ACS chemical biology

Letters

Cite This: ACS Chem. Biol. 2019, 14, 332−336

pubs.acs.org/acschemicalbiology

Defining and Exploiting Hypersensitivity Hotspots to Facilitate Abscisic Acid Agonist Optimization

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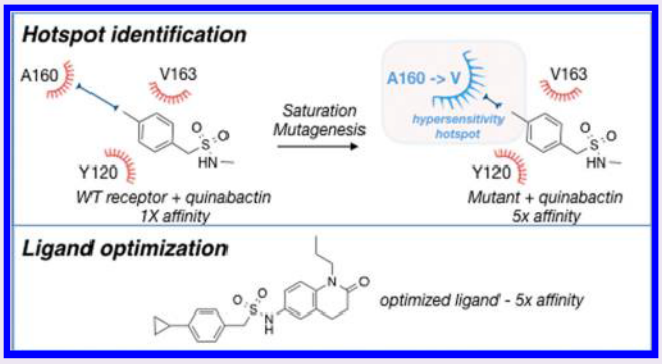
Supporting Information

ABSTRACT: Pyrabactin resistance 1 (PYR1) and related abscisic acid (ABA) receptors are new targets for manipulating plant drought tolerance. Here, we identify and use PYR1 hypersensitive mutants to define ligand binding hotspots and show that these can guide improvements in agonist potency. One hotspot residue defined, A160, is part of a pocket that is occupied by ABA's C6 methyl or by the toluyl methyl of the synthetic agonist quinabactin (QB). A series of QB analogues substituted at the toluyl position were synthesized and provide up to 10-fold gain in activity *in vitro*. Furthermore, we demonstrate that hypersensitive receptors can be used to improve the sensitivity of a previously described mammalian cell ABA-regulated transcriptional circuit by three orders of magnitude. Collectively, our data show that the systematic mapping of hypersensitivity sites in a ligand-binding pocket can help guide ligand optimization and tune the sensitivity of engineered receptors.



Abscisic acid (ABA) is a plant hormone that regulates many plant developmental processes and abiotic stress responses and is particularly important for mediating responses to water stress.[1,2] The receptors of ABA can hence be leveraged for "water banking" which has in turn promoted interest in the design of synthetic ABA mimics to control plant water use.[3−5] ABA acts through a core pathway that is highly conserved across land plants.[6] Biochemically, ABA's key role is to regulate the activity of a family of stress activated kinases in the AMPK superfamily called SnRK2s (for sucrose non-fermenting 1-related subfamily 2) via a negative regulatory pathway. It does so by binding to a family of soluble receptors that are members of the START (STeroidogenesis Acute Regulatory protein-related lipid Transferase) superfamily that share a conserved α-helix-β-grip architecture that forms a small globular protein with a central ligand-binding cavity.[7] ABA binding stabilizes closure of a mobile "gate" loop adjacent to the ligand binding pocket. This in turn creates an interaction surface that enables the ABA-bound receptor to dock into the active sites of most of the members of Clade A subfamily of type 2C phophatases (PP2Cs) and inhibit their enzymatic activity through a competitive mechanism.[8] The PP2Cs contain a loop with a central, highly conserved tryptophan residue that inserts between the gate and latch loops and makes a water-mediated H-bond to ABA's ketone. PP2C binding encloses the ligand binding cavity, which reduces ABA dissociation rates relative to the receptor in isolation;[9,10] *in vitro*, this is reflected by a >10-fold reduction in K_d for receptors in the presence of a PP2C.[11] The PP2Cs target the SnRK2s, dephosphorylating a critical activation loop serine and thus repress SnRK2 activity, and conversely, ABA-mediated PP2C inhibition removes this negative control of kinase activity and pathway activation.

