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 kinases), which are positive regulators (3–5). Several (+)-ABA (1) binding proteins have been reported (6–8) (Fig. 1A), however, their roles are not fully understood, and they do not appear to work via the genetically defined signaling pathway (9) or bind to the nonnatural but bioactive stereoisomer (-)-ABA (2) (10–12). The genetic dissection of ABA perception has not identified 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...
is not sufficient to account for pyrabactin’s agonist activity in seeds. In contrast to seed responses, seedling ABA and pyrabactin responses show poorer correlation ($r = 0.72$) (Fig. 1C), and few ABA-responsive genes significantly respond to pyrabactin (table S1). Thus, pyrabactin affects some, but not all, of the pathways regulated by ABA and is therefore a selective agonist.

Selective agonists have a long history as reagents for receptor identification, so we used pyrabactin for genetic dissection and isolated 12 PYRABACTIN RESISTANCE 1 (Pyr1) mutant alleles. Pyr1, isolated by map-based cloning, encodes a member of the cyclase subfamily of the START domain superfamily. START proteins share a conserved hydrophobic ligand-binding pocket (19-21). There are 13 genes in the Arabidopsis genome having marked similarity to Pyr1 (fig. S1), which we have named Pyl1 to Pyl13 (for PYR1-Like). This 14-member gene family (fig. 4B) has been independently identified as ABI1-interacting proteins (called “regulatory component of ABA receptor,” RCAR1 through RCAR14) by Ma et al. (22), and their role in ABA signaling is characterized there. The pyr1 mutant alleles we isolated are predicted to produce a variety of defects in PYR1 (fig. S3). Gene expression databases (23-27) show that Pyr and Pyl mRNAs are expressed at high levels in seeds and guard cells and respond to ABA (Fig. 2A), which is consistent with a role in ABA signaling. However, all of the pyr1 mutants isolated, including putative null alleles, reduce pyrabactin, but not ABA, sensitivity. To examine if functional redundancy might obscure a role for Pyr1 in ABA signaling, we isolated Pyl1, Pyl2, and Pyl4 insertion alleles and constructed multilocus mutants. Triple (pyr1;pyl1;pyl4) and quadruple (pyr1;pyl1;pyl2;pyl4) mutant lines display strong ABA insensitivity (Fig. 2B), which can be reversed by introducing Pyr1- or PYL4-expressing transgenes (fig. S3). The quadruple mutant is less sensitive to (+)-ABA as measured by seed germination, root growth, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 2C), and SnRK2 kinase assays (Fig. 2D and fig. S4). Collectively, our genetic data show that Pyr1 and Pyls are necessary for multiple ABA responses in vivo and illustrate the differential behavior that natural and synthetic agonists can display in genetic screens.

Because PyR1 is predicted to be a ligand-binding START protein necessary for pyrabactin activity, we hypothesized that pyrabactin might

Fig. 2. Pyr/Pyls are necessary for ABA signaling. (A) Pyr1 and Pyl1 to Pyl4 expression levels. Plots were made with the eFP browser (24); these heat maps show normalized ATH1 microarray expression values (divided by 100) according to the color scales shown; the right color scale is for the guard cell data only. (B) Genes for Pyr/Pyl act redundantly in ABA signaling. Genotypes shown were germinated on medium containing 0.9 M ABA (mixed stereoisomers) in (A) and (D), 10-μM (+)-ABA in (B) and (C), and 100-μM ABA (mixed stereoisomers) in (B) and (C).

