Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors

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The plant hormone abscisic acid (ABA) acts as a developmental signal and as an integrator of environmental cues such as drought and cold. Key players in ABA signal transduction include the type 2C protein phosphatases (PP2Cs) ABI1 and ABI2, which act by negatively regulating ABA responses. In this study, we identify interactors of ABI1 and ABI2, which have been named regulatory components of ABA receptor (RCARs). In Arabidopsis, RCARs belong to a family with 14 members that share structural similarity with class 10 pathogen-related proteins. RCAR1 was shown to bind ABA, to mediate ABA-dependent inactivation of ABI1 or ABI2 in vitro, and to antagonize PP2C action in planta. Other RCARs also mediated ABA-dependent regulation of ABI1 and ABI2, consistent with a combinatorial assembly of receptor complexes.

The phytohormone abscisic acid (ABA) serves as an endogenous messenger in biotic and abiotic stress responses (1–4). ABA responses include a redirection of gene expression, reduced transpiration, protection of photosynthesis, and control of plant growth (2, 4–6). A variety of proteins has been reported to function as ABA receptors (7–9), but their ability to bind to ABA, to transduce the ABA signal, and thereby to regulate diverse ABA responses has not been unequivocally established (10–15). Major players in ABA signaling are a subclass of Mg²⁺- and Mn²⁺-dependent serine-threonine phosphatases type 2C (PP2Cs) (16–21). Prototypes of these PP2Cs are ABI1 and its close structural homolog ABI2, which act in partially overlapping ways to repress ABA responses (22). ABI1 has emerged as a hub in the network of ABA signal transduction (23–25). The link between ABA perception and the regulation of ABI1 and ABI2 has not been elucidated.

As key regulators of ABA responses, the ABI1 and ABI2 protein phosphatases are of central importance for elucidating the integrative network of ABA signaling. We used the yeast two-hybrid system to screen for Arabidopsis proteins interacting with ABI2 (18). One of the interacting proteins (At1g01360.1) had no annotated function, and we named it regulatory component of ABA receptor 1 (RCAR1). The RCAR1 protein shares 75% and 74% amino acid identity to poplar and grape vine homologs, respectively, and its predicted structure is similar to the protein Bet v 1 of birch pollen (Fig. 1, A and B). RCAR1 belongs to a protein family with 14 members in Arabidopsis (Fig. 1C).

The interaction between RCAR1 and ABI2 (Fig. 2A) was almost completely abolished by the single–amino acid exchange present in abi2 (ABI2G180D) (26). The mutations in abi1 and abi1 (ABI1G180D) confer dominant ABA insensitivity. The single point mutation present in abi1 also impaired RCAR1 binding, and the interaction was abrogated with a carboxyl terminally truncated ABI1(1–160). There are more than 50 PP2Cs in Arabidopsis. Of these, HAB1, a PP2C involved in ABA signaling and structurally related to ABI1/2 (19), physically interacted with RCAR1 in the yeast assay. In contrast, two additional PP2Cs we...
tested, which belong to other subfamilies, did not significantly interact with RCAR1 (fig. S1). The protein interactions between RCAR1 and ABI1 or ABI2 were confirmed in plant cells by bimolecular fluorescence complementation (27).

Coexpression of RCAR1-YFPN and PP2C-YFPC in Arabidopsis protoplasts (27) yielded yellow fluorescent protein (YFP) signals both in the cytosol and in the nucleus (Fig. 2B). Fluorescence-activated cell distribution analysis of RCAR1-YFPN– and ABI1-YFPC–transfected protoplasts provides evidence for the formation of functional YFP complexes (fig. S2). Expression of a protein fusion between RCAR1 and green fluorescent protein (GFP) was localized to the same intracellular compartments as the RCAR1-PP2C complex (Fig. 2C).