There has been active work toward the development of ABA receptor agonists, in particular small molecules that target *Pyrabactin Resistance 1* (*PYR1*) and its homologues, given their potential to manage crop water productivity.[5,12−14] Lead optimization can be facilitated by structure-guided design and in depth structure activity relationship studies (SAR), which define critical receptor−ligand interactions. Alternately, lead optimization can also be guided by identifying target binding hotspots, which are defined as regions within a protein that contribute disproportionately to binding energy of a ligand. Hotspots can be identified by computational approaches such

Received: October 25, 2018
Accepted: January 22, 2019
Published: January 22, 2019

DOI: 10.1021/acschembio.8b00955
ACS Chem. Biol. 2019, 14, 332−336

as alanine scanning or directly through SAR by NMR approaches using fragment screening.[15] In practice, alanine screening identifies only a small subset of hotspots suitable for ligand binding. We reasoned that direct mutational scanning might complement the discovery of binding hotspots and have tested this using ABA receptors. We specifically hypothesized that mutations that increase ligand binding affinity—hypersensitivity mutations—could be useful for identifying hotspots valuable for ligand optimization.

RESULTS AND DISCUSSION

Hypersensitive Mutations Define the C6-Cleft as a Site for Tuning Receptor Sensitivity. To define sites that can be mutated to increase PYR1's ABA affinity, we exploited a library of previously constructed saturating mutations that possesses all possible mutations in PP2C-binding and ABA-binding residues.[16] We used a subset of 456 mutations in 24 ligand-proximal residues (i.e., within 5 Å of ligands or ligand-bound waters across multiple structures) and screened the collection for mutants with increased ABA sensitivity using an established yeast two hybrid assay that reports ABA-induced binding of PYR1 to the PP2C HAB1. This effort identified 18 mutations in 5 binding pocket residues (F61, V81, I110, E141, and A160) that improve ABA sensitivity in the Y2H assay (Figure 1A, Figure S1). To investigate if the observed increase

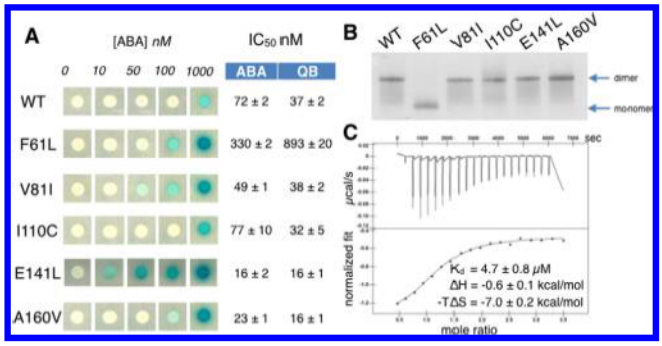


Figure 1. (A) Yeast two hybrid and in vitro assays of wild type and hypersensitive mutation ABA responses, all proteins used following tag cleavage (a data set for all mutants identified is shown in Figure S1). The left panel shows ABA-promoted binding of PYR1 (binding domain fusion) to HAB1 using β-galactosidase assay with x-gal. IC50 values report 50% inhibition values for inhibition of HAB1 phosphatase activity as inferred from dose−response curve, as described in the Supporting Information. (B) Native gel electrophoresis of wild type and mutant receptor oligomeric state (cathode at top) supporting gel filtration data is shown in Figure S2C. ITC of ABA binding reactions with the hypersensitive mutant PYR1 A160 V.

in sensitivity reflected *bona fide* alterations in ABA affinity, we produced recombinant proteins for representative mutants and measured their sensitivity to ABA using receptor-mediated PP2C inhibition; these data show that all mutants tested, with the exceptions of F61L and I110C, are indeed hypersensitive to ABA *in vitro* (Figure 1A).