Fig. 3. PYR/PYLS bind to group A PP2Cs in response to ABA. (A) Wild-type and mutant PYR1-PP2C interactions. PYR1 and three different pyrabactin-insensitive substitution mutants were constructed as binding-domain (BD) fusion proteins and were tested for their interactions with activation domain (AD)-fused HAB1 with the yeast two-hybrid assay by using the compounds shown at top (top panel). In the two bottom panels, AD fusions of HAB1, ABI1, ABI2, or ABI2G168D were tested for ABA- and pyrabactin-induced interactions with BD-PYR1. (B) ABA promotes PYR1 to PP2C interactions in planta. Total protein extracts (input) were made from N. benthamiana leaves transformed with the indicated constructs and/or treatments, immunoprecipitated with antibody against HA-agarose, and immunodetected with antibodies against green fluorescent protein (GFP) or HA. YFP-PP2Cs migrate at ~100 kD and HA-PYR1 at ~25 kD. (C) ABA-orfeome analysis of ABI1 interactions. Shown are subsets of an ABA-orfeome queried with ABI1; autoactivators are circled and ABI1-dependent interactors are indicated with arrows. (D) Reconstitution of ABA responses in vitro. Pull-down assays using glutathione S-transferase (GST)-HAB1 (-80 kD) and His6-tagged PYR1 (~25 kD) were conducted with purified recombinant proteins. GST-ABI1 and ABI2 tests were done with protein in crude lysates. Pyrabactin (10 μM, PyrA) was used in (A), 10 μM (+)-ABA was used in (A) and (D), and 100 μM ABA (mixed stereoisomers) in (B) and (C).
promote a protein-protein interaction between PYR1 and a downstream effector. To test this, ~2 million prey cDNA clones were screened against PYR1 yeast two-hybrid bait on medium containing pyrabactin. This revealed that the PP2C HAB1 (28, 29) interacts with PYR1 in response to pyrabactin and (+)-ABA, but not the inactive analog apyra-bactin or several other plant hormones (Fig. 3A and fig. S4). HAB1 resides in the group A subfamily of plant PP2Cs, which contains nine partially redundant members that negatively regulate ABA signaling (30, 31). To examine the relevance of the yeast two-hybrid data, we next performed a series of control experiments. First, we investigated the effects of defective PYR1 and PP2C amino acid substitution mutants on ABA-responsive interactions in the yeast two-hybrid assay (32). PYR1S152L and PYR1P88S, isolated because they cause pyrabactin insensitivity in planta, reduce ABA-induced PYR1-PP2C interactions (Fig. 3A); similarly, the dominant ABA-insensitive ABI2G168D mutant (Fig. 3B) disrupts the PYR1-ABI2 interaction. Second, we investigated in planta interactions by coexpressing a panel of yellow fluorescent protein (YFP)-tagged PP2Cs and haemagglutinin (HA)-tagged PYR1 constructs in N. benthamiana. Coimmuno-precipitation experiments performed on ABA or mock-treated plants recapitulated the PYR1-PP2C interactions observed in yeast and also showed that PYR1 does not interact with ABI1G168D, encoded by abi1-1 (Fig. 3C). Third, we used PYR1 and AB1 as bait in a yeast two-hybrid screen against a set of 258 ABA-responsive open reading frames and observed that interactions between PYR1 and group A PP2Cs occur only in the presence of ABA (Fig. 3D and table S2). Fourth, we successfully reconstituted the ABA-induced PYR1-PP2C interactions in vitro using recombinant proteins (Fig. 3E). Thus, we conclude that ABA promotes a biologically meaningful interaction between PYR1 and group A PP2Cs.

Given the redundancy observed in our genetic analyses, it is likely that other PYR/PYLs interact with PP2Cs in response to ABA. We therefore used the yeast two-hybrid assay to explore interactions of HAB1 and PP2CA (AHG3) (ABA-hypersensitive germination gene 3) with a panel of 12 PYR/PYLs, which shows that (+)-ABA promotes interactions between PYR1, PYL1 to PYL4, and HAB1 (Fig. 4A). We next used this PYR/PYL panel to examine ligand response selectivities, which shows that these five (+)-ABA-responsive PYR/PYLs do not all bind HAB1 in response to nonnatural agonists. For example, PYL2, PYL3, and PYL4 respond to both (+)-ABA and (-)-ABA (Fig. 4A), which makes these proteins candidates for the dual-stereoisomer receptors predicted by earlier studies (12). Consistent with this hypothesis, our quadruple mutant has greatly reduced (-)-ABA sensitivity (fig. S3). Ligand-selective interactions are also observed for pyrabactin, which promotes interactions between HAB1 and PYR1 and PYL1, PYL1, or PYL3 (Fig. 4A). Of these, only PYr1 is highly transcribed in seeds (Fig. 2B), which likely explains why mutations in Pyr1 cause the seeds to be insensitive to pyrabactin. Last, we observe that PYL12 interacts with PP2CA (AHG3) in response to ABA (fig. S4). Thus, at least 6 of the 14 PYR/PYLs confer ABA responsiveness to yeast. We hypothesize that the entire PYR/PYL gene family participates in ABA-promoted interactions, as these six genes are distributed across the PYR/PYL phylogenetic tree (Fig. 4B). The specificity with which pyr/pyl genes control which ligands trigger ABA signaling suggests that the ABA pathway may be dissected using selective PYR/PYL agonists.