In previous analyses, we consistently observed an up to 20% reduction of ABI1 phosphatase activity in the presence of micromolar levels of ABA, although no PP2C-bound ABA was detected (28). The inhibition indicated that the PP2C is to some extent capable of sensing ABA, but that an essential component that provides high affinity and stereo-selectivity for the ligand is missing. The missing constituent is RCAR1. In the presence of RCAR1, ABA instantaneously and almost fully blocked the phosphatase activity of purified ABI2 (Fig. 3A). Half-maximal inhibition of ABI1 and ABI2 occurred at ~60 nM and ~70 nM of physiologically active (S)-ABA, respectively (Fig. 3, B and C). In the absence of RCAR1, a residual PP2C inhibition of ~15 to 20% was observed at 1 to 30 μM (S)-ABA (Fig. 3C).

Fig. 1. Structural similarities between RCAR1 and the pollen allergen Bet v 1a. (A) Identical amino acid residues are shaded in gray, and predicted α-helical and β-sheet structures are highlighted in red and blue, respectively. The two RCAR1 homologs are from Populus (ABK92491) and Vitis (CA065816). (B) Superimposition of the RCAR1 primary structure (RCAR1128–7186) onto the three-dimensional structure of Bet v 1a. (C) Phylogenetic tree of the RCAR proteins of Arabidopsis with 14 members and three subfamilies (I, II, and III). Asterisks identify members of the family analyzed in the study.

Fig. 2. Physical interaction between RCAR1 and ABI1/2. (A) (Left) Yeast two-hybrid analysis with different bait variants of ABI1 and ABI2 (wild-type versus mutant). (Right) Binding of RCAR1 to the PP2Cs is seen as a stimulation of reporter activity above basal levels. The galactosidase activity is given in Miller units. (B) Interaction analysis in Arabidopsis protoplasts by bimolecular fluorescence complementation. RCAR1-YFPN was analyzed for YFP complementation with ABI1-YFPC and ABI2-YFPC (top and bottom, respectively). YFP complementation in the absence (left) and presence (right) of exogenous ABA. Administration of ABA (10 μM) did not detectably affect the interaction. Bright-field images (middle) correspond to the left images. Controls for the ABI1-RCAR1 interaction are shown in fig. S2C. (C) Confocal laser scanning microscope analysis of the GFP-RCAR1 fusion protein (green, middle), and chloroplasts (red autofluorescence, right). (Left) Bright-field pictures of the analyzed protoplasts. The arrows mark the nucleus. Scale bar, 10 μm.
The residual inhibition was also recorded in the presence of either (R)-ABA or trans-ABA and RCAR1. Supplementation of (R)-ABA and trans-ABA with (S)-ABA yielded the (S)-ABA-dependent inhibition, whereas heat-inactivation of RCAR1 abrogated ABA regulation of ABI2 (fig. S3A).

To ascertain that the ABA-stressselective inhibition of phosphatase activity by RCAR1 was not specific to the umbelliferylphosphate substrate, we also used an alternative phosphopeptide substrate and obtained comparable results (compare fig. S3A with S3B). The RCAR1-related proteins RCAR3, 8, and 12 (Fig. 1C) were able to block both PP2Cs in an ABA-dependent manner (fig. S3, C and D). In the presence of RCAR1, 30 μM (R)-ABA and trans-ABA were not able to inhibit the phosphatase activity of ABI1 and ABI2 to a level evoked by 30 nM (S)-ABA (Fig. 3, B and C).

Isothermal titration calorimetry revealed binding of (S)-ABA to RCAR1 and ABI2 with an apparent binding affinity (KD) of ~64 ± 8 nM ABA (Fig. 3D) and a single binding site (n = 1.08 ± 0.05). The analysis of binding of (S)-ABA to RCAR1 yielded lower energy changes and a higher apparent KD of ~0.66 ± 0.08 μM ABA (fig. S4, A and B).

