CAR family hereby identified represents a plant-specific C2 domain family of small proteins not associated to 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-YFPN* and *35S:YFPC-PYL4* constructs were delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration (Agroinfiltration) and, as a result, fluorescence was observed into the nucleus or visualized as a thin layer that could reflect plasma membrane or cytosolic localization (Figure 1C). 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 transmembrane domain that results in plasma membrane targeting (Batistic et al., 2012). Therefore, we co-expressed CAR1-YFPN, YFPC-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 (fluorescence emission spectra in the yellow range for reconstituted YFP and orange-red range for OFP). We found that Pearson-Spearman correlation coefficients indicated co-localization of OFP-TM23 and reconstituted YFP proteins, therefore a significant amount of the CAR1-PYL4 interaction was localized to plasma membrane (Figure 1D). In contrast, GFP did not show co-localization with OFP-TM23 when both proteins were co-expressed in tobacco cells. We also found that other PYR/PYLs, such as PYR1, PYL1, PYL6 and PYL8, also interacted with CAR1 in BiFC assays (Figure 1E); although we could not detect in Y2H assays the interaction of PYR1 with CAR1. 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 (Figure 1E). Although different expression levels of the YFP-tagged proteins were observed 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 1E).

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 1F). To avoid precipitation not mediated by antibody of the membrane fractions where CAR1-PYL4 interaction occurred, we used the soluble nuclear fractions where this interaction also occurs

(Figure 1C and 1E). To this end we precipitated nuclear CAR-GFP proteins using α-GFP 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 different extent (Figure 1F). The recovery of co-immunoprecipitated CAR1-GFP and HA-PYL6 was apparently higher than 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 other HA-tagged PYLs so the initial input was not comparable to them.

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 2A). 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 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 2B). 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 did 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 2A and 2B). 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 2C). First at all, we followed a fractionation technique to separate nuclei (pelleted at 1000g) from the soluble non-nuclear fraction (Figure 2D). 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. We could find 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 2E). 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 2E).

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

In order to obtain a molecular insight into CAR proteins, the X-ray structure of CAR4 in complex with Ca^{2+} was solved by molecular replacement at 1.6 Å resolution (Figure 3A, Table 1 and Methods section). We also solved the structure of CAR1, although in this case we could not obtain the complex with Ca2+ (Josan, no sé muy bien qué hacer con esto, ya lo hablamos, de poner algo irá a supplemental). 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 3A, see CAR4 topology). This insertion is conserved among the members of the CAR family (Supplemental Figure 2 and 3) 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 3A; Verdaguer et al., 1999). The 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 3A). 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 3B (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 $CAR1^{D22A\ D27A}$ and $CAR4^{D85A\ D87A}$ 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 3C). We calculated a half-maximal calcium concentration of 1.5 and 7.7 μM for in vitro phospholipid binding of CAR1 and CAR4, respectively, and a certain cooperative effect for Ca^{2+} binding (see inset showing Hill coefficient values, n_H). 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 it 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 3C).

One of the receptors that interacted well with CAR proteins in BiFC and coIP assays was PYL6 (Figure 1). Only a residual presence of PYL6 in the pellet was detected upon co-incubation with CAR1 and phospholipid vesicles lacking calcium (Figure 3D, 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 3D, lane 3). Such effect could be reversed by treating the pelleted vesicles with increasing concentrations of EGTA, a chemical acting as Ca^{2+} chelating agent (Figure 3D, 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 3E). 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 0.2-0.3 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.

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 at all, we analyzed ABA sensitivity of *35S:CAR1* lines with respect to ABA-mediated inhibition of seedling establishment and shoot growth (Figure 4). *35S:CAR1* lines showed enhanced sensitivity to ABA-mediated inhibition of seedling establishment and shoot growth compared to wt (Figure 4). Thus, establishment at 0.3-0.5 μM ABA was impaired in *35S:CAR1* lines compared to wt (Figure 4A and B). Those seedlings from *35S:CAR1* lines that were able to establish in 0.5 uM ABA showed a clear impairment of shoot growth compared to wt after 21 d growth in medium supplemented with ABA (Figure 4C). Likewise, seedlings from *35S:CAR1* lines transferred from MS plates to plates supplemented with 0.5 μM ABA showed reduced fresh weight after 21 d growth compared to Col wt (Figure 4D).

Next we identified loss-of-function knockout mutants for different members of the *CAR* family that were available at collections of T-DNA mutants. At the beginning of this work, we could identify T-DNA homozygous mutants for the loci At5g37740 (*car1*), At3g17980 (*car4*), At1g48590 (*car5*) and At1g70790 (*car9*) (Supplemental Figure 4). Since some functional redundancy might be expected among CAR proteins as it 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 5). 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 5A and 5B). 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 5C and D).

