Supporting Information

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SI Text

Table S3 lists all primer sequences of the mutagenic and quantitative RT-PCR (qRT-PCR) primers used in this study.

SI Methods

Yeast-Based Receptor Activation Assays. Mutant pBD GAL-PYR1 clones were individually transformed into *Saccharomyces cerevisiae* strain Y190 containing pACT-HAB1 (1), which expresses a GAL4 activation domain-HAB1 fusion protein. Yeast transformants were selected for the presence of plasmids on synthetic dextrose agar plates lacking Leu and Trp and examined for PP2C interactions using X-Gal staining to monitor β-Gal reporter gene expression levels. Individual clones were arrayed into 96-well plates and then spotted onto synthetic dextrose agar lacking Leu and Trp lawn (i.e., one-well) plates. Each assay plate contained 95 mutant clones and 1 wild-type PYR1 control clone. Plates were stained by the chloroform overlay X-Gal method after 2 d of incubation at 30 °C. Each assay plate was tested at least three times, and the activating mutations shown in Fig. 1 were observed as X-Gal$^+$ in all three separate experiments.

In Vitro Receptor Activation Assays. Full-length ABI1 and ABI2 were cloned into a modified pSUMO vector (LifeSensors, Inc.) yielding 6× His-SUMO fusion proteins; GST-HAB1 was expressed and purified as previously described (1). Mutant receptors were cloned into pET28, yielding 6× HIS-fusion proteins and expressed in BL21 (DE3) pLysS. Recombinant proteins were prepared as follows: 1 mL of an overnight culture was inoculated into 200 mL of Terrific Broth (for receptors) or 200 mL of LB (for PP2Cs). The cultures were preincubated for 2 h at 30 °C, and for PP2C expression, media were supplemented with 4 mM $MnCl_2$ 1 h after inoculation. After preincubation, isopropyl-β-D-thiogalactopyranoside was added (1 mM) and cells were induced at 15 °C for 16 h, after which they were collected by centrifugation, resuspended in 5 mL of buffer A (50 mM NaH_2PO_4, 300 mM NaCl) plus 10 mM imidazole (pH 8.0), and stored at −80 °C. For purification, cells were thawed and sonicated on ice (60 s); a cleared lysate was then applied to a 1-mL bed volume column of Ni-NTA (Qiagen) and washed with 20 column volumes of buffer A plus 30 mM imidazole and bound protein eluted with 1 mL of buffer A plus 250 mM imidazole. For receptors, the eluate was dialyzed against TBS, and for PP2Cs, fusion proteins were desalted by passage over a Sephadex G50 column equilibrated and eluted with 1× TBS; 10 mM $MnCl_2$ was added to peak PP2C-containing fractions to improve stability.

The purified receptors and phosphatases were used in PP2C assay reactions containing 600 nM PP2C and either 0, 600, 1,200, 2,400, or 4,800 nM receptor and 33 mM Tris·acetate (pH 7.9), 66 mM potassium acetate, 0.1% BSA, 10 mM $MnCl_2$, 0.1% β-ME, and 50 mM pNPP. Immediately after mixing proteins and substrates, reactions were monitored for hydrolysis of pNPP at A_{405} and specific activities were determined. The PP2C activity values shown in the figures are expressed as percentage of control phosphatase activity levels, as measured under identical reaction conditions in the absence of receptor protein. The average specific activity levels of GST-HAB1, 6× His-SUMO-ABI1, and 6× His-SUMO-ABI2 used in our experiments were ∼4,500 (GST-PP2C) or ∼2,500 (SUMO-PP2C) μmol·min·mg when assayed using the phosphatase substrate pNPP in the absence of PYR1 or other receptors.

Quantitative RT-PCR. Wild-type or transgenic lines were imbibed for 32 h in either water or 5 μM abscisic acid (ABA) at room temperature under continuous illumination, after which RNA was isolated using Concert Plant RNA Reagent (Invitrogen), followed by $LiCl_2$ precipitation, and DNase was treated using RNase-free DNase (Ambion). Purified RNA was used in qRT-PCR reactions utilizing primers for the ABA-regulated genes *Em6* (At2g40170), *LEA* (At2g21490), and *Rd29b* (At5g52300). Biological triplicate and triple technical replicate measurements were conducted. For qRT-PCR analyses of gene expression, cDNA was generated from 5 μg of total RNA using superscript reverse transcriptase II (Invitrogen) in a reaction mixture containing an oligo-dT_{20} and a ribosomal RNA primer. Real-time quantitative PCR analysis was performed by ΔΔCt method of relative quantification. PCR mixtures contained 2 μL of cDNA, 7.5 μL of 2× Maxima SYBR green/Fluorescein qPCR master mix (Fermentas), and 330 nM each gene-specific primer in a final reaction volume of 15 μL. The RT-PCR assays were done using the BioRad CFX96 Real-Time PCR System, and data were processed using BioRad CFX Manager software. PCR assays were performed under the following cycling conditions: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 55 °C, and 30 s at 72 °C in 96-well optical reaction plates (BioRad). The specificity of amplicons was verified by melting curve (disassociation) analysis (60–95 °C) after 40 cycles. Imbibed seed input cDNA was normalized using rRNA primers, whereas green seed and leaf samples were normalized by PEX4 (AT5G25760). Primer sequences are listed in Table S3.