To define the stoichiometry of the RCAR1-PP2C interaction, a fixed concentration of ABI2 was titrated with increasing levels of RCAR1 in the presence of 1 mM ABA. Half-maximal inhibition occurred at an RCAR1 to ABI2 ratio of ~0.5 (Fig. 3E). This value ranged between 0.4 and 0.8 for different protein preparations. Combined, the data are indicative of a one-to-one ratio of the heteromeric protein complex. The PP2C inhibition imposed by ABA and RCAR1 is independent of substrate concentration (fig. S5A). The Michaelis-Menten constant of the ABI2-catalyzed reaction was not affected by increasing RCAR1-enzyme ratios, whereas vmax was reduced (fig. S5B). Thus, the mode of inhibition relies on a noncompetitive inactivation of the enzyme. The Scatchard analysis of the PP2C inactivation in the presence of RCAR1 tentatively indicates a nanomolar dissociation rate for ABA with an apparent KD of ~35 and 26 nM (S)-ABA for ABI1 and ABI2, respectively (Fig. 3F and fig. S5C). The KD values are somewhat lower than the data from thermal measurements but fall within a comparable range. In the presence of saturating ABA levels, serial dilutions of an RCAR1-containing ABI2 solution maintained a constant inhibition level of up to 5 nM ABI2 (fig. S5D).

The transient expression of RCAR1 in Arabidopsis protoplasts resulted in an enhanced ABA-response at the level of gene regulation (Fig. 4A). An eightfold stimulation of ABA signaling was observed in the absence of exogenous ABA. Analyses of the ABA-deficient aba2-1 mutant supported our conclusion that RCAR1 expression activates ABA signaling in response to endogenous ABA levels (Fig. 4B). Coexpression of a construct targeting RCAR1 and related transcripts with RNA interference (RNAi) counteracted the ABA response (Fig. 4, C and D), whereas cytokinin signaling was not affected (fig. S6). The concomitant expression of PP2Cs antagonized the RCAR1- and ABA-stimulated reporter expression (Fig. 4E) in a dose-response-dependent manner, as shown for ABI2 (Fig. 4F).

Regulation of stomatal aperture, seed dormancy, and growth are major physiological control nodes of ABI1 and ABI2 action. Transgenic Arabidopsis plants with high transcript levels of RCAR1 were generated, and these plants were hypersensitive to ABA. The characterization of two independent lines, in which RCAR1 transcript levels were up-regulated (Fig. 4G), is depicted in Fig. 4, H to J. Regulation of stomatal aperture was impaired in the RCAR1 transgenic plants. In the absence of exogenous ABA, stomatal aperture ratios differed marginally between the RCAR1-2 and RCAR1-5 lines (0.42 ± 0.06) and control plants (0.45 ± 0.06) (Fig. 4H). Stomatal closure in response to ABA was enhanced in the RCAR1 overexpressors (compare an aperture of 0.05 ± 0.07 in RCAR1-2 to one of 0.33 ± 0.03 in the control line at 0.3 μM ABA, P < 0.001). Higher ABA levels (~10-fold) were required to close the stomata of the control to the extent observed at 0.3 μM ABA in the overexpressors. Similarly, the RCAR1–overexpressing lines were ABA-hypersensitive with respect to seed germination and root elongation. The median inhibitory concentration (IC50 value) for (S)-ABA–mediated inhibition of germination was shifted from 0.6 ± 0.15 μM ABA in the control to 0.2 ± 0.07 μM in the RCAR1–overexpressing line (Fig. 4I). ABA (1 μM) inhibited root growth by more than 80% in RCAR1-2 and RCAR1-5 plants, as compared with 40% in control plants (Fig. 4J). The IC50 value of (S)-ABA to inhibit root growth was 0.4 ± 0.2 μM for RCAR1-2 seedlings as compared with 3 ± 0.3 μM for the control line. RCAR1 is expressed in various parts of Arabidopsis including roots, stomata, and parenchymal cells of the vasculature (fig. S7, A to F).

