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Peer Review File

Stabilization of dimeric PYR/PYL/RCAR family members relieves abscisic acid-induced inhibition of seed germination

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript presents highly novel insights validated through rigorous experimentation. The authors identified an extended binding pocket on the PYL1 surface, crucial for dimerization. Using virtual screening, they targeted this pocket to discover conformational change inhibitors (dimer stabilizers) for dimeric PYLs. Notably, DBSA exhibited stronger binding to PYR1 compared to ABA. X-ray crystallography confirmed the predicted binding mode of DBSA. Furthermore, crystal structure analysis and MD simulations suggest that DBSA binding promotes closer proximity between PYL1 monomers while increasing hydrogen bonds, leading to a stabilizing effect on the dimer. This work successfully establishes a novel molecule capable of regulating ABA signaling.

P8 L196-L208: How the difference in affinity of DBSA to PYR1 and to PYL1 is explained?

P9 L220-L222: If DBSA directly inhibits HAB1, it seems possible that high concentration treatments may inhibit seed germination. The effect of DBSA at concentrations higher than 1 μM on seed germination should be evaluated.

Figure 2A&2C: Why do the thermal changes upon dropping ABA differ between PYL1 (endothermic reaction) and PYR1 (exothermic reaction), even though both PYL1 and PYR1 are dimeric PYLs?

ITC experiments: If a ΔH change is observed after dropping ABA into a cell containing PYL1 and DBSA, it is likely to be a non-competitive inhibition, whereas if no ΔH change is observed, it is likely to be a mode of inhibition as shown in the model in Figure 5B. Such experiments would make it much clearer whether the binding of DBSA to PYL prevents ABA from binding to PYL.

Figure 2E: Does ABA inhibition by DBSA result solely from stabilizing the PYL dimer (is DBSA targeting dimeric PYLs only)? Although DBSA undoubtedly stabilizes the dimeric conformation of PYL1, it seems possible that DBSA interferes with ABA entry into the ligand-binding pocket, as DBSA showed antagonist activity not only for dimeric PYLs but also for monomeric PYLs. Data confirming the ABA antagonist activity of DBSA against multiple deletion mutants of monomeric PYLs or showing that DBSA does not target monomeric PYLs using monomeric PYL overexpressing mutants would be more convincing for DBSA to target only dimeric PYLs, but since it is unlikely that mutants can be prepared in a short time, please consider this in the future.

Figure 4A&4B legend: Please indicate the colors of the crystal and the predicted model structures (which is which).

Figure 4D: Only the results of DBSA and ABA treatments alone are shown here; to prove that DBSA stabilizes the dimer and inhibits the ABA response, it is important to show that ABA and DBSA co-treatment inhibits ABA-induced dimer dissociation.

P16 L392-L393: Please cite the paper on AS6. Nat Chem Biol 2014, 10, 477-482.

Reviewer #2 (Remarks to the Author):

Wang et al. describe the discovery of a new antagonist molecule of ABA (DBSA). This molecule relieves ABA-induced inhibition of Arabidopsis seed germination. The key point in this work is the notion that the action of DBSA depends on the stabilization of the quaternary structure of dimeric ABA receptors, while other antagonist molecules, such as ANT and ABA derivatives, bind to the receptors and hinder their interaction with the downstream PP2C phosphatases.

Both the pipeline employed for the discovery of DBSA and the "post-Morten" crystallographic analyses to uncover the basis of its activity are interesting. However, while the crystallography and in-plant studies are solid and clearly presented, there is a lack of care in the description of the pipeline for the in silico work. The biochemical data to prove the in vitro activity of DBSA are incomplete. It would be excellent if the authors made more efforts to enhance DBSA until achieving sub-micromolar affinities.

The results are nicely discussed. The reference list is complete.

There are several questions that could be addressed to enhance the quality of the data presented in this study.

Line 115 and 116: The assertion that "Due to the lack of dimeric ABA-bound PYL1 and apo-PYR1 structures..." is incorrect. Please consult PDB codes 3K3K (PYR1, with one protomer bound to ABA and the other in the apo form), 3KAY, and 3JRS (PYL1, with ABA bound and in apo forms). This is likely to affect the subsequent section until line 144. I will restrict the description of the transition from the open to the closed conformation of the PYL receptors to either PYL1 or PYR1. This

adjustment should also extend to the details regarding the reduction in the volume of the binding pocket and to Figures 1 A, B, and C.

Lines 149. I think authors should call Figure 1D instead of Figure 1C.

Figure 1D lacks clarity and does not provide useful information. Could you please overlap the binding pockets of PYL1 ABA and PYL2 PYRA (antagonist conformation), and provide the rationale for designing the new antagonist molecules? I may be mistaken, but to my knowledge, the sulfonamide moiety of PYRA is located at the bottom of the ABA binding pocket, far from the dimerization interface. In this conformation, growing the fragment from the sulfonamide molecule will not reach the dimerization interface. Please clarify this.

Lines 163 to 175 could be omitted or moved to the supplementary material since the crystal structure already provides the experimental details of the agonist binding mode.

The Kd values of DBSA for PYR1 and PYL1 are much lower than those observed for ANT (which are in the low pM range). Therefore, considering that the affinity of the ternary complex PYL1:ABA:HAB1 for ABA is around 100nM, it is unexpected to observe the effect of 50 microM DBSA on PP2C activity in the presence of 5 microM ABA. The authors should evaluate the potency of DBSA by quantifying the antagonist-mediated restoration of PP2C activity in the presence of saturating ABA, and compute an IC50 value, as shown in Vaidya et al 2021.

It is also surprising to observe the antagonist activity of DBSA when using monomeric receptors (Figure 2E). The authors should provide an explanation for this unexpected result, or discuss it in the corresponding section. Based on these data, the title of the section should be revised. DBSA stabilizes the dimers but it might also compete with ABA in the monomers. Additionally, data on the HAB1 activity without receptor and without receptor and DBSA should be included in Figure 2E.

Figure 4A should be omitted or sent to the supplementary materials as it represents the comparison between the experimental and theoretical models of the complex PYL1 and DBSA. Please include the RMSD for the backbone and heavy atoms in the figure caption. The authors might discuss the accuracy of their predictions in the discussion section. Instead, a new figure comparing the complexes of PYL1 with DBSA and with ABA should be included.

The molecular dynamic simulations were performed with ABA-bound PYR1, apo-PYL1, and DBSA-bound PYL1. I believe this is because the authors are not aware of the structure of ABA-bound PYL1 (3JRS) (see above). To provide a complete picture of the mechanism, I would suggest repeating the calculations using this structure. This adjustment affects the rest of the section.

Figure 5A: Please display all the complexes in the same orientation.

It would be interesting to discuss that while DBSA's antagonist activity is based on stabilizing the PYL dimers, AS6 obstructs PPase interaction by occupying the 3' tunnel, and ANT blocks the Trp lock. This insight could be incorporated into the infogram displayed in igure 5B.

To properly evaluate the experimental approach, the methods section should provide protocols for the purification of all the receptors used for the PPase activity assays. In addition, it should include the protocol for ITC, including buffer composition for ligand and protein samples.

Please include figures showing a section of the final 2Fo-FC map and an unbiased Fo-Fc map obtained after omitting the ligand from the model in the supplementary material

Line 32 (abstract) and Line 439 (discussion). What are the genetic approaches used in this study to discover DBSA?

Dear Reviewers,

Thank you for your reviewing of our manuscript NCOMMS-24-18245A (NCOMMS-24-18245-T), entitled "**Stabilization of dimeric PYR/PYL/RCAR family members relieves abscisic acid-induced inhibition of seed germination**", which we previously submitted for consideration of publication in *Nature Communications*. We appreciate you for the helpful comments and suggestions, which may help us to improve our manuscript. The manuscript has been revised in response to your suggestions. Please notice below our responses to your comments. Here is a summary of our changes and responses point by point:

RE Comments from Reviewer #1:

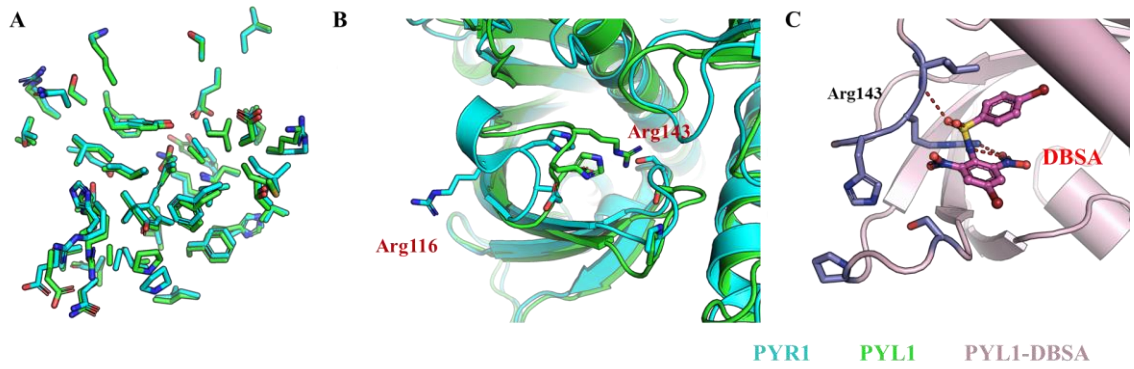
Comments 1: This manuscript presents highly novel insights validated through rigorous experimentation. The authors identified an extended binding pocket on the PYL1 surface, crucial for dimerization. Using virtual screening, they targeted this pocket to discover conformational change inhibitors (dimer stabilizers) for dimeric PYLs. Notably, DBSA exhibited stronger binding to PYR1 compared to ABA. X-ray crystallography confirmed the predicted binding mode of DBSA. Furthermore, crystal structure analysis and MD simulations suggest that DBSA binding promotes closer proximity between PYL1 monomers while increasing hydrogen bonds, leading to a stabilizing effect on the dimer. This work successfully establishes a novel molecule capable of regulating ABA signaling.