Western Blot Analyses. Proteins were extracted in TBS buffer [10 mM Tris·HCl (pH 7.4) 150 mM NaCl] supplemented with 1% protease inhibitor mixture (Sigma), separated by 10% acrylamide (wt/vol) SDS/PAGE, blotted onto nitrocellulose membranes, and probed with monoclonal anti-GFP (Clontech) or anti–α-Tubulin (Sigma) antibody at 1:10,000 dilution. Anti-mouse–HRP (1:10,000) was the secondary antibody, and ECL (GE Healthcare) was used for immunoreactive protein detection.

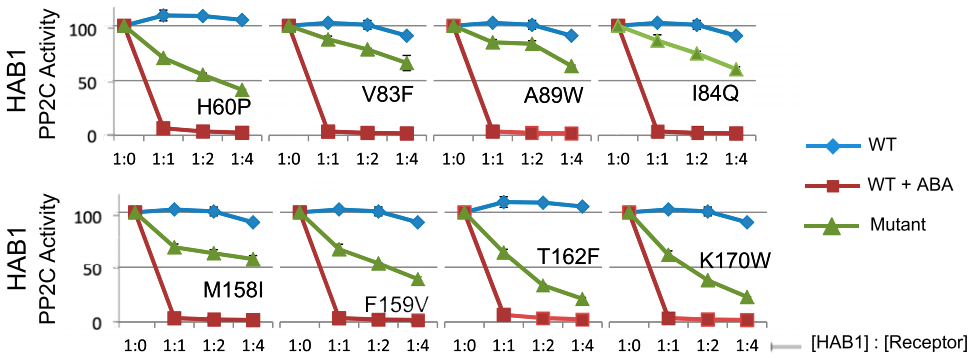

Fig. S1. Mutations identified by site-saturation mutagenesis increase Pyrabactin resistance-1's (PYR1) basal activity. Recombinant wild-type 6× His-PYR1 or mutants were prepared as described in *Methods* and assessed in in vitro type 2C protein phosphatases (PP2C) assays using 600 nM GST-HAB1 and varying concentrations of receptor (0, 600-, 1,200-, 2,400-, or 4,800-nM receptor). PP2C activity is expressed as percentage of control, (i.e., activity of PP2C in the absence of receptor and ABA but otherwise identical reaction conditions). For comparison with the degree of activation elicited by ABA on wild-type receptor, each graph shows wild-type PYR1 reactions run with either 0 μM (blue) or 10 μM (red) ABA; mutant proteins are shown in green and were assayed in the absence of ABA. Mutant proteins were analyzed in two separate experiments (V83F, A89W, I84Q, M158I, F159V, and K170W, H60P, and T162F), with each experimental set containing one wild-type control. Graphs plot average values from three technical replicates, and error bars show 1 SD.

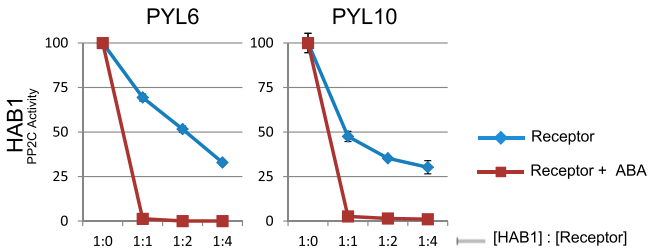

Fig. S2. PYR1-like (PYL)6 and PYL10 do not possess full constitutive activity. Recombinant wild-type 6× His-PYL6 or 6× His-PYL10 was prepared as described in *Methods* and assessed in in vitro PP2C assays using 600 nM GST-HAB1 and varying concentrations of receptor (0, 600-, 1,200-, 2,400-, or 4,800-nM receptor). PP2C activity is expressed as percentage of control (i.e., activity of PP2C in the absence of receptor and ABA but otherwise identical reaction conditions). Graphs show reactions run with either 0 μM (blue) or 10 μM (red) ABA.