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 6). Seedling establishment of *car1car5car9* triple mutant was less sensitive to ABA-mediated inhibition than wt (Figure 6A 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 6B). Root length of the seedlings that established in

0.5 μM ABA was larger in *car1car5car9* compared to wt (Figure 6C). Interestingly, 0.5 μM ABA supplementation enhanced root length of *car1car5car9* seedlings compared to medium lacking ABA, an effect previously described in some *pyr/pyl* ABA-insensitive mutants (Figure 6C and D; Gonzalez-Guzman et al., 2012). 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 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 148 triple mutant (Figure 6E and F). Therefore, both *CAR* and *PYR/PYL* genes regulate additively root sensitivity to ABA. Lateral root growth is also depending 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 6G). Therefore, taken together these results indicate that CAR proteins regulate ABA sensitivity both in primary and lateral roots. Reporter gene analysis of *CAR1* promoter showed predominant expression of *CAR1* in the vascular bundle of the primary root as well as in the cortex of the upper part of the root (Supplemental Figure 5). In lateral roots, *CAR1* expression was also detected in epidermis and root tip (Supplemental Figure 5).

Discussion

In this work we describe a family of calcium sensors harboring a C2 domain that interact with PYR/PYL ABA receptors and mediate their approaching to 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, could be membrane-recruited in a Ca^{2+}-dependent manner (Figure 3). We have also

demonstrated that PYL4 and CAR1 interact in plasma membrane of plant cells; however, the precise membrane association between the full set of CAR and PYR/PYL proteins remains to be investigated. 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. Currently, genetic evidence obtained with combined *car* mutants supports that CAR proteins regulate at least a subset of ABA responses, so one function of CAR proteins appears to be regulation of ABA signaling. Thus, triple mutants impaired in CAR proteins show a reduced sensitivity to ABA-mediated inhibition of seedling establishment and root growth, which suggests that CAR-dependent transient interactions of ABA receptors with membranes affect ABA signaling.

Different abiotic stresses induce Ca^{2+} fluctuations that serve as a second messenger to elicit plant responses to changing environment (McAinsh and Pittman, 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 (Schapire et al., 2008; Yamazaki et al., 2008; Dodd et al., 2010). Members of the *CAR* family are transcriptionally regulated by different abiotic stresses (Kilian et al., 2007; Supplemental Figure 6) and an orthologous rice gene of the *CAR* family, *OsSMCP1*, was previously identified by conferring 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 response to abiotic stress involving calcium fluctuations. ABA signaling involves increases of intracellular [Ca^{2+}], which has been mostly studied in guard cells (Kim et al., 2010). However, it is likely that other plant tissues responsive to ABA, such as root, also involve 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 root response to abiotic stress involves increases in cytoplasmic free calcium (Kiegle et al., 2000). Osmotic and salt stress cause Ca^{2+} increases 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 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 (Figure 6).

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 function of several Ca^{2+} associated proteins such as CDPKs/CPKs, calmodulins (CaMs), calcineurin B-like (CBLs) and CBL-interacting protein kinases (CIPKs), which mediate plant response to environmental stress (Hubbard et al., 2010). Indeed, some of these components show interplay for signaling of abiotic stress and ABA (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 plasma membrane (Cherel et al., 2002; Lee et al., 2007; Lee et al., 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 it remains to be investigated the role of nuclear CAR-PYR/PYL interactions as well as the putative role of Ca^{2+} for it. Interestingly, other small C2 domain proteins described in plants also show a dual localization at 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 exclusive from the cytosol, since also exist in noncytosolic locations, such as mitochondria, chloroplast and nucleus (McAinsh and Pittman, 2009; Dodd et al., 2010). In this 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 salt stress response has

been reported in *Arabidopsis* and it is well known the role of calcium/CaM binding transcription activators (CAMTAs) to mediate abiotic stress responses (Galon et al., 2010; Guan 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 might regulate yet unidentified nuclear targets. 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 (Davletov et al., 1998; Medkova and Cho, 1998; Verdaguer et al., 1999; Frazier et al., 2003; Kohout et al., 2003) allowed us to simulate the CAR4 interaction with a phospholipid bilayer (Figure 7). 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 it 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 at membrane proximity. Interestingly, the protein face opposite to Ca^{2+}-binding loops has been previously 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 that 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 by limited deletion analysis (Figure 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 7). 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 events.

Material and methods

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}.

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 Supplemental Table1. 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). 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. 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).