Answer: Thank you for your kind comments on our manuscript to improve the quality of our work. We have modified our manuscript according to your comments. Please see the following modifications point by point.

Comments 2: P8 L196-L208: How the difference in affinity of DBSA to PYR1 and to PYL1 is explained?

Answer: Thank you for your kind and professional suggestion, and we think it was interesting to explore the reason to cause such differences. We aligned the PYR1 and PYL1 the binding pocket of PYR1 and PYL1, and found that they share almost the same sequences. Therefore, the differences of binding affinity might be not caused by interaction with residues in binding pockets. Then we further

superposed a gate open monomer of PYR1 dimer (PDB code 3K3K) and the gate open monomer of PYL1 dimer (PDB code 3KAY), and found that a pair of key residues Arg116 of PYR1 and Arg143 of PYL1 shared a quite difference conformation. Arg116 of PYR1 was exposed in the solvent, but Arg143 was located in the entrance of PYL ligands, which might hinder the entrance of DBSA. And from the co-crystal structure of PYL1-DBSA we could find that Arg143 was moved deeper into binding pocket. Therefore, we guess that the movement of Arg143 of PYL1 might cost some binding free energy loss for DBSA and therefore it showed a higher K_d value. If you still have any questions about this problem, we are pleased to further discuss it with you.



Comments 3: P9 L220-L222: If DBSA directly inhibits HAB1, it seems possible that high concentration treatments may inhibit seed germination. The effect of DBSA at concentrations higher than 1 μM on seed germination should be evaluated.

Answer: Thank you for your kind and professional suggestions. We performed the seed germination experiments of different concentration of DBSA (Figure S4). It could be noticed that in the concentration of 10 μM and 20 μM, the seed germination rate of WT *Arabidopsis thaliana* was more than 90%. But in the higher concentration, such as 50 μM, the seed germination rate was decrease to about 30%. These results are coincided with our hypothesis. In addition, the directly inhibitory activity of DBSA to HAB1 was evaluated. The HAB1 activity was obviously inhibited at the concentration higher than 200 μM, but is no effect on low concentration (Figure S13). Therefore, we believe that the concentrations of DBSA used in this manuscript would not affect the accuracy of our experiments. And we added the text in the third graph of our Discussion part to introduce this potent limitation. If you still have any questions about this problem, we are pleased to further discuss it with you.

Added Text
In addition, high concentration of DBSA might inhibit the seed germination due to its HAB1 directly inhibition (Figure S13).

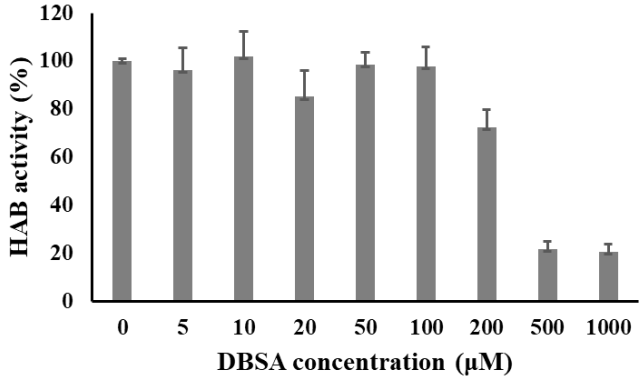


Figure S4. The directly inhibition of HAB1 activity by DBSA. The concentration of HAB1 was 0.4 μM; n = 3; error bars represent SD value.

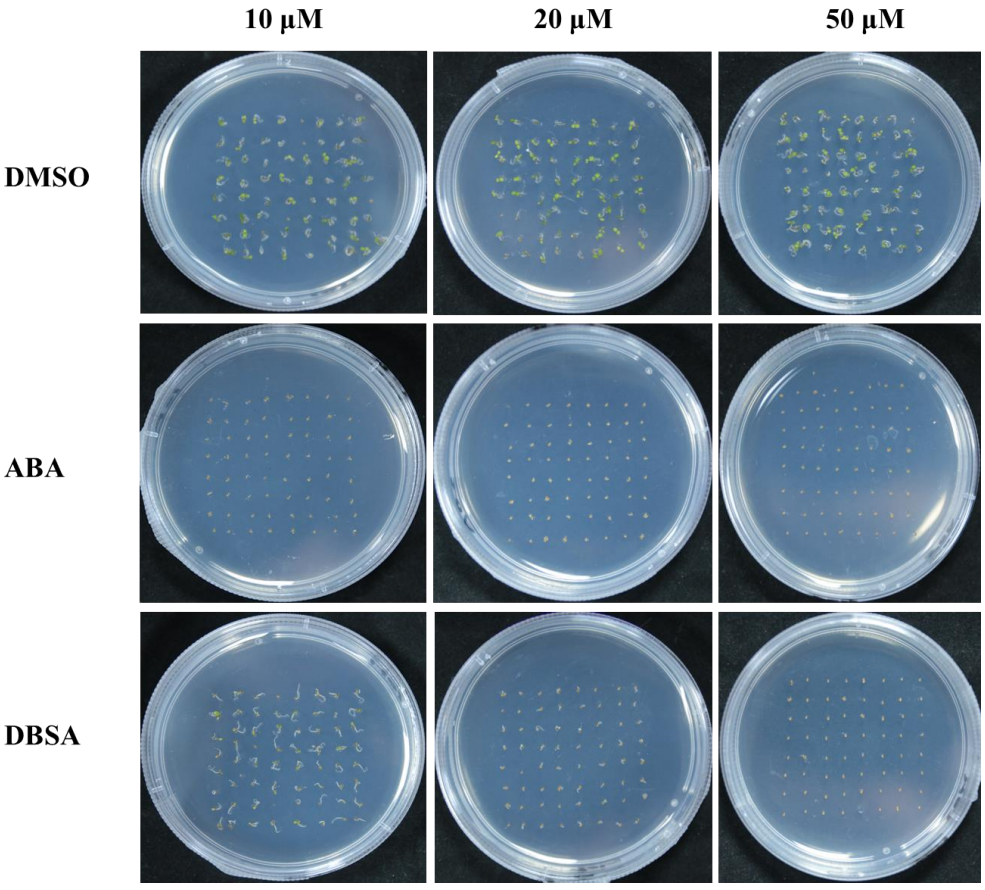


Figure S13. Seed germination experiment of different concentration of ABA and DBSA on WT

Arabidopsis thaliana seeds.

Comments 4: Figure 2A&2C: Why do the thermal changes upon dropping ABA differ between PYL1 (endothermic reaction) and PYR1 (exothermic reaction), even though both PYL1 and PYR1 are dimeric PYLs?

Answer: Thank you for your kind and professional suggestions, and we apologize for our confused results. It was noticed that in most of reported cases (*Nature*, 2009, 462, 665-668; *EMBO J.* 2011, 30, 4171-4184), ABA binding to PYL dimers, such as PYR1 or PYL1, was an endothermic reaction. Therefore, the ITC experiments of ABA to PYR1 was reperformed, and an endothermic reaction was observed. The K_d value of ABA to PYR1 was 21.95±1.18 μM. We apologize for our mistakes again. Please see the modified figure below.

Modified Figure

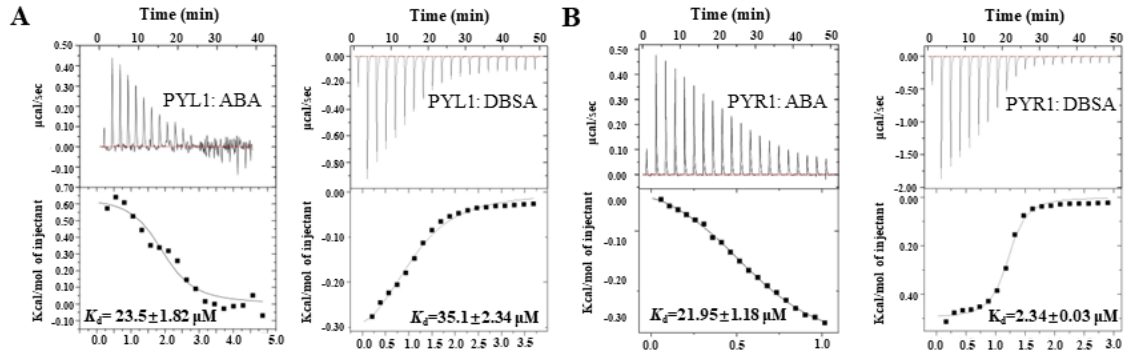


Figure 2. DBSA shows low ABA receptor binding affinity and is a potent antagonist of multiple ABA receptors. (A) The K_d values of ABA and DBSA to PYL1 using ITC. (B) The K_d values of ABA and DBSA to PYR1 using ITC. (n = 3; error bars = SE).

Comments 5: ITC experiments: If a ΔH change is observed after dropping ABA into a cell containing PYL1 and DBSA, it is likely to be a non-competitive inhibition, whereas if no ΔH change is observed, it is likely to be a mode of inhibition as shown in the model in Figure 5B. Such experiments would make it much clearer whether the binding of DBSA to PYL prevents ABA from binding to PYL.

Answer: Thank you for your kind suggestions. To make it much clearer whether the binding of DBSA to PYL prevents ABA from binding to PYL, we performed ITC experiments in two ways. First, the ITC experiment was performed by dropping ABA into a cell containing PYR1 and DBSA. Second, another ITC experiment was performed by dropping DBSA into a cell containing PYR1 and ABA.

