Supporting Online Material for

Molecular Mimicry Regulates ABA Signaling by SnRK2 Kinases and PP2C Phosphatases


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Materials and Methods

Protein preparation

Wild type kinase SnRK2.6 from Arabidopsis thaliana were expressed as H6Sumo fusion proteins from the expression vector pSUMO (LifeSensors). The modified fusion protein contains a H6-tag at the N terminus and a SUMO protease site between Sumo and SnRK2.6. BL21(DE3) cells transformed with the expression plasmid were grown in LB broth at 16°C to an OD600 of ~1.0 and induced with 0.1 mM IPTG for 16 hr. Cells were harvested, resuspended in 100 ml extract buffer (20 mM Tris, pH 8.0, 200 mM NaCl, and 10% glycerol) per six liters of cells, and passed three times through a French press with pressure set at 1000 Pa. The lysate was centrifuged at 18,000 rpm in a Sorvall SS34 rotor for 30 min, and the supernatant was loaded on a 50 ml Nickel HP column. The column was washed with 10% buffer B (20 mM Tris, pH 8.0, 200 mM NaCl, 500 mM imidazole, and 10% glycerol) for 600 ml and eluted with 100 ml of buffer B. The eluted H6Sumo-SnRK2.6 was dialyzed against extract buffer and cleaved overnight with SUMO protease at a protease/protein ratio of 1:1000 at 4°C. The cleaved H6Sumo tag was removed by passing through a Nickel HP column, and the protein was further purified by chromatography through a HiLoad 26/60 Superdex 200 gel filtration column in 25 mM Tris, pH8.0, 200 mM ammonium acetate, 1mM dithiotreitol and 1mM EDTA.

Purification of PYL2 (residues 14-188) and ABI2 (residues 101–423) were as described previously (1, 2). To prepare the PYL2–ABA–ABI2 ternary complex, we added (+)-ABA and purified PYL2 to purified ABI2 at a 5:1:1 molar ratio in the presence of 5 mM MgCl2.

The SnRK2.6–HAB1 complex was constructed as a fusion protein with a H6-tag (MAHHHHHHHA) at the N-terminus of SnRK2.6 (residues 11–362) fused to HAB1(172–511) with a GSGSAGSAAGS linker. We also generated fusion proteins with HAB1 at the N-terminus and SnRK2.6 at the C-terminus as well as fusion proteins with different linker lengths. Only H6SnRK2.6– GSGSAGSAAGS–HAB1 produced crystals that could be further improved by surface entropy reduction mutagenesis (3, 4). The modified protein was expressed in E. coli BL21 (DE3) and purified using a Nickel HP column and a HiLoad 26/60 Superdex 200 column following the same method and using the same buffers as the kinase proteins except that all buffers contained 5mM MgCl2 and that no cleavage and removal of H6-tag was performed. SnRK2s, ABI2, ABI2–ABA–PYL2, and the SnRK2.6– HAB1 complex all eluted as monomeric proteins or monomeric protein complex from size exclusion chromatography columns.

Crystallization

We generated more than a dozen different surface entropy reduction mutants of the SnRK2.6-HAB1 fusion protein. Only the D296A/E297A mutation in SnRK2.6, which was predicted in a solvent exposed loop (fig. S1), yielded better crystals. The H6-tagged SnRK2.6 D296A/E297A – HAB1 fusion protein crystals were grown at room temperature in sitting drops containing 0.2 ul of purified protein at 7 mg/ml and 0.2 ul of well solution containing 0.1 M HEPES, pH 8.0, 1.2 M ammonium sulphate, 2% (w/v) PEG 1000 and 3% (w/v) trimethylamine N-oxide dihydrate. Crystals of about 100 µm in length appeared in 1-2 days. Crystals were soaked in 20% ethyl glycol before flash freezing in liquid nitrogen.
ABI2 crystals were grown at room temperature in hanging drops containing 1 μl of the purified protein at a concentration of 10.9 mg ml\(^{-1}\) and 1 μl of well solution containing 0.2 M sodium formate, 18% w/v PEG 8000 and 10% w/v sucrose. Crystals of about 80 μm length appeared within 1–2 days. Crystals were serially transferred to well buffer with increasing sucrose concentration (29.5% (w/v) final) before flash freezing in liquid nitrogen.