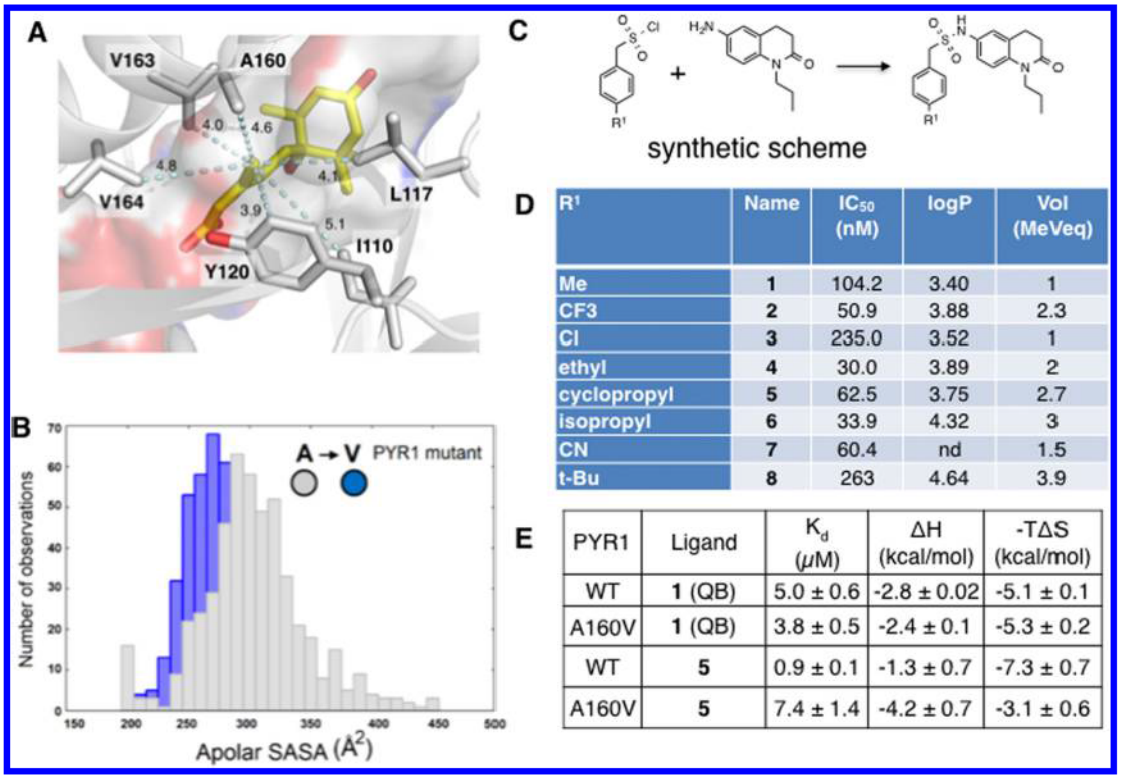
PYR1 is a homodimer that exists in an autoinhibited state because its homodimer interface that involves many residues that are also required for PP2C binding. ABA binding promotes PYR1 monomerization and PP2C binding,[17] which increases ABA affinity by reducing K_{off}. Thus, mutants that disrupt PYR1 homodimerization may be hypersensitive *in vivo* without altering PYR1's intrinsic ABA binding affinity.[17] We therefore investigated the oligomeric state of representative hypersensitive mutants using a combination of native gel electrophoresis and gel filtration. These experiments showed that all mutants except F61L are stable homodimers (Figure 1B and Figure S2). Furthermore, we used ITC to examine the binding affinity of ABA to the hypersensitive mutant A160 V and observe that PYR1 A160 V possesses an increased affinity (K_d 4.7 μM, Figure 1C) in comparison to wild type PYR1, which has a K_d that has been previously estimated to be >50 μM.[17] We therefore conclude that the A160 V and the other hypersensitive mutations isolated (excluding mutations at F61 and I110) are likely to increase ABA binding affinity.

ABA receptors are encoded by a large gene family with three subtypes that are highly conserved across angiosperms. PYR1 belongs to subfamily III, whose receptors possess, in general, lower ABA binding affinity than subfamily I and II receptors.[18] Two of the hypersensitive residues defined by our screen (I110 and A160) differ in sequence between receptor subtypes[19] (Figure S3), and prior work has demonstrated that sequence differences at these sites contribute to differences in both ABA sensitivity and agonist selectivity between receptor subtypes.[20−22] The other two hypersensitivity sites that we defined (V81 and E141) are invariant across receptor subtypes (Figure S3) and would not be predicted to alter sensitivity based on evolutionary considerations alone. Saturation mutagenesis can therefore be useful for defining hotspot residues that do not naturally contribute to differences in agonist sensitivity between receptor subtypes and/or that are highly conserved.