Y2H assays

Protocols were similar to those described previously (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 Supplemental Table1. 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. 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/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.

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. 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. Thus, +1 value indicates co-localization, values close to +1 indicate partial co-localization and negative values indicate lack of co-localization.

Biochemical fractionation, protein extraction, analysis and immunoprecipitation

Constructs to express GFP or HA-tagged proteins were done 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 (~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 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. 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.

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.

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. $CAR1^{D22A\ D27A}$ and $CAR4^{D85A\ D87A}$ mutants were generated using the PCR-overlap extension procedure and cloned into pETM11. PYR/PYL proteins were prepared as described previously (Santiago et al., 2009).

Data Collection and Structure determination and refinement

CAR4 X-ray diffraction data was collected in a ADSC detector using the European Synchrotron Radiation Facility (Grenoble, France) radiation source at 0.94 Å wavelengths at the ID14.4 beamline. Diffraction data was processed with XDS (Kabsch, 2010) and scaled with SCALA from the CCP4 package (Collaborative Computational Project, Number 4) (Winn et al., 2011). A summary of the data-collection statistics is given in Table 1. The X-ray structure of CAR4 was solved by molecular replacement using the coordinates of the X-ray structure of the C2 domain of Munc13-C2b (Protein Data Bank code 3KWT) as the search model (Shin et al., 2010; Vagin and Isupov, 2001). The electron density map calculated using these phases was good enough to manually build and refine the residues of CAR4. Several cycles of restrained refinement with REFMAC5 (Murshudov et al., 2011) and PHENIX (Adams et al., 2010) and iterative model building with COOT (Emsley et al., 2010) were carried out. The

stereochemistry of the model was verified with PROCHECK (Laskowski et al., 1996). Ribbon figures were produced using PyMOL (http://www.pymol.org). The refinement statistics are summarized in Table 1.

Accession numbers

The coordinates and structure factor amplitudes of CAR4 in complex with calcium been deposited in the Protein Data Bank (PDB ID ****). Arabidopsis Genome Initiative (AGI) locus identifiers for *CAR1, CAR2, CAR3, CAR4, CAR5, CAR6, CAR7, CAR8, CAR9* and *CAR10* are At5g337740, At1g66360, At1g73580, At3g17980, At1g48590, At1g70800, At1g70810, At1g23140, At1g70790 and At2g01540, respectively. AGI identifiers for *PYR/PYL* genes have been published previously (Gonzalez-Guzman et al., 2012).

Supplemental data

The following materials are available in the online version of this article

Supplemental Table 1. List of oligonucleotides used in this work.

Supplemental Figure 1. Scheme of some representative proteins harboring C2 domains in *Arabidopsis*.

Supplemental Figure 2. Amino acid sequence and secondary structure alignment of *Arabidopsis* CAR proteins.

Supplemental Figure 3. Amino acid sequence alignment of representative members of the CAR family in *Arabidopsis*, tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*)

Supplemental Figure 4. Scheme of *CAR1, CAR4, CAR5 and CAR9* genes and location of the corresponding T-DNA insertion in *car* mutants.

Supplemental Figure 5. Photographs showing GUS expression driven by *ProCAR1:GUS* gene in different tissues and developmental stages.

Supplemental Figure 6. Induction of *CAR* genes by cold, osmotic- and salt-stress in shoot or root tissues.

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Author contributions (falta JAM, RA and D)

PLR conceived the project; LR, MGG, MD, AR, AA and PLR designed research; LR, MGG, MD, AR, ACI, AA and PLR performed research; LR, MGG, MD, AR, ACI, JMM, AA and PLR analyzed data; AA and PLR wrote the paper