And the experiment results were showed in Figure S3. According to the results, we could find that a ΔH change is not observed after dropping ABA into a cell containing PYR1 and DBSA, which indicated that DBSA is competitive to ABA like described in Figure 5B. In addition, an exothermic reaction was identified after dropping DBSA into a cell containing PYR1 and ABA, which suggested that DBSA showed stronger binding affinity than ABA. In summary, our ITC results showed that DBSA antagonize ABA signaling pathway by preventing the binding of ABA to PYLs with a higher binding affinity as the mode shown in the model in Figure 5B.

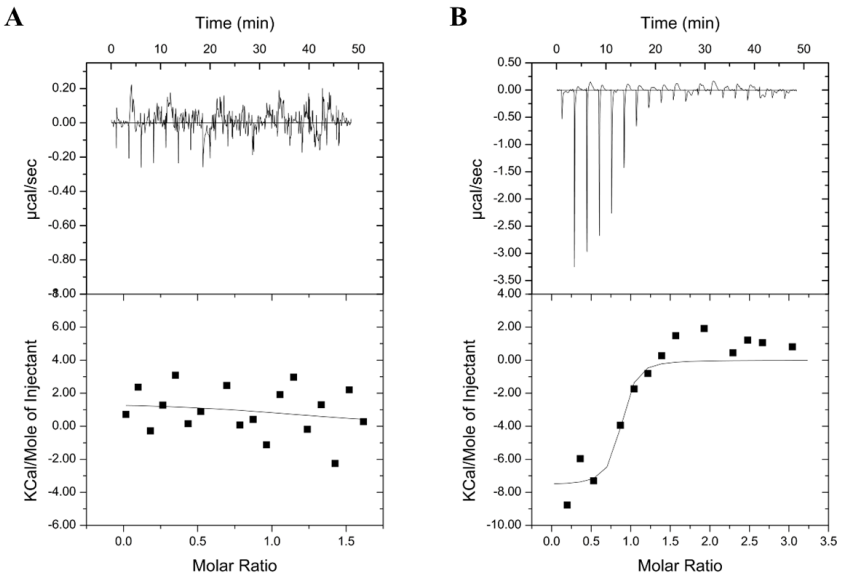


Figure S3. (A) ITC experiment results of dropping ABA into a cell containing PYR1 and DBSA. (B) ITC experiment results of dropping DBSA into a cell containing PYR1 and ABA.

Comments 6: Does ABA inhibition by DBSA result solely from stabilizing the PYL dimer (is DBSA targeting dimeric PYLs only)? Although DBSA undoubtedly stabilizes the dimeric conformation of PYL1, it seems possible that DBSA interferes with ABA entry into the ligand-binding pocket, as DBSA showed antagonist activity not only for dimeric PYLs but also for monomeric PYLs. Data confirming the ABA antagonist activity of DBSA against multiple deletion mutants of monomeric PYLs or showing that DBSA does not target monomeric PYLs using monomeric PYL overexpressing mutants would be more convincing for DBSA to target only dimeric PYLs, but since it is unlikely that mutants can be prepared in a short time, please consider this in the future.

Answer: Thank you for your kind and professional suggestions. The selectivity of DBSA between PYL dimeric receptors and monomeric receptors was also the most important issues that we concerned

in this study. But the PP2C inhibitory activity results were easy to be affected by the concentration of proteins and substrates. Therefore, a series of *in vitro* and *in vivo* experiments was performed again to verify the selectivity of DBSA. For the *in vitro* experiments, we first reperformed the PP2C inhibitory activity experiments again for dimeric and monomeric PYL members, and found that DBSA exhibited different antagonistic effect. For monomers (PYL5, PYL6 and PYL10), DBSA showed almost no antagonistic effect to the HAB1 inhibition induced by ABA (Figure 2C). While for dimeric members, DBSA could antagonize the HAB1 inhibition induced by ABA except for PYL3 as it is a cis-homodimer (*Structure* 2012, 20, 780-790). In addition, DBSA antagonized the HAB1 inhibitory activity of ABA in dose-depend way on PYR1, but it could not antagonize PYL5 and PYL10 even in a high concentration (Figure 2D). To further prove the reliability of our PP2C inhibitory activity results, the ITC experiments were performed between DBSA and some PYL monomeric members. It could be noticed that DBSA showed no obvious binding to PYL5, PYL6 and PYL10 (Figure S2). These results support our goal that DBSA antagonize the ABA signaling by stabilizer PYL dimers.

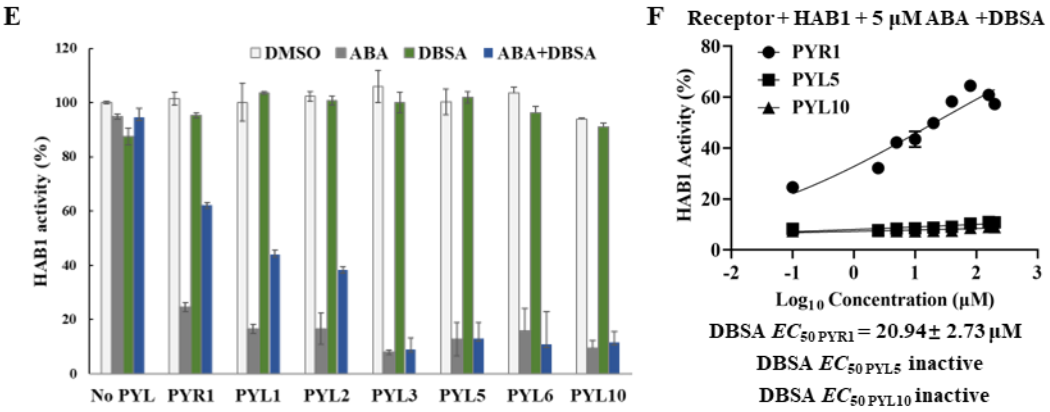


Figure 2. (C) Antagonistic effect of 50 μM DBSA on HAB1 activity through a phosphatase assay. PYLs and HAB1 were present at a molar ratio of 1:1 (0.4 μM: 0.4 μM) for PYR1/PYL1/PYL2/PYL3 and 2:1 (0.8 μM: 0.4 μM) for PYL5/PYL6/PYL10. Various PYL-HAB1 combinations were incubated with the indicated chemicals (5 μM ABA, 50 μM DBSA or 5 μM ABA and 50 μM DBSA); $n = 3$; error bars represent s.t.d. (D) Antagonistic effect of various concentrations of DBSA on HAB1 activity through a phosphatase assay. EC_{50} values were obtained by nonlinear fits of dose-response data. DBSA were tested at 0 μM to 200 μM, and the concentration of ABA was 5 μM. The concentration of PYR1, PYL5 and PYL10 were 0.4 μM, 0.8 μM and 0.8 μM, respectively, while HAB1 proteins were used at the molar ratio of 0.4 μM; $n = 3$; error bars represent s.t.d value.

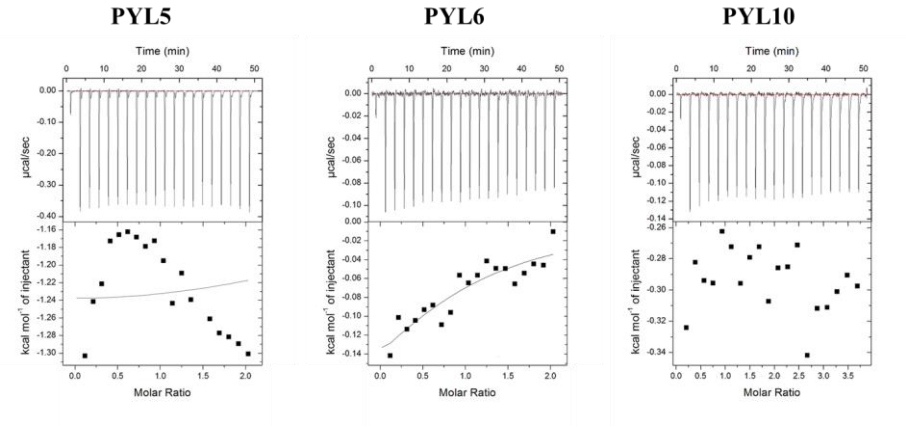


Figure S2. The ITC results of DBSA to PYL5, PYL5 and PYL10.

In addition, the in vivo experiments might be more convincing to explain the mechanism of studied compounds. For the extra seed germination experiments, we agree with you that it might be a promising way to verify the how DBSA antagonize ABA during seed germination process and greatly appreciate you for your kind understanding of the difficulty to obtain mutants. As you say, the preparation of mutants needs a relative long time. But in order to reduce the doubt of you and readers, we try to obtain mutant seeds from our collaborators or some scientists. Finally, we obtained the *pyl4* overexpression (*4OE*) seeds from Prof. Yuan Zheng at Henan University and PYL monomer *pyl3/pyl7/pyl9/pyl11/pyl12* (*3791112*) multiple deletion mutants from Prof. Yang Zhao at Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences. The results showed that DBSA could relieve ABA-induced seed germination inhibition of *3791112* seeds, but could not for *4OE* seeds. These results indicated that DBSA might antagonize ABA induced seed germination inhibition mainly via dimeric ABA receptor members.