The PYL2–ABA–ABI2 complex crystals were grown at 4 °C in hanging drops containing 1.6 μl of the above protein–ligand–PP2C solutions and 2.4 μl of well solution (0.1 M HEPES, pH 7.0, 6% (w/v) PEG 8000 and 10% (w/v) sucrose). Crystals appeared the next day and grew to a dimension of ~300 μm in length on the sixth day. Crystals were serially transferred to well buffer with increasing sucrose concentration (39% (w/v) final) before flash freezing in liquid nitrogen.

**Data Collection and Structure Determination**

All diffraction data sets were collected at 100 K using an X-ray beam at 0.97872 Å wavelength with Rayomics300 or Rayomics225 CCD detectors at the ID-D and ID-F beamlines of sector-21(LS-CAT) at the Advanced Photon Source at Argonne National Laboratory. The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package (5). The resolution cutoff is based on the criteria of I/σ ratio >2.0 and Chi square <1.5 in the highest resolution shell as guided by HKL2000 package. Molecular replacement was performed by using the Collaborative Computational Project 4 (CCP4) program Phaser (6) with the mammalian AMPK kinase domain structure (PDB:2H6D) and HAB1 structure from PYL2-HAB1 complex (PDB:3KB3) as the initial models. Programs O and Coot were used to manually fit the protein models (7, 8). Model refinement was performed with CNS and the CCP4 program Refmac5 (9, 10) with isotropic B-factors. Most residues of the refined structures are in the most favorable regions of Ramachandran plot and no residues are in disallowed regions based on Protein Check (11). (SnRK26-HAB1: Residues in most favorable regions 86.9%, Residues in additional allowed regions 12.4%, Residues in generously allowed regions 0.8%, Residues in disallowed regions 0%; ABI2: Residues in most favorable regions 92.2%, Residues in additional allowed regions 7.8%, Residues in generously allowed regions 0%, Residues in disallowed regions 0%; PYL2/ABA/ABI2: Residues in most favorable regions 83.9%, Residues in additional allowed regions 16.1%, Residues in generously allowed regions 0%, Residues in disallowed regions 0%.) The statistics of data sets and refined structures are listed in Table S1.

**Hydrogen/Deuterium Exchange and Mass Spectrometry**

Solution phase HDX experiments were performed with a LEAP Technologies Twin HTS PAL liquid handling robot interfaced with an Orbitrap mass spectrometer (Exactive, ThermoFisher Scientific) (12). When the HDX of HAB1 was studied in the presence of SnRK2’s, HAB1 was at 10uM and SnRK2’s were at 10uM. When the HDX of SnRK2’s were studied in the presence of HAB1, SnRK2’s were at 15uM and HAB1 was at 50uM.
Complexes were allowed to equilibrate for 1 hour at 4°C before initiating deuterium exchange by diluting protein or protein-protein complexes 1:5 into D$_2$O buffer (or H$_2$O buffer for “0 second” samples) and incubated for predetermined times (10, 30, 60, 300, 900 and 3600 seconds) at 4°C before quenching. Digestion was performed in line with chromatography using an in-house packed pepsin column (13) at 50 μl/min and peptides were captured and desalted on a 2 mm i.d. C8 trap (Thermo Fisher Scientific, San Jose, CA). Peptides were then separated across a 10x1 mm (5μm) Hypersil Gold C8 column (Thermo Fisher Scientific, San Jose, CA) with a linear gradient of 12-40% acetonitrile in 0.3% formic acid over five minutes. Peptide ion signals were confirmed if they had a MASCOT score of 20 or greater and had no ambiguous hits using a decoy (reverse) sequence in a separate experiment using a 60 minute gradient. The intensity weighted average m/z value (centroid) of each peptide’s isotopic envelope was calculated with the in-house developed software HD Desktop (14) and corrected for back-exchange.