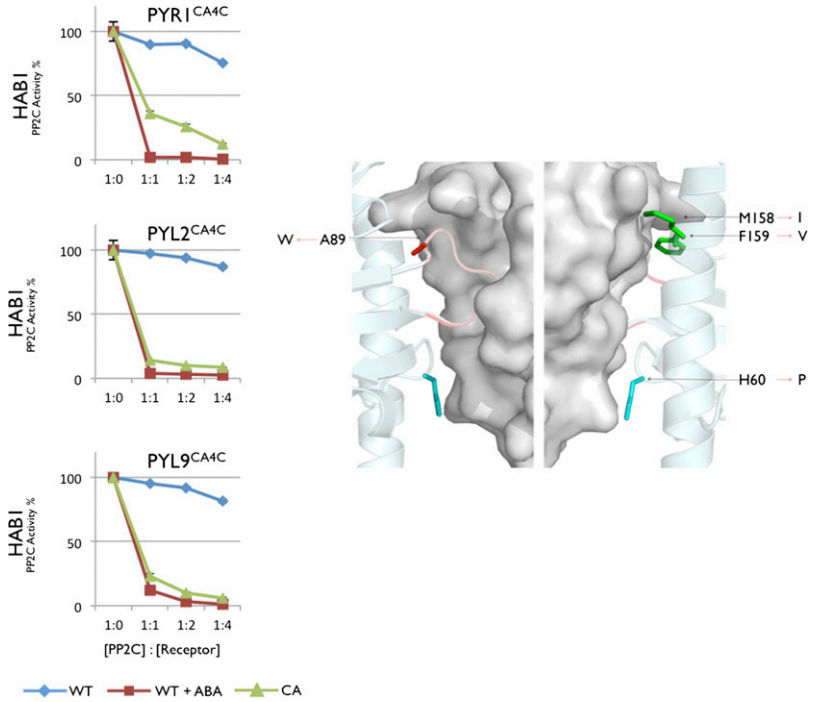

Fig. S3. CA4C mutations activate multiple receptors. Recombinant wild-type or mutant 6× His receptors were prepared as described in *Methods* and assessed in in vitro PP2C assays using 600 nM GST-HAB1 and varying concentrations of receptor (0, 600-, 1,200-, 2,400-, or 4,800-nM receptor). PP2C activity is expressed as percentage of control (i.e., activity of PP2C in the absence of receptor and ABA but otherwise identical reaction conditions). For comparison with the degree of activation elicited by ABA on wild-type receptor, each graph shows wild-type PYR1 reactions run with either 0 μM (blue) or 10 μM (red) ABA; mutant proteins are shown in green and were assayed in the absence of ABA. Values plotted in graphs are the average of three independent measurements, and error bars show 1 SD.