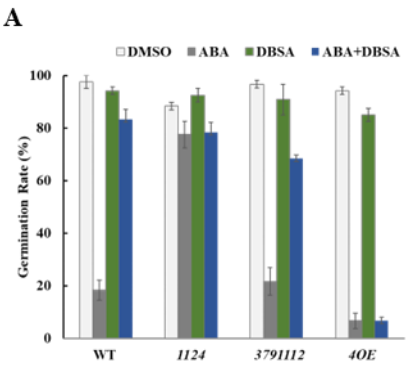


Figure 3. (A) Germination rate of seeds exposed to ABA (1 μM), DBSA (1 μM) or ABA and DBSA (1 μM:1 μM). DMSO (0.05%) was used as a control. WT and PYL dimer quadruple deletion mutant

(*pyr1/pyl1/pyl2/pyl4, 1124*), PYL monomer multiple deletion mutant (*pyl3/pyl7/pyl9/pyl11/pyl12, 3791112*), PYL4 overexpression (*4OE*) seeds are considered germinated when green cotyledons expand.

We believe the *in vitro* and *in vivo* study along with the crystal structure could prove that DBSA antagonize ABA signaling pathway mainly via dimeric ABA receptors. If you still have any problems about our manuscript, we are pleased to further discuss them with you to improve our manuscript.

Comments 7: Figure 4A&4B legend: Please indicate the colors of the crystal and the predicted model structures (which is which).

Answer: We apologize for our mistakes. We have modified our manuscript and verified the colors of the crystal and the predicted model in Figure 4A&4B in our revised manuscript. But according to the comments of Reviewer 2, Figure 4A was moved into the supplementary information, and the RMSD value for backbone and heavy atoms was included in the figure caption. A new Figure 4A was added about the comparison the complexes of PYL1 with DBSA and with ABA should be included. Please see the following modifications.

Modified Text
X-ray crystallography was performed to verify the binding mode between DBSA and PYL1 dimer. The structure of dimeric PYL1-DBSA complex was obtained at a resolution of 2.29 Å (PDB code 7DND, Table S8). Consistent with our hypothesis, DBSA is located at the binding pocket of dimer interface with a gate open and latch open conformation compared with ABA bind to a gate close and latch close conformation (Figure 4A and Figure S6). In addition, the binding mode of DBSA was similar to our predicted binding mode (Figure S7): the root-mean square deviation (RMSD) values were 1.06 Å (protein backbone atoms), 1.48 Å (protein heavy atoms) and 0.86 Å (ligand heavy atoms), respectively, which indicated that our computational model was reliable. For the detailed binding mode of DBSA, the gate loop of PYL1-DBSA complex was in its open state, and the key residues Ser112, Pro115 and His142 were in conformations similar to those in the *apo*-PYL1 structure. Furthermore, as predicted, hydrogen bond was observed between DBSA and the side chain of Arg143, but the hydrogen bond between DBSA and Ser112 was not found (Figure 4B).

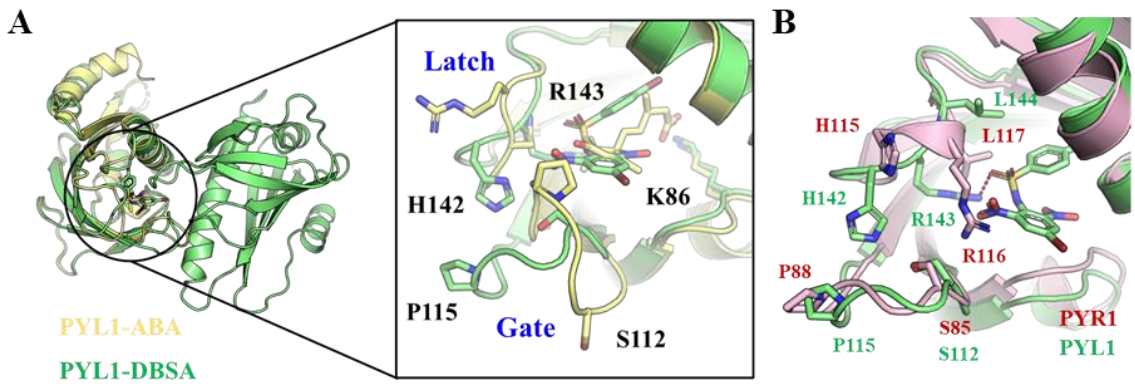


Figure 4. DBSA targets PYL1 dimer interface and stabilizes the PYL1 dimer via hydrogen bond networks. (A) Crystal structure of PYL1 with DBSA (PDB code 7DND, green) and ABA (PDB code 3JRS, yellow). ABA induced a gate-close and latch-close conformation, which DBSA bind to a DBSA induced a gate-open and latch- open conformation. (B) PYL1 was maintained in the latch-open and gate-open state in the crystal structure (shown in green stick model). DBSA forms hydrogen bonds with the side chain of Arg143. DBSA was predicted to bind to latch-open and gate-open PYR1 and interact with R116 (shown in pink stick model).

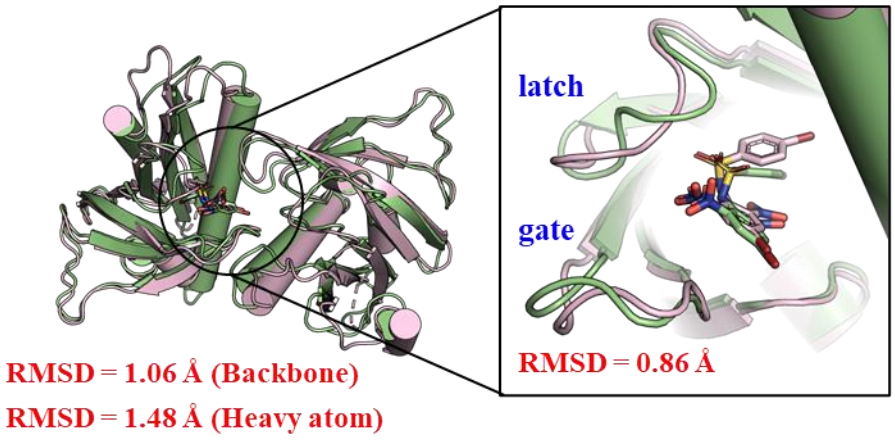


Figure S2. The comparison between predicted model and x-ray crystal structure of PYL1-DBSA complex (PDB code 7DND). The crystal structure shared a similar structure with our predicted model. The prediction model was shown in pink stick model, and X-ray crystal structure was shown in green stick model.

Comments 8: Only the results of DBSA and ABA treatments alone are shown here; to prove that DBSA stabilizes the dimer and inhibits the ABA response, it is important to show that ABA and DBSA

co-treatment inhibits ABA-induced dimer dissociation.

Answer: Thank you for your comment, and we apologize for our mistake. We have performed the SEC-MALLS experiment in the condition of ABA and DBSA co-treatment, and we found that the co-treatment inhibits ABA-induced dimer dissociation (see the following Figure). And we have modified our description and Figure 4D. Please see the following modifications.

Modified Text and Figure
To further verify the effect of DBSA on PYR1 dimer stabilization, the protein oligomeric state was detected using SEC-MALLS approach. The elution volume and detected molecular weight of apo-PYR1 was 14.74-15.45 mL and 44±2 kDa (Figure 4D), respectively, which is similar to literature and indicated that it was in the dimeric state (theoretical mass of 43 kDa). After the treatment of ABA, the elution volume was changed to 16.02-17.05 mL, and the molecular mass was 22±2 kDa, which is existing in PYR1 monomer state. While for DBSA treatment and DBSA/ABA co-treatment, the elution volumes were 14.82-15.47 mL and 14.66-15.51 ml, respectively. The molecular weight of 42±1 kDa for DBSA-PYR1 and 45±1 kDa for DBSA/ABA-PYR1 indicated that proteins were in dimeric states. These results suggested that ABA led to a dimer dissociation, and DBSA reached our goal to stabilize the PYL dimer.

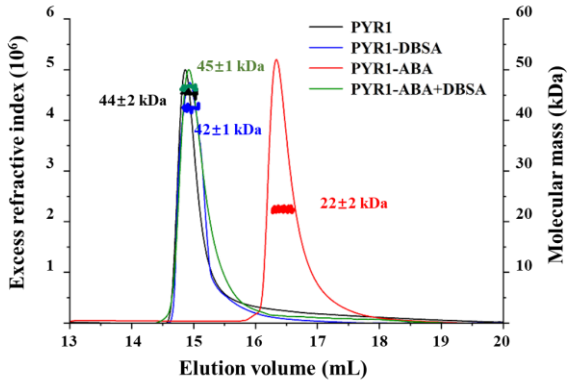



Figure 4. DBSA targets PYL1 dimer interface and stabilizes the PYL1 dimer via hydrogen bond networks. (D) The aggregation states of PYR1 dimer induced by ABA, DBSA or ABA/DBSA co-treatment were detected by SEC-MALLS.

Comments 9: P16 L392-L393: Please cite the paper on AS6. Nat Chem Biol 2014, 10, 477-482.

Answer: We apologize for our negligence. We have added the reference of AS6 in our manuscript.

Please see the following modifications.

Previous text	Modified text
A gate-closed conformation is observed for PYR1-AS6, and AS6 antagonize ABA signaling pathway by blocking PYL-PP2C interaction.38	A gate-closed conformation is observed for PYR1-AS6, and AS6 antagonize ABA signaling pathway by blocking PYL-PP2C interaction.[38] 38 Takeuchi, J. et al. Designed abscisic acid analogs as antagonists of PYL-PP2C receptor interactions. Nature Chemical Biology 10, 477-482, doi:10.1038/nchembio.1524 (2014)

We thank you very much for the constructive comments to improve our manuscript. If you still have comments on our manuscript, we are pleased to discuss them with you!