In conclusion, the manipulation of RCAR1 transcript levels via RNAi or overexpression affected all facets of ABA signaling examined: gene expression, adjustment of stomatal aperture, seed germination, and vegetative growth. RCAR-related proteins from Vigna and lupine have been linked to phytohormone homeostasis (29–31), and the crystal structure analyses of the proteins revealed a cavity in the center encaged by seven β sheets and two α-helical domains. Ligands such as the steroid deoxycholate and cytokinins can be bound within the cavity and, by analogy, possibly ABA. Our analyses indicate binding of a single ABA molecule per RCAR1 and a dissociation constant in the nanomolar range for the complex of RCAR1-PP2C with ABA. The considerably lower KD value of the heteromeric protein–ABA complex argues for a ligand-induced complex stabilization similar to FLS2 and BAK1 receptor stabilization by flagellin (32). The heteromeric complex of PP2C and RCAR1 protein has the hallmark of a receptor, i.e., selective recognition and transmission of the signal, in this case, via ABA-exerted control on protein phosphatase activity. The ABA receptor complex was detected both in the cytosol and in the nucleus. There is strong evidence for a cytosolic perception site of ABA (5) and a control of ABA signaling by nuclear ABI1 (27).
Recently, two homologous G proteins, GTG1 and GTG2, have been characterized as plasma membrane-localized ABA receptors that are structurally related to a mammalian anion channel (9). Hence, ABA receptors seem to display a diversity not known for any other phytohormone. The capacity of RCAR1 and other RCAR1-like proteins to regulate ABI2 and ABI3 in an ABI-dependent manner indicates a combinatorial complexity of ABA receptors. The RCAR proteins examined are from different clades within the protein family, which supports the notion that all 14 members might represent ABA receptor components. This 14-member RCAR gene family has been independently identified by Park et al. (33), who analyzed the genes’ role in ABA signaling and named the genes PYRABACTIN RESISTANCE and PYR1-Like (PYRI and PYL1) through PYL13, respectively. Members of the RCAR family seem to interact with other P2Cs, such as HAB1, involved in ABA signaling (33). The functional knockout of RCAR1 in Arabidopsis did not reveal altered ABA responses consistent with functional redundancy among the RCAR proteins. However, multiple knockouts of RCAR1-PYR1 and other RCARs-PYLs generated an ABA-insensitive phenotype (33). The regulation of the receptor complexes for (S)-ABA at the nanomolar level is in the range of ABA levels under conditions of mild stress, which already limit transpiration (34, 35). Future studies will reveal whether a combinatorial assembly of different receptor complexes provides a mechanism to adjust the sensitivity of ABA perception for optimal gas exchange and performance.

Fig. 4. ABA responses and RCAR1. (A to F) The ABA-induced up-regulation of gene expression was monitored with the ABA-responsive reporter constructs pRD29B::LUC (A, B, and D to F) and pRAB18::LUC (C) in Arabidopsis protoplasts and was measured as relative light units (RLU/RFU; n > 3). (A) Activation of the ABA response by ectopic expression of RCAR1 (1 μg, filled squares) in comparison with the control (open circles). (B) Regulation of gene expression by the effector RCAR1 (3 μg) in the absence (white bars) and presence of 3 μM (S)-ABA (black bars). (C and D) The RCAR1-mediated stimulation of the ABA response is antagonized by coexpression of an RNAi construct (1 μg) targeting RCAR1. CRNAi: control RNAi. Control, empty vector plasmid, open circles; CRNAi, filled squares; RNAi, filled triangles. (E) The RCAR1- and ABA-stimulated reporter expression is antagonized by concomitant expression of various P2PCs (1 μg) in the absence (white bars) and presence of 3 μM (S)-ABA (black bars). (F) The RCAR1-mediated stimulation of the ABA response is antagonized by coexpression of ABI2. The levels of RCAR1 effector constructs were 0, 1, and 10 μg plasmid (open circles, filled squares, and triangles, respectively). (G) Comparative reverse transcriptase polymerase chain reaction analysis of transgenic lines RCAR1-2, RCAR1-5, and of a control line for transcripts of RCAR1 and RCAR2 (At4g01020) in the presence and absence of 3 μM (S)-ABA. (H and I) Analysis of transgenic lines overexpressing RCAR1. (H) Stomatal response of 5-day-old seedlings exposed to (S)-ABA (n > 50). (I) Seed germination after 5 days of incubation (n > 140). (J) Inhibition of root growth of the transgenic lines in the presence of (S)-ABA (3 days, n > 60). In the absence of ABA, root growth equaled 15.4 ± 1.8 mm, 15.2 ± 1.6 mm, and 14.9 ± 1.2 mm for RCAR1-2, RCAR1-5, and the control line, respectively. (G) RCAR1-2 black bar in (H) and filled circles in (I and J); RCAR1-5 (gray bar, filled squares), control line (white bar, open circles).