Given the role of PYR/PYLs in controlling ligand responses, we explored whether (+)-ABA binds PYR1 by using 15N-labeled PYR1 and PYR1P88S in heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) experiments, which probe chemical shifts of protein amide-NH bonds in response to ligands (33). Because START proteins contain a conserved ligand-binding cavity (34), binding should selectively perturb residues lining this cavity. Addition of (+)-ABA altered the HSQC signals for many PYR1 and PYR1P88S residues (Fig. 4C and figs. SS and S6), which showed that ABA binds PYR1 and likely induces a conformational change. We also investigated the PYR1-HAB1 interaction in the presence of ABA, because our NMR experiments showed that PYR1P88S is not defective in ABA binding. Addition of unlabeled HAB1 caused peak broadening for PYR1, but not PYR1P88S HSQC signals (fig. S7), which localized the PYR1P88S defect to binding HAB1 after ABA perception. Because PP2Cs are negative regulators of ABA signaling, we hypothesized that ABA-promoted PYR/PYL–PP2C interactions would inhibit phosphatase activity. To test this, we examined the effects of (+)-ABA on PP2C enzyme

Fig. 4. PYR1 is an ABA-binding protein that regulates PP2C activity. (A) PYR/PYL proteins determine selectivities for responses to different ligands. A panel of PYR/PYL genes were constructed as BD fusions and tested in yeast for interactions with HAB1 and AHG3 in response to (+)-ABA, (-)-ABA, pyrabactin, or apyra-bactin (all 10 μM), dimethyl sulfoxide (DMSO) (carrier solvent, 1%). Shown are results for five PYR/PYLs that interact with HAB1 in response to ABA. (B) ABA response activity is distributed throughout the PYR/PYL family. Shown is a neighbor-joining tree of the PYR/PYL family. (Middle) Ligand selectivity data derived from yeast two-hybrid experiments. (+)-ABA–responsive PYR/PYLs are colored red, Arabidopsis Genome Initiative (AGI) annotations are shown at right; PYL9 colored red, on the basis of data from Ma et al. (22). (C) ABA binds to PYR1 and PYR1P88S. Shown are subregions of HSQC spectra for 15N-labeled PYR1 and PYR1P88S in response to increasing amounts of ABA. Arrows indicate amide protons whose chemical environments shift in response to ABA. (D) PYR1 inhibits PP2C2 activity in the presence of ABA. Initial reaction velocities of recombinant GST-HAB1 were tested in the presence of PYR1 or PYR1P88S and differing ABA concentrations using the colorimetric substrate p-nitrophenyl phosphate (pNPP). The measured IC50 values are 125 nM for PYR1 and 50 μM for PYR1P88S. (E) Hypothesized model for PYR1 control of ABA signaling. We propose the following model: in the absence of ABA (left), PYR/PYL proteins are not bound to PP2Cs, and therefore, PP2C activity is high, which prevents phosphorylation and activation of SnRK2s and downstream factors (DFs). In the presence of ABA, PYR/PYLs bind and inhibit PP2Cs. This allows accumulation of phosphorylated downstream factors and ABA transcriptional responses. The regulation of SnRK2s by PYR/PYLs may be indirect or may involve other factors.
kinetics using recombinant HAB1, PYR1, or PYR1\(^{1988}\). These experiments show that (+)-ABA acts as a potent and saturable inhibitor of phos- 
phatase activity in the presence of PYR1 [median inhibitory concentration (IC\(_{50}\) = 125 nM], but not PYR1\(^{1988}\) (IC\(_{50}\) = 50 μM) (Fig. 4D and fig. S8). Similarly, ABA displays saturable inhibition of 
HAB1 PP2C activity in the presence of recombinant 
PYR1-4 (fig. S8). Thus, PYR/PYLs regulate 
PP2Cs in response to ABA, which defines an 
unprecedented mechanism for ligand-mediated regu-
lation of PP2C activity.