RE Comments from Reviewer #2:

Comment 1: Wang et al. describe the discovery of a new antagonist molecule of ABA (DBSA). This molecule relieves ABA-induced inhibition of Arabidopsis seed germination. The key point in this work is the notion that the action of DBSA depends on the stabilization of the quaternary structure of dimeric ABA receptors, while other antagonist molecules, such as ANT and ABA derivatives, bind to the receptors and hinder their interaction with the downstream PP2C phosphatases.

Both the pipeline employed for the discovery of DBSA and the "post-Morten" crystallographic analyses to uncover the basis of its activity are interesting. However, while the crystallography and in-plant studies are solid and clearly presented, there is a lack of care in the description of the pipeline for the in silico work. The biochemical data to prove the in vitro activity of DBSA are incomplete. It would be excellent if the authors made more efforts to enhance DBSA until achieving sub-micromolar affinities.

The results are nicely discussed. The reference list is complete.

There are several questions that could be addressed to enhance the quality of the data presented in this study.

Answer: We sincerely thank you for your interest on this topic and constructive suggestions. We

apologize for our mistakes in writing the manuscript. And we have improved our manuscript according to your suggestions. Please see the following comments and answers one-by-one.

Comment 2: Line 115 and 116: The assertion that "Due to the lack of dimeric ABA-bound PYL1 and *apo*-PYR1 structures…" is incorrect. Please consult PDB codes 3K3K (PYR1, with one protomer bound to ABA and the other in the apo form), 3KAY, and 3JRS (PYL1, with ABA bound and in apo forms). This is likely to affect the subsequent section until line 144. I will restrict the description of the transition from the open to the closed conformation of the PYL receptors to either PYL1 or PYR1. This adjustment should also extend to the details regarding the reduction in the volume of the binding pocket and to Figures 1 A, B, and C.

Answer: We apologize for our misleading description. In this manuscript, our aim is to design a stabilizer of PYL dimers to antagonize the ABA signaling thus reveal the relationship between PYL dimmer stabilization and seed germination. Therefore, we are more interested about the conformational change of PYL dimers before and after ABA binding, especially the interaction differences between two monomers of a PYL dimer. To achieve this goal, we want to find the apo-PYL dimer and ABA-binding PYR dimer. But unfortunately, only ABA-binding PYR1 dimer (PDB core 3K3K) and apo-PYL1 dimer (PDB core 3KAY) was suitable for analysis as they share almost the same binding pocket (although apo-PYL2 dimer was also found but it showed amino acid difference and cause pyrabactin exhibited different effect, see *J. Biol. Chem.* 2010, 285, 28953-28958). Indeed, we have noticed the crystal structure 3JRS (ABA-bound PYL1 (3JRS), but it was a monomer PYL1, which lacks the interaction information between two PYL1 monomers after ABA binding. In fact, in this sentence, we want to emphasized that we want found dimeric ABA-bound PYL and dimeric apo-PYL, and compared their differences for the stabilizer design, but due to the lacking of dimeric ABA-bound PYL1 and dimeric *apo*-PYR1, we chose the dimeric ABA-bound PYR1 and dimeric apo-PYL1 for analysis. We apologize to you again that we did not explain this issue clearly. We have modified our description to explained why chose these two structures. The description homologous residues of PYR1 and PYL1 might be hard, but we have tried to clearly describe them in our text and figures. Please see the following modifications. If you still have question about this problem, we are pleased to further discuss it with you.

Previous text	**Modified text**

To design a chemical probe that stabilizes PYL dimers, a possible binding cavity on the dimer interface was identified first. Due to the lack of dimeric ABA-bound PYL1 and apo-PYR1 structures, the binding pockets in ABA-bound PYR1 and apo-PYL1 were detected.	To design a chemical probe that could stabilize PYL dimers, a possible binding cavity on the dimer interface was identified first. As the conformational changes of PYL dimers caused by ABA was essential for the activation of ABA signaling pathway, the comparison of dimeric ABA-bound PYL and dimeric *apo*-PYL might give the important guidance for stabilizer design. But due to the lack of dimeric ABA-bound PYL1 or dimeric *apo*-PYR1 structures, the binding pockets in ABA-bound PYR1 and apo-PYL1 were detected as they shared highly conserved ligand binding pockets among dimeric PYL members.

Comment 3: Lines 149. I think authors should call Figure 1D instead of Figure 1C.

Answer: Thank you for your kind suggestions. We have modified our manuscript according to your comment. Please see the following modifications.

Previous text	**Modified text**
Based on the extended binding pocket in the apo-PYL1 dimer interface, chemical probes that may stabilize the PYL1 dimer were designed by computational virtual screening (Figure 1C).	Based on the extended binding pocket in the apo-PYL1 dimer interface, chemical probes that may stabilize the PYL1 dimer were designed by computational virtual screening (Figure 1D).

Comment 5: Figure 1D lacks clarity and does not provide useful information. Could you please overlap the binding pockets of PYL1 ABA and PYL2 PYRA (antagonist conformation), and provide the rationale for designing the new antagonist molecules? I may be mistaken, but to my knowledge, the sulfonamide moiety of PYRA is located at the bottom of the ABA binding pocket, far from the dimerization interface. In this conformation, growing the fragment from the sulfonamide molecule will not reach the dimerization interface. Please clarify this.

Answer: We apologize for any inconvenience caused to you. In this study, we want to design a compound to stabilize the PYL dimer to antagonize ABA signaling pathway. Therefore, the stabilizer is need to bind to gate-open and latch-open conformation of PYR1, PYL1 and PYL2. We aligned the gate-open PYL1 (PDB code 3KAY), pyrabactin with PYL2 (PDB code 3NR4, antagonist conformation). It was reported that due to the differences between a homologues Ile of PYR1/PYL1 and Val of PYL2, pyrabactin could not be able to bind to PYR1/PYL1 as an antagonist as in PYL2 due to steric clash of its pyridine ring (*J. Biol. Chem.* 2010, 285, 28953-28958). Therefore, to avoid steric clash and find a proper conformation for dimer stabilizer design, the pyridine group should be removed, and molecular docking was performed on the rest of molecules (Figure S1). It could be noticed that conformation towards dimer interface was easy to cause steric clash with gate open and latch open PYL1. Therefore, molecule was finally optimized into 4-bromobenzenesulfonamide group. More importantly, molecular dynamics simulations indicated that 4-bromobenzenesulfonamide group was binding tightly to gate-open and latch-open PYL1 by forming hydrogen bonds with Arg143. Through computational-based optimization, 4-bromobenzenesulfonamide group was selected as a starting structure for fragments growing to discover dimer stabilizer. Hence, the fragment growing was performed to discover NBSA and DBSA. We apologize for our description and figures that cause you confusing. We have modified our manuscript and figure 1D to explain the design of DBSA, and Figure S1 was added to explain the detailed optimization progress of starting fragments. Please see the following modifications. If you still have any problems about this issue, we are pleased to further discuss it with you.

Modified text
Computationally designed ligand to stabilize PYL dimer Based on the extended binding pocket in the *apo*-PYL1 dimer interface, chemical probes that may stabilize the PYL dimer were designed by computational virtual screening (Figure 1D). Pyrabactin could act as ABA receptor agonist (PYR1 and PYL1) or antagonist (PYL2). We analyzed the binding mode of pyrabactin as antagonist, and found steric clash was occurred to PYR1 and PYL1 (Figure S1). To avoid steric clash and find a proper conformation for dimer stabilizer design, the pyridine group should be removed, and molecular docking was performed on the rest of molecule. It could be noticed that conformation towards dimer interface was easy to cause steric clash with gate open and latch open PYL1. Therefore, molecule was finally optimized into 4-bromobenzenesulfonamide group. More importantly, molecular dynamics simulations indicated that 4-

bromobenzenesulfonamide group was binding tightly to gate-open and latch-open PYL1 by forming hydrogen bonds with Arg143. Through computational-based optimization, 4-bromobenzenesulfonamide group was selected as a starting structure for fragments growing to discover dimer stabilizer. Subsequently, fragments from PADFrag, a library of high-frequency fragments from approved drugs and pesticides, were linked to 4-bromobenzenesulfonamide using ACFIS 2.0 web server. Newly generated chemical probes were sorted according to their binding free energy. Nitrobensulfamide (NBSA) exhibited the lowest binding free energy (ΔG = -12.23 kcal/mol, Table S1). The predicted binding mode of NBSA revealed that it might form hydrogen bonds with Ser112, Arg143 and Leu144 (Figure 1E). A series of structural modifications of NBSA were made using AILDE web server. We found that DBSA, which contains an additional nitro group, exhibited the largest improvement in the binding free energy (ΔG = -18.63 kcal/mol). The additional intramolecular hydrogen bonds of DBSA were predicted to enhance the conformation of DBSA (Figure 1F). Moreover, DBSA was predicted to interact with Ser112, and its binding at the dimer interface pocket was hypothesized to hinder the Pro115 ring flip and the conformational change of His143. Thus, DBSA might bind to the pocket at the dimer interface, and thus hinder the conformational changes of the gate loop and latch loop by interacting with the key residue Ser112 to stabilize the PYL dimer.