References and Notes

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26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
27. Materials and methods are available as supporting material on Science Online.
Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins

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Type 2C protein phosphatases (PP2Cs) are vitally involved in abscisic acid (ABA) signaling. Here, we show that a synthetic growth inhibitor called pyrabactin functions as a selective ABA agonist. Pyrabactin acts through PYRABACTIN RESISTANCE 1 (PYR1), the founding member of a family of START proteins called PYR/PYLs, which are necessary for both pyrabactin and ABA signaling in vivo. We show that ABA binds to PYR1, which in turn binds to and inhibits PP2Cs. We conclude that PYR/PYLs are ABA receptors functioning at the apex of a negative regulatory pathway that controls ABA signaling by inhibiting PP2Cs. Our results illustrate the power of the chemical genetic approach for sidestepping genetic redundancy.

Abscisic acid (ABA), identified in plants in the 1960s, is a small molecule that functions to inhibit growth and to regulate plant stress responses. Genetic analyses have identified many factors involved in ABA signaling (1), including the group A type 2C protein phosphatases (PP2Cs), which negatively regulate ABA signaling at an early step in the pathway (2), and the SNF1-related kinase 2 (SnRK2 proteins resembling receptors, which suggests that the ABA receptor(s) may be functionally redundant or may be required for viability (13). We therefore pursued a chemical genetic strategy (14), because chemicals can bypass redundancy by inducing phenotypes not revealed by single-locus mutations (15). For example, an antagonist with low selectivity can perturb the function of an entire protein family, whereas a selective agonist can illuminate the function of one member of normally redundant receptors, as we describe here with pyrabactin (3) (Fig. 1A), a synthetic seed germination inhibitor (14). The analysis of analogs revealed that pyrabactin’s activity requires its pyridyl nitrogen, because the analog apyrabactin (4) is biologically inactive (Fig. S1) (16). Further investigation of pyrabactin’s action revealed reduced sensitivity in ABA-insensitive mutants, but not ABA biosynthesis or gibberellic acid–perception mutants (Fig. S2), which suggests it is an agonist of ABA signaling that inhibits germination in response to environmental stress (17). Aside from ABA analogs, no synthetic agonists of this stress pathway are known. Microarray analyses of the ABA and pyrabactin responses of seeds and seedlings revealed that, in seeds, both compounds induce highly correlated transcriptional responses (r = 0.98; Fig. 1B; table S1). Three unrelated germination inhibitors (18) failed to induce ABA-like effects (Fig. S2), which demonstrates that an indirect germination effect

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Fig. 1. Pyrabactin is a seed-selective ABA agonist. (A) Structures of molecules described in this study. (B) ABA/ATH1 microarray comparison of pyrabactin and ABA effects on seeds and seedlings. The axes plot log2-transformed values for probe responses to pyrabactin (y axis) or ABA (x axis), relative to control samples. The Pearson correlation coefficient (r) for each comparison is shown within the graph. Probes selected for analyses were those significantly responsive to either ABA or pyrabactin. Germination-responsive transcripts were removed for seed analyses. Detailed methods are provided in (16).
ERRATUM

Reports: “Regulators of PP2C phosphatase activity function as abscisic acid sensors” by Y. Ma et al. (22 May, p. 1064; published online 30 April). The date of receipt was 27 October 2008, not the later date in the original Science Express publication. The date has been corrected both online and in print.