Collectively, we have shown that PYR1 binds 
(+)-ABA, PYR/PYLs bind to and inhibit PP2Cs in 
response to (+)-ABA, and PYR/PYLs control 
which ligands trigger PP2C interactions. We 
conclude that the PYR/PYLs are a family of ABA 
receptors. However, the precise site of ABA 
binding remains unclear, because the ABA-binding 
site may be shared with the PP2C. Discriminat-
ning between these receptor and co-receptor 
models will require structural studies of cocystalized 
PYR/PYLs, PP2Cs, and ligands. Note that the 
PYR/PYLs interact directly with PP2Cs, which 
are core components of the ABA signaling path-
way. Because SnRK2 activity is decreased in the 
PYR/PYL quadruple mutant, we propose a hypo-
thesical model (Fig. 4D) for ABA action in which 
ABA and PYR/PYLs inhibit PP2Cs, which in turn 
releases repression of positive factors, such as the 
SnRK2s. Consistent with this model, we observed 
interaction of SnRK2.2 with PP2CA (AHG3), 
AHG1, and ABI1 when we used the yeast two-
hybrid assay (fig. S4). This suggested that the low 
SnRK2 activity observed in the PYR/PYL quadrupl-
le mutant may be a direct consequence of PP2C-
SnRK2 interactions. Understanding of the role of 
PP2Cs in ABA signaling has been complicated by 
observations from abi1-1 and abi2-1 mutants. Their 
dominant phenotypes suggest that they en-
code hypermorphic proteins (35), but they paradox-
ically reduce, but do not abolish, PP2C activity 
(36). Our data show that these mutants do not bind 
PYR1 in response to ABA. We therefore hypo-
thesize that ABA normally lowers wild-type PP2C 
activity through PYR/PYL proteins, but abi 
PP2Cs escape this and disrupt signaling because of their 
residual activity. Consistent with this model, a sec-
ond site mutation that abolishes abi1-1’s catalytic 
activity suppresses its dominant ABA-insensitive 
phenotype (36). 

The redundancy in the Pyr/Pyl gene family, 
typical of many plant genes, has kept these genes 
from emerging as factors necessary for ABA 
response. We leveraged pyrabactin’s selectivity 
for a subset of the PYR/PYL family to bypass the 
genetic redundancy that masks ABA phenotypes 
in single mutants. Thus, our results demonstrate 
the power of synthetic molecules to expose phe-
notypes for otherwise redundant genes.

References and Notes

16. Materials and methods are available as supporting material on Science Online.
22. Y. Ma et al., Science 324, 1064 (2009); published online 30 April 2009 (10.1126/science.1172408).
32. 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.

Understanding the Spreading Patterns of Mobile Phone Viruses

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We modeled the mobility of mobile phone users in order to study the fundamental spreading patterns that characterize a mobile virus outbreak. We find that although Bluetooth viruses can reach all susceptible handsets with time, they spread slowly because of human mobility, offering ample opportunities to deploy antiviral software. In contrast, viruses using multimedia messaging services could infect all users in hours, but currently a phase transition on the underlying call graph limits them to only a small fraction of the susceptible users. These results explain the lack of a major mobile virus outbreak so far and predict that once a mobile operating system’s market share reaches the phase transition point, viruses will pose a serious threat to mobile communications.

Lacking a standardized operating system, traditional cellphones have been relatively immune to viruses. Smart phones, however, can share programs and data with each other, representing a fertile ground for virus writers (1–4). Indeed, since 2004 more than 420 smart phone viruses have been identified (2,3), the newer ones having reached a state of sophistication that took computer viruses about two decades to achieve (2). Although smart phones currently represent less than 5% of the mobile market, given their reported fast annual growth rate (4) they are poised to become the dominant communication device in the near future, raising the possibility of virus breakouts that could overshadow the disruption caused by traditional computer viruses (5).

References and Notes

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