A160 is located at the top of a hydrophobic binding pocket that favorably accommodates ABA's C6 methyl and quinabactin's toluyl methyl and is delimited by the residues I110, V117, Y120, V163, and V164; we refer to this pocket as the C6-cleft (Figure 2A). The A160 V mutation recapitulates naturally occurring variation present in many higher affinity receptors (Figure S3), which together with our hypersensitivity mutant data suggests that the C6-cleft is a binding hotspot that might be exploited to tune agonist affinity. We additionally chose to focus our efforts on this region because of the synthetic ease with which modifications to quinabactin's toluyl substituent could be made and tested. To better understand the mechanistic basis for A160 V's effect, we created a homology model for PYR1 A160 V based on a wild type PYR1−HAB1−ABA ternary complex (Figure 2; PDB ID: 3QN1), and this model was consistent with the simple hypothesis that the bulkier Val residue in A160 V favors ABA binding through increased hydrophobic contacts. We also compared the solvent accessible surface area in WT and A160 V receptors to gain insight into the druggability of the two binding pockets. Because pocket accessibility is dependent on receptor flexibility, we performed molecular dynamics (MD) simulations and analyzed the time dependent variation in the apolar solvent accessible surface area (SASA) of the ABA-bound WT and mutant A160 V pockets. These dynamics simulations revealed a shift toward reduced apolar SASA in the A160 V binding pocket (Figure 2B), consistent with the notion that increased hydrophobic contacts stabilize the interaction; the reduced SASA may also favor binding by decreasing local flexibility of neighboring residues in the binding pocket.[23]

On the basis of the combination of our molecular dynamics and hypersensitivity data, we hypothesized that we might increase agonist potency by increasing the size of the p-toluyl substituent of quinabactin, as its toluyl methyl overlaps with ABA's C6 methyl in crystal structures.[12,13] We therefore

DOI: 10.1021/acschembio.8b00955
ACS Chem. Biol. 2019, 14, 332−336



R¹	Name	IC50 (nM)	logP	Vol (MeVeq)
Me	1	104.2	3.40	1
CF3	2	50.9	3.88	2.3
Cl	3	235.0	3.52	1
ethyl	4	30.0	3.89	2
cyclopropyl	5	62.5	3.75	2.7
isopropyl	6	33.9	4.32	3
CN	7	60.4	nd	1.5
t-Bu	8	263	4.64	3.9

PYR1	Ligand	K_d (μM)	ΔH (kcal/mol)	-TΔS (kcal/mol)
WT	1 (QB)	5.0 ± 0.6	-2.8 ± 0.02	-5.1 ± 0.1
A160V	1 (QB)	3.8 ± 0.5	-2.4 ± 0.1	-5.3 ± 0.2
WT	5	0.9 ± 0.1	-1.3 ± 0.7	-7.3 ± 0.7
A160V	5	7.4 ± 1.4	-4.2 ± 0.7	-3.1 ± 0.6

Figure 2. (A) C6-cleft. (B) Pocket accessibility investigation performed using molecular dynamics simulations (binned data). (C) Synthetic scheme for the synthesis of quinabactin analogues; full details are provided in the Supporting Information. (D) IC50 values for agonist mediated inhibition of HAB1, using recombinant SUMO-PYR1 (uncleaved tag); logP and Vol were determined as described in the Supporting Information. (E) Summary of ITC data for QB and cyclopropyl-QB (5) with wild type PYR1 or A160 V mutant receptors.

synthesized a series of analogues by coupling para-substituted benzylsulfonyl chlorides with 6-amino-1-propyl-1,2,3,4-tetarhydroquinolin-2-one building block (Figure 2C) and tested the resultant ligands for ABA receptor agonist activity *in vitro* using established receptor-mediated PP2C inhibition assays against PYR1. These data suggest that the best increases in affinity are realized with small alkyl/cycloalkyl substituents that explore this lipophilic pocket with an optimal volume of 2 to 3 methyl equivalents and enable favorable contacts between analogues and A160 V in PYR1. Thus, occupying the C6-cleft with hydrophobic substituents mimics the increased affinity observed between ABA and mutant A160 V receptor. We selected the cyclopropyl-QB analogue (5) for downstream biological characterization as its strained three-membered ring does not markedly alter logP (Figure 2D) but is also likely to be more stable than simple alkyl substituents, while enabling the exploration of the PYR1 binding cavity.