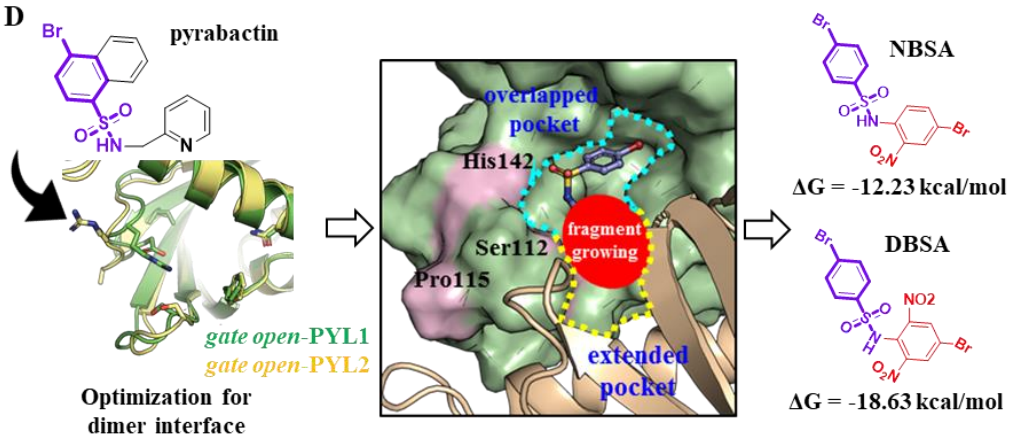


Figure 1. (D) Fragment screening was performed to discover chemical probes targeting PYL1 dimer interface. Structural optimization and molecular docking were performed on pyrabactin to suit gate open and latch open PYL1 (PDB code: 3KAY) and PYL2 (PDB code: 3NR4), and find a

conformation towards dimer interface. NBSA exhibited the lowest binding free energy. Based on structural optimization, DBSA showed the largest improvement of binding free energy in structural optimization.

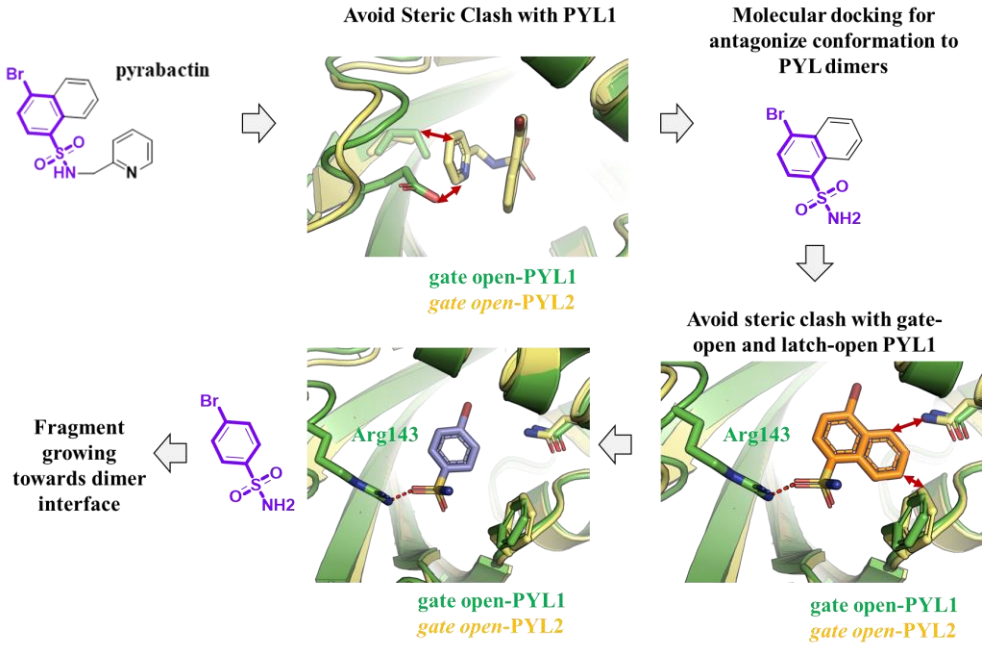


Figure S1. The detailed optimization of pyrabactin for fragment growing. Pyrabactin could act as ABA receptor agonist (PYR1 and PYL1) or antagonist (PYL2). Binding mode of pyrabactin as antagonist to gate open PYL2 (PDB code 3NR4) was analyzed, and steric clash was occurred to gate open PYL1 (PDB code 3KAY). To avoid steric clash and find a proper conformation for dimer stabilizer design, the pyridine group should be removed, and molecular docking was performed on the rest of molecules. It could be noticed that conformation towards dimer interface was easy to cause steric clash with gate open and latch open PYL1. And molecule was finally optimized into 4-bromobenzenesulfonamide group, which was binding tightly to gate-open and latch-open PYL1 by forming hydrogen bonds with Arg143.

Comment 6: Lines 163 to 175 could be omitted or moved to the supplementary material since the crystal structure already provides the experimental details of the agonist binding mode.

Answer: Thank you for your kind suggestions. We agree with you that lines 163-175 in our original version might be to redundant, therefore we combine these lines with the previous paragraph because we thought that the prediction binding mode should be introduced to make readers understand. Please see

the following modifications. If you still have any problems about the writing of this part, we are willing to further discuss it with you.

Previous text	Modified text
Computationally designed ligand to stabilize PYL dimer Based on the extended binding pocket in the *apo*-PYL1 dimer interface, chemical probes that may stabilize the PYL1 dimer were designed by computational virtual screening (Figure 1D). Pyrabactin can act as ABA receptor agonist or antagonist for different PYLs. We analyzed the binding mode of pyrabactin as antagonist in the overlapping portion of the extended binding pocket using molecular docking, and the optimized 4-bromobenzenesulfonamide was selected as a starting structure for fragments growing. Subsequently, fragments from PADFrag, a library of high-frequency fragments from approved drugs and pesticides, were linked to 4-bromobenzenesulfonamide using ACFIS 2.0 web server. A total of 4,000 newly generated chemical probes were sorted according to their binding free energy with PYL dimer. Nitrobensulfamide (NBSA) exhibited the lowest binding free energy (ΔG = -12.23 kcal/mol, Table S1). A series of structural modifications of NBSA were made using AILDE web server. We found that DBSA, which contains an additional nitro	**Computationally designed ligand to stabilize PYL dimer** Based on the extended binding pocket in the apo-PYL1 dimer interface, chemical probes that may stabilize the PYL1 dimer were designed by computational virtual screening (Figure 1D). Pyrabactin could act as ABA receptor agonist (PYR1 and PYL1) or antagonist (PYL2). We analyzed the binding mode of pyrabactin as antagonist, and found steric clash was occurred to PYR1 and PYL1 (Figure S1). To avoid steric clash and find a proper conformation for dimer stabilizer design, the pyridine group should be removed, and molecular docking was performed on the rest of molecule. It could be noticed that conformation towards dimer interface was easy to cause steric clash with gate open and latch open PYL1. Therefore, molecule was finally optimized into 4-bromobenzenesulfonamide group. More importantly, molecular dynamics simulations indicated that 4-bromobenzenesulfonamide group was binding tightly to gate-open and latch-open PYL1 by forming hydrogen bonds with Arg143. Through computational-based optimization, 4-bromobenzenesulfonamide group was selected as a starting structure for fragments growing to discover dimer stabilizer. Subsequently, fragments

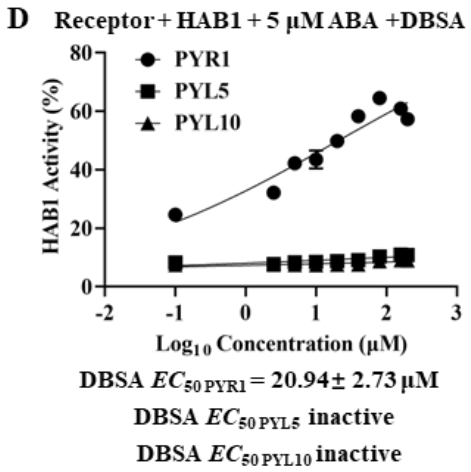
group, exhibited the largest improvement in the binding free energy (ΔG = -18.63 kcal/mol). Thus, NBSA and DBSA might bind to the PYL dimer with high affinity. To further evaluate the potential of NBSA and DBSA to stabilize the PYL1 dimer, we analyzed their binding modes. The predicted binding mode of NBSA revealed that it might form hydrogen bonds with Ser112, Arg143 and Leu144 (Figure 1C). The nitro group of NBSA forms two hydrogen bonds with the side chain of Ser112 and the positively charged side chain of Arg143. The sulfonamide group of NBSA forms a hydrogen bond with the main chain of Leu144. The additional intramolecular hydrogen bonds of DBSA were predicted to enhance the conformation of DBSA (Figure 1E). Moreover, DBSA was predicted to interact with Ser112, and its binding at the dimer interface pocket was hypothesized to hinder the Pro115 ring flip and the conformational change of His143. Thus, in theory, DBSA could strongly bind to the pocket at the dimer interface, and thus might hinder the conformational changes of the gate loop and latch loop by interacting with the key residue Ser112 to stabilize the PYL	from PADFrag, a library of high-frequency fragments from approved drugs and pesticides, were linked to 4-bromobenzenesulfonamide using ACFIS 2.0 web server. Newly generated chemical probes were sorted according to their binding free energy with PYL dimer. Nitrobensulfamide (NBSA) exhibited the lowest binding free energy (ΔG = -12.23 kcal/mol, Table S1). The predicted binding mode of NBSA revealed that it might form hydrogen bonds with Ser112, Arg143 and Leu144 (Figure 1E). A series of structural modifications of NBSA were made using AILDE web server.35 We found that DBSA, which contains an additional nitro group, exhibited the largest improvement in the binding free energy (ΔG = -18.63 kcal/mol). The additional intramolecular hydrogen bonds of DBSA were predicted to enhance the conformation of DBSA (Figure 1F). Moreover, DBSA was predicted to interact with Ser112, and its binding at the dimer interface pocket was hypothesized to hinder the Pro115 ring flip and the conformational change of His143. Thus, DBSA might bind to the pocket at the dimer interface, and thus hinder the conformational changes of the gate loop and latch loop by interacting with the key residue Ser112 to stabilize the PYL dimer.

dimer.	