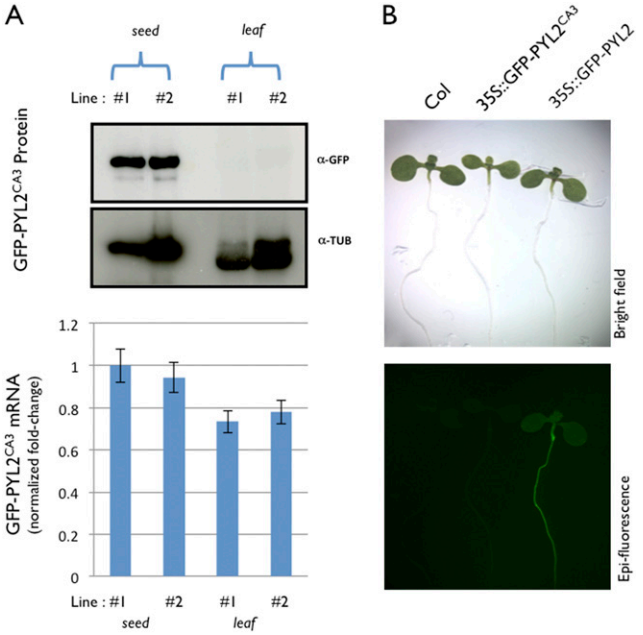

Fig. S4. GFP-PYL2^{CA3} RNA, but not protein, accumulates in vegetative leaf tissue. (*A*) Protein or total RNA was isolated from two independent 35S::GFP-PYL2^{CA3} transgenic lines from either green seeds isolated from stage 17B siliques [according to the method of Ferrándiz et al. (1)] or leaves of 3-wk-old plants. Western blot analyses were performed on 15 μg of SDS/PAGE-separated total proteins probed with either a 1:10,000 dilution of a GFP monoclonal antibody (Clontech) or a 1:10,000 dilution of an α-Tubulin antibody (Sigma), followed by a 1:10,000 secondary HRP-conjugated anti-mouse IgG antibody (Sigma), and detected by ECL (GE Healthcare). *GFP-PYL2^{CA3}* transcript levels were measured by qRT-PCR, performed and analyzed as described in *Methods* using GFP primers. The fold induction plotted was calculated with respect to mRNA levels measured in seeds of *35S::GFP-PYL2^{CA3}* line 1. The graphs plot average values from four technical replicates, and error bars show 1 SD. (*B*) Epifluorescence and bright-field images of seedlings of the wild-type Columbia (Col), *35S::GFP-PYL2*, or *35S::GFP-PYL2^{CA3}* (line 1) GFP fluorescence are markedly lower in *35S::GFP-PYL2^{CA3}* relative to *35S::GFP-PYL2*.

1. Ferrándiz, Pelaz S, Yanofsky MF (1999) Control of carpel and fruit development in *Arabidopsis*. *Annu Rev Biochem* 68:321–354.

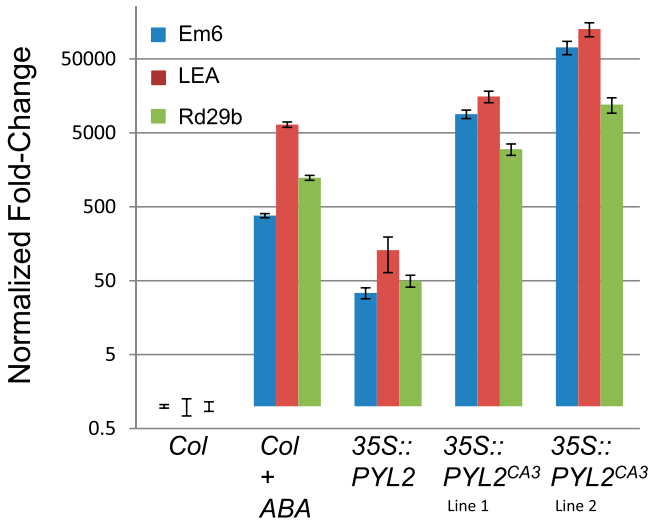

Fig. S5. Expression of PYL2^{CA3} elevates levels of ABA-regulated mRNAs. Seeds of the wild-type Columbia (Col), 35S::GFP-PYL2, or two independent 35S::GFP-PYL2^{CA3} lines were imbibed for 32 h at room temperature; RNA was prepared and qRT-PCR reactions were performed using primers for the Em6 (blue), LEA (red), or Rd29b (green) as described in *Methods*; and wild-type Columbia seeds were treated with either 0 or 5 μM ABA. The fold-induction plotted was calculated with respect to wild-type expression (arbitrarily set to a value of 1 using BioRad CFX Manager software); note that the *y* axis is displayed in log-scale. Graphs plot average values from three technical replicates, and error bars show 1 SD; the same trends in gene expression were seen in three separate biological replicates.

Table S1. Summary of site-saturation mutagenesis results

Table S1 (DOCX)

Shown are the sites mutated in this study and the mutations isolated as positive in the yeast two-hybrid assay, as described in the main text.

Table S2. Summary of receptor activation levels for wild-type and mutant receptors characterized in this study

Table S2 (DOCX)

Recombinant wild-type or mutant 6× His receptors were prepared as described in *Methods* and assessed in in vitro PP2C assays using 600 nM GST-HAB1, 600 nM 6× His-Sumo-ABI1, or 600 nM 6× His-Sumo-ABI2 and either 0 or 600 nM receptor. PP2C activity is expressed as percentage of control (i.e., activity of PP2C in the absence of receptor and ABA but otherwise identical reaction conditions). To enable comparison with the degree of activation elicited by ABA on wild-type receptors, the data in the upper part of the table summarize wild-type receptor (PYR1, PYL2, PYL6, PYL9, and PYL10) reactions run with either 0 μM or 10 μM ABA. In the table, homologous mutations are indicated in a column and an X indicates the presence of the mutation. The red type indicates the names assigned to particular mutant proteins; these names are used in the main body of the text, i.e. "CA4C."

Table S3. Summary of mutagenic and qRT-PCR primers used in this study

Table S3 (DOCX)