To determine the phosphorylation status of SnRK2.6, peptides corresponding to phosphorylated and unphosphorylated ion signals were sequenced in a separate MS/MS experiment (1 hour ATP incubation). Ratios of phosphorylated to unphosphorylated peptide were calculated from area under the curve measurements of monoisotopic ion signals.

**Kinase Assays**

SnRK2 kinases were either pre-incubated with HAB1 in kinase buffer (25 mM Tris, pH 7.4, 12 mM MgCl$_2$, 2 mM DTT) for 30 minutes at room temperature or were directly incubated with 0.2 mM unlabelled ATP, 2.5 μCi [$^{32}$P]-γATP, and 1 μM (Suppl. figures) or 2 μM (Fig. 4A&B) GST-ABF2(73-120) for 30 min at room temperature in a total volume of 15 μl. Reactions were terminated by addition of SDS sample buffer and subjected to Tricine SDS-PAGE. Gels were stained with Coomassie and subjected to autoradiography using a FLA-5000 phosphor imager (Fuji).

**Phosphatase Assays**

Phosphatase assays were performed by colorimetric determination of phosphate release from phospho-S175 of a SnRK2.6 activation loop phosphopeptide as described previously (1). Reactions contained 200 nM recombinant HAB1 protein and 100 μM SnRK2.6 phosphopeptide.

**AlphaScreen assays for SnRK2 PP2C interactions**

Interactions between PYL2 and PP2Cs and SnRK2s and PP2Cs were assessed by luminescence-proximity AlphaScreen technology as described previously (1). Reactions contained 100 nM recombinant H6GST-SnRK2.6 or –PYL2 proteins bound to nickel-acceptor beads and 100 nM recombinant biotin-PP2C bound to streptavidin donor beads.

**Mutagenesis**
Site-directed mutagenesis was carried out using the QuikChange method (Agilent). Mutations and all plasmid constructs were confirmed by sequencing.
**Supplemental Table 1:**  
**Statistics of data sets and structure refinement**