We next used ITC to characterize interactions of QB and ligand 5, the cyclopropyl version of QB with wild type and A160 V mutant receptors. These data reveal that 5, cyclopropyl-QB possesses a higher affinity for wild type PYR1, with an apparent K_d of 0.9 μM, almost 5-fold lower than QB itself, with binding primarily driven by entropy (ΔH = −1.3; $-T\Delta S$ = −7.3 kcal/mol; Figure 2E) resulting from additional hydrophobic contacts within the C6-cleft. Conversely, the PYR1 A160 V mutant, which presents steric clash for binding to 5, results in 7-fold decrease in dissociation constant for ligand 5. To further rationalize this, we modeled cyclopropyl-QB in the A160 V pocket, which suggests that steric clash explains the upper size limit for substituents. Consistent with this observation, the bulky tBu-QB analogue (compound 8) is less active on the wild type PYR1 receptor (Figure 2D).

We next investigated if this improved *in vitro* activity of toluyl-modified quinabactin analogues could be translated to the whole plant level by evaluating the evapotranspiration of soybean plants treated with quinabactin (1) or cyclopropyl-QB (5). Due to the relatively high cost and effort of conducting whole plant assays, we chose a single new compound, cyclopropyl-quinabactin (5), for analysis because cyclopropyl substitutions are generally considered to increase the metabolic stability of probe molecules.[24] The water use of the plants was then followed by regular weighing over three days. The evapotranspiration of the treated plants was reduced compared to the control plants, presumably as a result of stomata closure induced by these ABA agonists (Table S1). The effect was stronger for cyclopropyl-QB on the second and third day. This is in agreement with the *in vitro* experiments, although an increase of metabolic stability as compared to QB cannot be ruled out.

Applications to an ABA Induction System. To further explore the utility of the hypersensitive interactions, we investigated if the mutants could be used to improve the sensitivity of an engineered transcriptional circuit where ABA-induced dimerization of PYL1 and ABI1 (close relatives of PYR1 and HAB1) brings fused Gal4 DNA binding and VP16 activation domains into proximity and drives transcription.[25] This system is useful for precise control of gene expression in mammalian cells because it spans several hundred-fold in dynamic range of activation, can be applied orthogonally to other induction systems, and lacks toxicity from ABA. However, it does possess relatively low ABA sensitivity when measured in a 293T luciferase reporter system (EC_{50} = 36.5 ±

DOI: 10.1021/acschembio.8b00955
ACS Chem. Biol. 2019, 14, 332−336

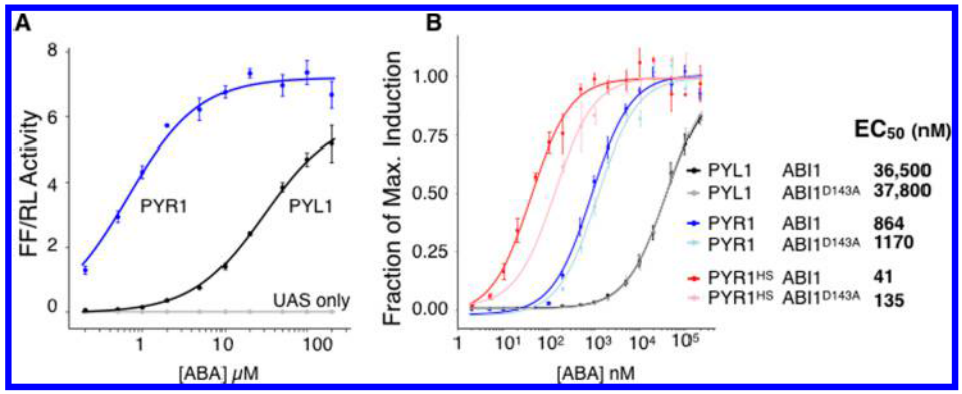


Figure 3. (A) Activation of a 6xGal4-UAS firefly (FF) luciferase reporter by PYL1- or PYR1-VP16AD association with ABI-Gal4DBD as a function of ABA concentration. FF luciferase activity was normalized to cotransfected Renilla luciferase (RL) controls. Replicates measured in parallel on the same day are plotted (n = 3). (B) Activation of luciferase activity by PYL1, PYR1, and PYR1^{HS} constructs together with WT or D143A ABI1, expressed as a fraction of maximal activation for each construct. Error bars represent standard error of the mean.