Comment 7: The Kd values of DBSA for PYR1 and PYL1 are much lower than those observed for ANT (which are in the low pM range). Therefore, considering that the affinity of the ternary complex PYL1:ABA:HAB1 for ABA is around 100nM, it is unexpected to observe the effect of 50 microM DBSA on PP2C activity in the presence of 5 microM ABA. The authors should evaluate the potency of DBSA by quantifying the antagonist-mediated restoration of PP2C activity in the presence of saturating ABA, and compute an IC50 value, as shown in Vaidya et al 2021.

Answer: Thank you for your kind comments. We could understand your concerns, and we are also interested about these problems. Therefore, the EC_{50} value of antagonist-mediated restoration of PP2C activity in the presence of saturating ABA was evaluated according to the protocol provided by Vaidya et al 2021 at PNAS. And the result showed that the EC_{50} value of DBSA on PYR1 was 20.95 μM, which indicated that DBSA antagonize ABA induced HAB1 inhibition in a dose-depend way. Besides, the EC50 value of DBSA to PYL5 and PYL10 were also tried to evaluated, but the results indicated that DBSA was inactive to antagonize ABA via PYL5 and PYL10. Please see the following modifications.

Added Text and Figure
In addition, DBSA showed the highest antagonistic effect to PYR1 with an EC_{50} value of 20.94 μM (Figure 2F), but could not antagonize ABA for PYL5 and PYL10 even at high concentration.



(D) Antagonistic effect of various concentrations of DBSA on HAB1 activity through different

PYL members. EC_{50} values were obtained by nonlinear fits of dose-response data. DBSA were tested at 0 μM to 200 μM, and the concentration of ABA was 5 μM. The concentration of PYR1 and PYL5 were 0.4 μM and 0.8 μM, respectively, while HAB1 proteins were used at the molar ratio of 0.4 μM; $n = 3$; error bars represent s.t.d value.

Comment 8: It is also surprising to observe the antagonist activity of DBSA when using monomeric receptors (Figure 2E). The authors should provide an explanation for this unexpected result, or discuss it in the corresponding section. Based on these data, the title of the section should be revised. DBSA stabilizes the dimers but it might also compete with ABA in the monomers. Additionally, data on the HAB1 activity without receptor and without receptor and DBSA should be included in Figure 2E.

Answer: Thank you for your kind and professional suggestions. Thank you for your hard working to improve the quality of our manuscript. We agree with you that it was important to clarify the antagonistic effects of DBSA on dimeric PYL receptors and monomeric ABA receptors. But the PP2C inhibitory activity results were easy to be affected by the concentration of proteins and substrates. Therefore, a series of *in vitro* and *in vivo* experiments was performed again to verify the selectivity of DBSA. For the *in vitro* experiments, we first reperformed the PP2C inhibitory activity experiments again for dimeric and monomeric PYL members, and found that DBSA exhibited different antagonistic effect. For monomers (PYL5, PYL6 and PYL10), DBSA showed almost no antagonistic effect to the HAB1 inhibition induced by ABA (Figure 2C). While for dimeric members, DBSA could antagonize the HAB1 inhibition induced by ABA except for PYL3 as it is a cis-homodimer (*Structure* 2012, 20, 780-790). In addition, DBSA antagonized the HAB1 inhibitory activity of ABA in dose-depend way on PYR1, but it could not antagonize PYL5 and PYL10 even in a high concentration (Figure 2D). To further prove the reliability of our PP2C inhibitory activity results, the ITC experiments were performed between DBSA and some PYL monomeric members. It could be noticed that DBSA showed no obvious binding to PYL5, PYL6 and PYL10 (Figure S2). These results support our goal that DBSA antagonize the ABA signaling by stabilizer PYL dimers.

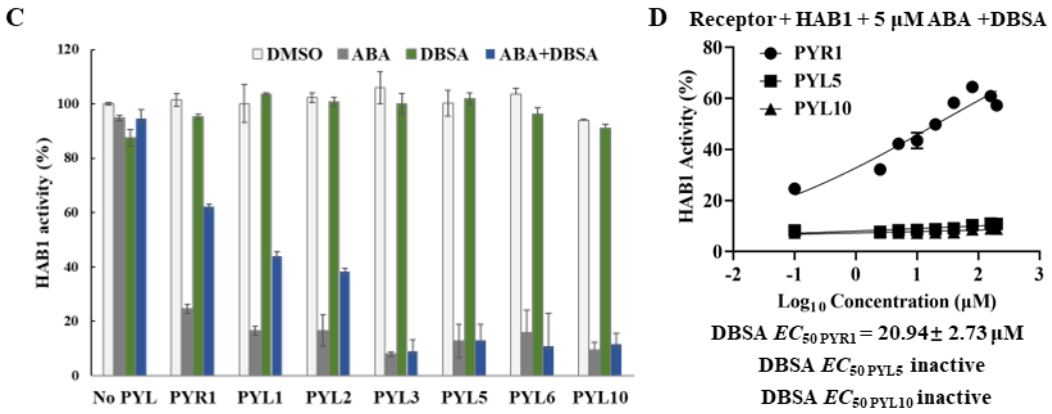


Figure 2. (C) Antagonistic effect of 50 μM DBSA on HAB1 activity through a phosphatase assay. PYLs and HAB1 were present at a molar ratio of 1:1 (0.4 μM: 0.4 μM) for PYR1/PYL1/PYL2/PYL3 and 2:1 (0.8 μM: 0.4 μM) for PYL5/PYL6/PYL10. Various PYL-HAB1 combinations were incubated with the indicated chemicals (5 μM ABA, 50 μM DBSA or 5 μM ABA and 50 μM DBSA); n = 3; error bars represent s.t.d. (D) Antagonistic effect of various concentrations of DBSA on HAB1 activity through a phosphatase assay. EC_{50} values were obtained by nonlinear fits of dose-response data. DBSA were tested at 0 μM to 200 μM, and the concentration of ABA was 5 μM. The concentration of PYR1, PYL5 and PYL10 were 0.4 μM, 0.8 μM and 0.8 μM, respectively, while HAB1 proteins were used at the molar ratio of 0.4 μM; n = 3; error bars represent s.t.d value.

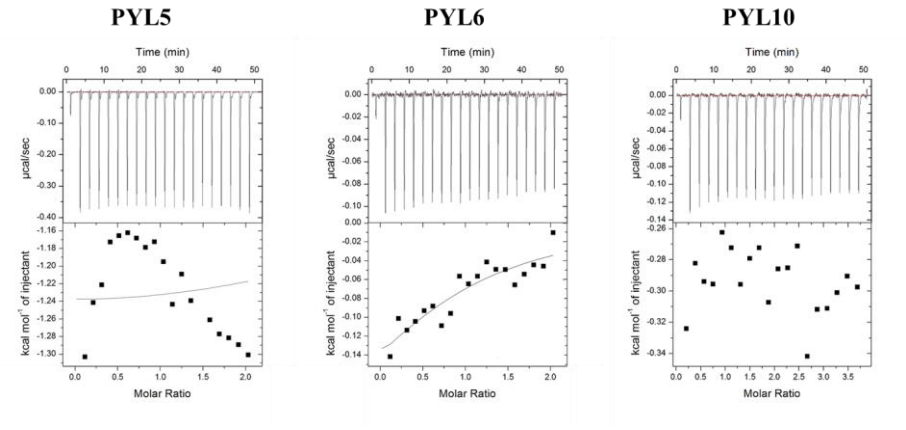


Figure S2. The ITC results of DBSA to PYL5, PYL5 and PYL10.

In addition, to further prove DBSA antagonize ABA signaling pathway through stabilizer PYL dimers, the *pyl4* overexpression (*4OE*) seeds from Prof Yuan Zheng at Henan University and *pyl3/pyl7/pyl9/pyl11/pyl12* multiple deletion mutants (*3791112*) seeds from Prof. Yang Zhao at Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences were used to performed seed

germinations experiments. The results showed that DBSA could relieve ABA-induced seed germination inhibition of *3791112* seeds, but could not for *4OE* seeds. These results indicated that DBSA might antagonize ABA induced seed germination inhibition mainly via dimeric ABA receptor members.

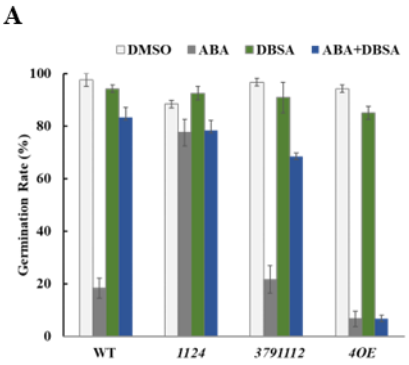


Figure 3. (A) Germination rate of seeds exposed to ABA (1 μM), DBSA (1 μM) or ABA and DBSA (1 μM:1 μM). DMSO (0.05%) was used as a control. WT and PYL dimer quadruple deletion mutant (*pyr1/pyl1/pyl2/pyl4, 1124*), PYL monomer multiple deletion mutant (*pyl3/pyl7/pyl9/pyl11/pyl12, 3791112*), PYL4 overexpression (*4OE*) seeds are considered germinated when green cotyledons expand.

We believe the *in vitro* and *in vivo* study along with the crystal structure could prove that DBSA antagonize ABA signaling pathway mainly via dimeric ABA receptors. We have also modified our manuscript according to the results. If you still have any problems about our manuscript, we are pleased to further discuss them with you to improve our manuscript.