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Supplemental Figure 1: Structure-based sequence alignment of the three SnRK2s with the α1 and α2 subunits of human AMPK and S. cerevisiae and S. pombe Snf1. Secondary structure elements and the ATP binding loop and activation loop are indicated. PP2C-interacting residues are enclosed by black boxes, including the complete ABA box (shown as shaded rectangle). The surface entropy reduction mutation sites are indicated by black asterisks. The additional E341 in the SnRK2.6 ABA box is marked with a red arrow.
Supplemental Figure 2: Schematic representation of the amplified luminescent proximity assay (AlphaScreen). H6GST-SnRK2 (SnRK2-H6) proteins are immobilized on Ni-chelating acceptor beads and biotinylated PP2C (b-PP2C) proteins on streptavidin-coated donor beads. Donor beads contain a photosensitizer that upon activation at 680 nm converts ambient oxygen to singlet oxygen. If acceptor beads are brought into close proximity of the donor beads by SnRK2–PP2C interaction, energy is transferred from singlet oxygen to thioxene derivatives in the acceptor beads resulting in light emission at 520-620nm. Addition of untagged SnRK2 competes acceptor bead-immobilized SnRK2-H6 away from b-PP2C.
Supplemental Figure 3: SnRK2.6 and HAB1 are active in the context of the SnRK2.6–linker–HAB1 fusion protein. Kinase assay using 1 µM H6GST-SnRK2.6 and biotin-MBP-HAB1 (right) or 1 µM H6-SnRK2.6–11 amino acid linker–HAB1 wildtype (middle) and D296E/E297A (left) fusion protein in the presence of GST-ABF2(73-120) and the presence or absence of ~5µM PYL2/50 µM (+)-ABA. *: Degradation product, M=marker.
Supplemental Figure 4: HDX perturbation map comparing protection of the SnRK2.6–HAB1 complex formed in the fusion protein with the complex formed by separate proteins. Heat map displaying changes in HDX protection. The bars below the sequence represent the peptide fragments resolved by mass spectrometry and the color of the bars the relative change in HDX protection. All peptides resolved show no significant change in protection (color code in lower right corner; ns: no significant change in protection) between the SnRK2.6–HAB1 complex formed in the fusion protein and the complex formed by separate proteins.
Supplemental Figure 5: Details of the binding interfaces of the SnRK2.6–HAB1 complex. 2Fo–Fc composite omit map (1σ) of three regions of the interaction surface between SnRK2.6 and HAB1 as indicated in Figure 2A and 2B. SnRK2.6 is shown in green, the activation loop is light blue. HAB1 is colored pink and its PYL2-interaction loop orange. The catalytic Mg²⁺ ions are presented as gray spheres, and the sulfate ions are shown as ball-stick models.
Supplemental Figure 6: Similarity between PYL2–HAB1 and the SnRK2.6–HAB1 interfaces. HAB1 is colored pink, PYL2 cyan, and SnRK2.6 green. The catalytic HAB1 Mg$^{2+}$-ions are shown as ball model, the lock residue W385 and the HAB1 catalytic cleft-inserting Ser89 (gate loop) and Ser175 (activation loop) residues as stick models. A. Docking of the PYL2 gate loop with Ser 89 (left) and the SnRK2.6 activation loop with Ser175 (right) into the catalytic cleft of HAB1. HAB1 is shown as translucent surface. B. Insertion of the HAB1 lock residue W385 into pockets of PYL2 and SnRK2.6. PYL2 and SnRK2.6 are shown as solid surfaces.
Supplemental Figure 7: The SnRK2.6 activation loop residue pS175 is preferentially dephosphorylated by HAB1. Ratios of phosphorylated and unphosphorylated peptides from the N-terminus of SnRK2.6 (VKDIGSNFGVARL, including Ser29) and from the activation loop of SnRK2.6 (HSQPKSTVGTAPAYIAPEVL, including residue S175). Phosphorylation ion signals were detected using mass spectrometry. Peptides corresponding to phosphorylated and unphosphorylated ion signals were sequenced in a separate MS/MS experiment following a one hour incubation with ATP. Unphosphorylated peptide ion signal was detected in every sample. A ratio of 0 corresponds to non-detected phosphorylated ion signal.
Supplemental Figure 8: Structures of apo ABI2 and ABI2–ABA–PYL2. (A) Apo ABI2 structure. The PYL2-inserting residue W290 (equivalent of W385 of HAB1) is shown as stick presentation, the three catalytic Mg$^{2+}$ ions as solid spheres. (B) Structure of ABI2(orange-brown)–ABA(ball presentation)–PYL2(light blue) overlaid with the structure of apo ABI2(dark red). W290 is shown in stick presentation and the Mg$^{2+}$ ions as grey balls. The PYL2 gate loop is colored pink and the PYL2 latch loop magenta.
Supplemental Figure 9: PYL2/ABA- and SnRK2.6-binding do not induce significant conformational changes in the PP2Cs ABI2 and HAB1. (A) Side-by-side structure comparison of apo ABI2, ABI2 from the ABI2–ABA–PYL ternary complex, HAB1 from the HAB1–SnRK2.6 binary complex, and HAB1 from the HAB1–ABA–PYL2 ternary complex (PDB code: 3KB3). (B) Overlay of the structures of apo ABI2 (dark red), ABI2 from the ternary ABI2–ABA–PYL complex (pink), and HAB1 from the binary HAB1–SnRK2.6 complex (orange). Mg$^{2+}$-ions are shown as spheres.
Supplemental Figure 10: Competition of the PYL2/ABA–HAB1 interaction by SnRK2.6 kinase domain. Inhibition of the interaction between H6GST-PYL2/ABA and biotin-HAB1 by untagged SnRK2.6 lacking the ABA box (amino acids 1-319) and the untagged SnRK2.6 ABA box peptide (amino acids 333-362). Error bars indicate SD (n = 3).
Supplemental Figure 11: SnRK2.6 HDX perturbation map. Heat map displaying the change of SnRK2.6 HDX protection upon HAB1 binding. The bars below the sequence represent the peptide fragments resolved by mass spectrometry and the color of the bars indicates the relative decrease in deuterium exchange (i.e. relative increase in local conformational stabilization) upon HAB1 binding (color code in lower right corner; ns: no significant change in protection). Red boxes indicate regions of protection in the presence of HAB1. Amino acid positions of the SnRK2.6 protein are indicated above the sequence.
Supplemental Figure 12: Mutational and biochemical analysis of the ABA box-interaction site in HAB1. (A) AlphaScreen assays for HAB1-ABA box interaction. (B) HAB1 phosphatase assay (n=3, error bars, s.d.). HAB1 mutant proteins with strongly reduced ABA box-binding activity are shown in red (compare Fig. 3A).
Supplemental Figure 13: ABA box alanine scanning mutagenesis.  (A) AlphaScreen interaction between SnRK2.6 H6GST-ABA box proteins and biotin-HAB1.  Error bars indicate SD (n = 3).  (B) Protein stain of wildtype H6GST-SnRK2.6 ABA box proteins and the 30 mutant proteins.  All amino acids of the ABA boxes were converted to alanine residues with the exception of Ala361, which was altered to Gly.  All constructs expressed to similar levels as wildtype with the exception of the Met362 mutant.
Supplemental Figure 14: The interaction between SnRK2.6 and HAB1 is not competed by PYL2. Inhibition graph of the AlphaScreen interaction between H6GST-SnRK2.6 and biotin-HAB1 by untagged PYL2 in the absence or presence of 10µM (+)-ABA (n=3, error bars=s.d.).
Supplemental Fig. 15