Figure legends

Figure 1. PYL4 and other PYR/PYL receptors interact with CAR1 in Y2H assays. BiFC and coimmunoprecipitation (coIP) assays show interaction of CAR1/CAR4 and PYR/PYLs in tobacco epidermal cells. (A) ABA-independent interaction of CAR1 and different PYR/PYLs. Interaction was determined by growth assay on media lacking His and Adenine (-H, -A), which were supplemented or not with 10 mM ABA (+ABA). Dilutions (10^{-1}, 10^{-2} and 10^{-3}) of saturated cultures were spotted onto the plates. GAL4 DNA-binding domain (GBD) and GAL4 activation domain (GAD). (B) Deletion of the N-terminal region of PYL4, PYL6 and PYL8 impairs the interaction with CAR1. (C) CAR1 and PYL4 interact in the plasma membrane and nucleus of tobacco cells. Confocal images of transiently transformed tobacco epidermal cells co-expressing CAR1-YFP^N/YFP^C-PYL4 interacting proteins and the plasma membrane marker OFP-TM23 (upper panels) or GFP and OFP-TM23 (lower panels). BiFC interaction of CAR1-YFP^N and YFP^C-PYL4 was observed and this interaction co-localizes with the plasma membrane marker OFP-TM23 (see merge panel). (D) Statistical analysis of colocalization of CAR1-YFP^N/YFP^C-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/GFP and OFP fluorescence (French et al., 2008). The degree of colocalization between the two fluorescent signals was analyzed using Zeiss software. (E) BiFC assays show both nuclear and non-nuclear interactions of CAR1/CAR4 and PYR/PYLs 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 CAR and YFPC-tagged PYR/PYL proteins in tobacco epidermal cells. (F) Coimmunoprecipitation assays demonstrate the interaction of CAR1-GFP or CAR4-GFP and PYR/PYLs. Nuclear protein extracts obtained from tobacco leaves infiltrated with Agrobacterium suspensions harbouring the indicated constructs were analyzed using anti-HA or anti-GFP antibodies. Input levels of epitope-tagged proteins in crude protein extracts (20 µg of total protein) were analyzed by immunoblotting. Immunoprecipitated (IP) CAR1-GFP or 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 loading control.

Figure 2. CAR-GFP fusion proteins localize to nucleus, plasma membrane and cytosol upon transient expression in *Nicotiana benthamiana*. (A) Confocal images of transiently transformed tobacco epidermal cells co-expressing GFP, GFP-tagged CAR/PYL4 proteins or the CAR1-YFP^N/YFP^C-PYL4 interacting proteins, and the plasma membrane marker resulting of 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. (B) Pearson-Spearman correlation coefficients indicate co-localization of CAR-GFP proteins or the CAR1-YFP^N/YFP^C-PYL4 interaction 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/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. (C) Biochemical fractionation and immunoblot analyses of protein extracts prepared from tobacco 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. Position of the molecular mass standards (kDa) is indicated. (D) Scheme of the fractionation protocol. A description of the abbreviations used for nuclear fraction (N), non-nuclear soluble fraction (S), cytosolic fraction (C) and total microsomal fraction (M) is indicated. (E) Quantification of the subcellular localization of GFP and GFP-tagged proteins transiently expressed in tobacco epidermal cells. Immunoblot signals obtained in section C were captured using the image analyzer LAS3000 and quantification of the protein signal was done using Image Guache V4.0 software.

Figure 3. Calcium-dependent phospholipid-binding of CAR proteins. Ca2+ and CAR-dependent recruitment of PYR/PYLs to phospholipid vesicles. (A) Crystal structure, Ca2+ 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 a1bAbB extradomain is highlighted in orange. (B) Scheme of the biochemical assay to detect Ca2+-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. (C) CAR1, CAR4, $CAR1^{D22A\ D27A}$ and $CAR4^{D85A\ D87A}$ proteins were incubated with phospholipid vesicles in the presence of increasing concentrations of Ca2+ 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. (D) Ca2+ and CAR1-dependent recruitment of PYL6 to phospholipid vesicles. Ca2+ and CAR1-dependent vesicle pelleting of PYL6 can be reversed by EGTA-treatment. Pelleted vesicles bound to PYL6 and CAR1 were EGTA-treated, precipitated again by centrifugation and analysed by SDS-PAGE and immunoblot. (E) Ca2+-dependent vesicle pelleting assay of CAR1 and CAR4 using different PYR/PYLs. The Ca2+ 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.

Figure 4. 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 were taken 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.

Figure 5. 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 were taken 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.

Figure 6. ABA sensitivity of the *car1car5car9* triple mutant compared to *pyr1pyl4pyl8* triple mutant. Additive effect of the *car5car9pyr1pyl4pyl8* pentuple 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. (E) 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. Next, 20 plants were transferred to new MS plates lacking or supplemented with 20 mM 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. (F) The photographs show representative seedlings removed at 20 d from MS plates lacking or supplemented with 20 μM ABA and rearranged on agar plates. (G) 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 *car1car5pyr1pyl4pyl8* pentuple to *pyr1pyl4pyl8* triple mutant. Representative seedlings were removed at 10 d and rearranged on agar plates.

Figure 7. A working model is presented 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 structures of the apo PYL1 receptor (PDB code 3KAY) and its complex with ABA and ABI1 phosphatase (PDB code 3JRQ) are displayed as wheat and pale cyan ribbons respectively. The CAR4 α1β1β2 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.

Table1. Crystallographic data collection and refinement statistics

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