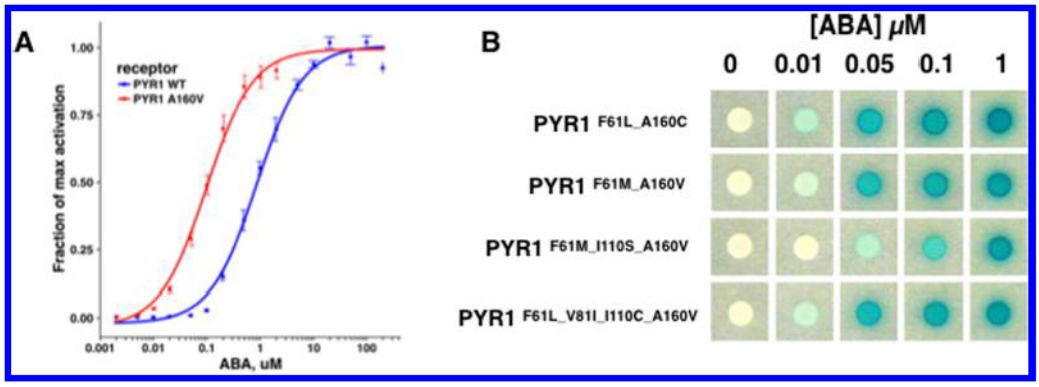


Figure 4. (A) ABA dose response curves for WT and A160 V PYR1 receptors. Activation of a 6xGal4-UAS FF luciferase reporter by PYL1- or PYR1-VP16AD association with ABI-Gal4DBD as a function of ABA concentration and normalized to fraction of maximal activation. (B) Yeast two hybrid assays of wild type and mutant receptors on different concentrations of ABA.

4 μM; Figure 3A). Because our hypersensitive mutants were isolated using PYR1, we first modified the ABA chemically induced proximity (CIP) system by replacing PYL1 with wild type PYR1. Surprisingly, this simple modification resulted in a ~40-fold increase in ABA sensitivity (EC_{50} of 864 nM; Figures 3A and B) without loss of dynamic range. When a panel of single hypersensitive substitutions was assayed, sensitivity was improved to varying extent (Figure S4B) with additional ~10× increases for the A160 V and I110C mutations (EC_{50} of 98 and 87 nM respectively; Figure 4A and 4B). We next sought to see if sensitivity could be increased further by combining hypersensitive mutants. We used combinatorial mutagenesis to make libraries of receptors harboring multiple combinations of all mutations isolated in the primary screen and used yeast selections to identify strains harboring mutants that would enable growth on selective medium at low concentrations. This led to the identification of four mutants with increased ABA sensitivity in yeast two hybrid assays (Figure 4B). One mutant (F61L, A160C; PYR1^{HS}) was tested in 293T cells in combination with wild type ABI1 or a catalytic mutant (D134A) that avoids potential side effects of introducing an active phosphatase. The dose response curves of PYR1^{HS} indicate that this combination further improved sensitivity ~2.5 fold relative to A160 V alone (EC_{50} = 41 nM; Figure 3B). Together the PYL1, PYR1, and PYR1^{HS} variants provide sensitivities that span three orders of magnitude.

In summary, we demonstrated that hypersensitivity of mutants can be used to define regions of ligand binding pockets that make a disproportionate contribution to binding affinity (i.e., hotspots). Moreover, we demonstrate that ligand modifications targeted to the hotspot region improve binding affinity. Thus, we document that a binding hotspot on a receptor is proximal to a binding hotspot on its ligand. This suggests that the systematic mapping of hypersensitivity regions in a ligand binding pocket may be a productive approach to help guide ligand optimization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00955.

Details of synthesis, experimental procedures, compound characterization, and other supplementary data including figures and tables (PDF)

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DOI: 10.1021/acschembio.8b00955
ACS Chem. Biol. 2019, 14, 332−336

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Notes

The authors declare the following competing financial interest(s): Multiple authors on this manuscript are listed as inventors in patent applications covering compounds and mutants described in this application. The rights to these inventions have been assigned by the inventors to the University of California or Syngenta Crop Protection.

■ ACKNOWLEDGMENTS

Supported by NSF (Grant 1656890), Syngenta Crop Protection AG. We thank Jayati Mandal for valuable technical assistance with identification of PYR1 mutants.

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