The two-step mechanism makes two predictions:

1. A catalytically inactive HAB1 mutant should be unable to inhibit SnRK2.6 at sub-stochiometric levels and below K_d, but should be capable to abolish SnRK2.6 activity at higher concentrations. This is indeed the case for HAB1 D492A, which is a completely inactive phosphatase (see panel C below), but inhibits SnRK2.6 as efficiently as wild type HAB1 at 3.0 and 10 µM concentrations (Fig. 4B).

2. Combining the catalytic HAB1 mutation with mutations that disrupt the interaction with the SnRK2 kinase domain should cause HAB1 to lose its ability to inhibit SnRK2.6 even at high concentrations, which we show in panels A and B below. Note that the loss of SnRK2.6 inhibition of the HAB1 double mutants is not due to incorrectly folded or aggregated mutant proteins since the proteins completely maintained their ability to interact with the SnRK2.6 ABA box (panel D below).

Supplemental Figure 15: HAB1 inhibits SnRK2 activity both enzymatically and by blocking the catalytic cleft. (A,B) Combination of the catalytically inactive HAB1 mutant D492A with mutations in key HAB1–SnRK2.6 kinase domain interaction residues [E323R (A) and W385R (B), see Fig. 2B] do not inhibit SnRK2.6 even at high concentrations. Reactions also contained 10 mM of the PP2C inhibitor NaF. Note that mutated HAB1 also becomes substrate of SnRK2.6. (C) HAB1 D492A does not dephosphorylate ABF2. ABF2 was incubated with SnRK2.6 and [32P]-γATP, affinity-purified, and then incubated with 10 µM of either wildtype HAB1 or HAB1 D492A under the same conditions as in (B). Note that HAB1 dephosphorylates not only the SnRK2.6 activation loop, but also ABF2. (D) HAB1 D492A/E323R and HAB1 D492A/W385R retain full activity in ABA box interaction as determined by AlphaScreen interaction assay. Error bars indicate SD (n = 3).
References and Notes