Comment 9: Figure 4A should be omitted or sent to the supplementary materials as it represents the comparison between the experimental and theoretical models of the complex PYL1 and DBSA. Please include the RMSD for the backbone and heavy atoms in the figure caption. The authors might discuss the accuracy of their predictions in the discussion section. Instead, a new figure comparing the complexes of PYL1 with DBSA and with ABA should be included.

Answer: Thank you for your kind suggestion. We have sent Figure 4A to the supplementary materials as Figure S2, and the RMSD for the backbone and heavy atoms were shown in the figure caption. The low RMSD value indicated that our calculation was reliable. And we discussed the accuracy of

predictions in the discussion section. Besides, a new figure comparing the complexes of PYL1 with DBSA and with ABA was included in our revised manuscript as Figure 4A. Please see the following modifications.

Modified Figure

Results

DBSA binds to the pocket of PYL1 dimer interface

X-ray crystallography was performed to verify the binding mode between DBSA and PYL1 dimer. The structure of dimeric PYL1-DBSA complex was obtained at a resolution of 2.29 Å (PDB code 7DND, Table S8). Consistent with our hypothesis, DBSA is located at the binding pocket of dimer interface with a gate open and latch open conformation compared with ABA bind to a gate close and latch close conformation (Figure 4A and Figure S6). In addition, the binding mode of DBSA was similar to our predicted binding mode (Figure S7): the root-mean square deviation (RMSD) values were 1.06 Å (protein backbone atoms), 1.48 Å (protein heavy atoms) and 0.86 Å (ligand heavy atoms), respectively, which indicated that our computational model was reliable. For the detailed binding mode of DBSA, the gate loop of PYL1-DBSA complex was in its open state, and the key residues Ser112, Pro115 and His142 were in conformations similar to those in the *apo*-PYL1 structure. Furthermore, as predicted, hydrogen bond was observed between DBSA and the side chain of Arg143, but the hydrogen bond between DBSA and Ser112 was not found (Figure 4B).

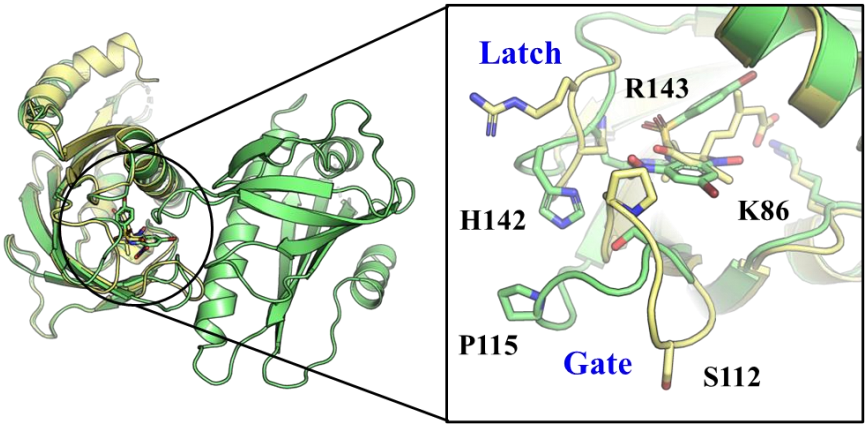


Figure 4. DBSA targets PYL1 dimer interface and stabilizes the PYL1 dimer via hydrogen bond networks. (A) Crystal structure of PYL1 with DBSA (PDB code 7DND, green) and ABA (PDB code

3JRS, yellow). ABA induced a gate-close and latch-close conformation, which DBSA bind to a DBSA induced a gate-open and latch- open conformation.

Discussion

We designed a PYL high affinity ligand DBSA via computational approach, which is the first ABA receptor antagonist by stabilizing dimeric PYL receptors. The high similarity between experiment and predicted model indicated our computational methods were reliable, which could be used for the discovery of more ligands with unique functions.

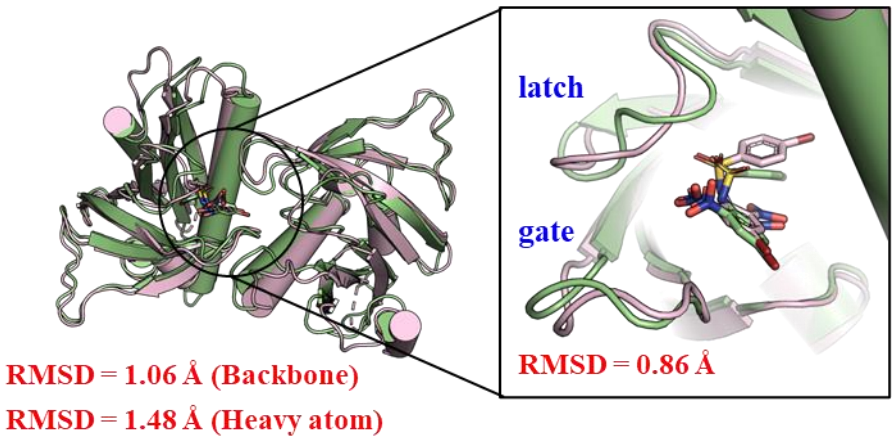


Figure S7. The comparison between predicted model and x-ray crystal structure of PYL1-DBSA complex (PDB code 7DND). The crystal structure shared a similar structure with our predicted model. The prediction model was shown in pink stick model, and X-ray crystal structure was shown in green stick model.

Comment 10: The molecular dynamic simulations were performed with ABA-bound PYR1, apo-PYL1, and DBSA-bound PYL1. I believe this is because the authors are not aware of the structure of ABA-bound PYL1 (3JRS) (see above). To provide a complete picture of the mechanism, I would suggest repeating the calculations using this structure. This adjustment affects the rest of the section.

Answer: Thank you for your kind suggestion. In this manuscript, our aim is to design a stabilizer of PYL dimers to antagonize the ABA signaling thus reveal the relationship between PYL dimmer stabilization and seed germination. Therefore, we are more interested about the effect and molecular mechanism of DBSA or ABA to the stability of PYL dimers. Indeed, we have noticed the crystal structure 3JRS (ABA-

bound PYL1 (3JRS), but it was a monomer PYL1, which lacks the interaction information between two PYL1 monomers after ABA binding. Therefore, the ABA-bound PYR1 (PDB core 3K3K), apo-PYL1 (PDB core 3KAY), and DBSA-bound PYL1 (PDB core 7DND, reported in this work) were selected for the molecular dynamics simulations as they are both PYLs dimers, and PYR1 and PYL1 shared a high sequence similarity. If you still have any questions about this problem, we are willing to discuss it with you.

Comment 11: Figure 5A: Please display all the complexes in the same orientation.

Answer: Thank you for your kind comments. We apologize for our mistakes. We have modified our figures to enable that all the complexes in the same orientation. Please see the following modifications.

Modified Figure

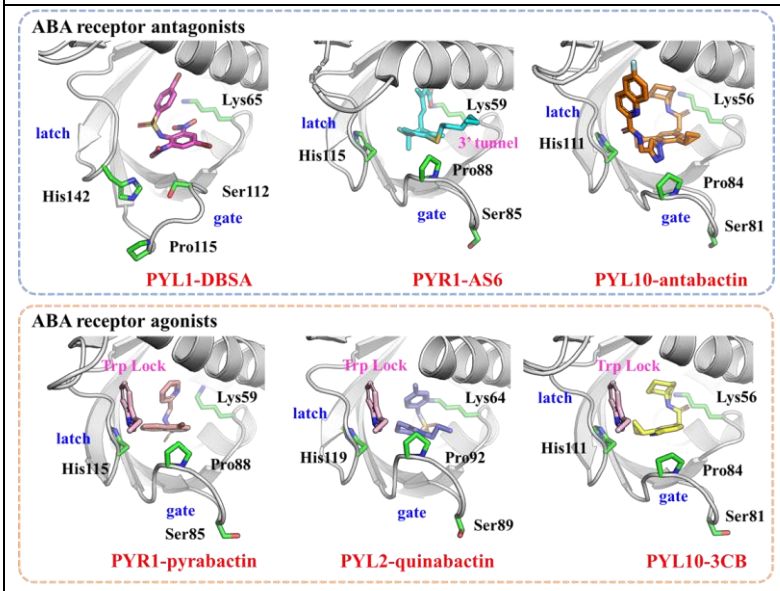


Figure 5. Mode of action of DBSA compared with those of reported ABA receptor agonists and antagonists. (A) Binding mode of different ligands with PYLs. PYL1-DBSA (PDB code 7DND), PYR1-AS6 (PDB code 3WG8), PYL10-antabactin (PDB code 7MLD), PYR1-pyrabactin (PDB code 5UR4), PYL2-quinabactin (PDB code 4LA7) and PYL10-3CB (analog of opabactin, PDB code 6NWC). PYR1-pyrabactin, PYL2-quribaction, PYL10-3CB, PYL10-antabactin and PYL5-AS6 are in latch-closed and gate-closed conformations, whereas PYL1-DBSA is in latch-open and gate-open conformation. For the latch-closed and gate-closed agonist binding conformations, a conserved Trp lock of PP2Cs were insert into the PYL pockets.

Comment 12: It would be interesting to discuss that while DBSA's antagonist activity is based on stabilizing the PYL dimers, AS6 obstructs PPase interaction by occupying the 3' tunnel, and ANT blocks the Trp lock. This insight could be incorporated into the infogram displayed in Figure 5B.

Answer: Thank you for your kind comment. There are several antagonism mechanisms reported: DBSA stabilizes the PYL dimers, AS6 obstructs PPase interaction by occupying the 3' tunnel, and ANT blocks the Trp lock. These mechanisms showed advantages in different aspects. Therefore, we have modified our figure to explain such mechanisms. Please see the following modifications:

Modified Figure

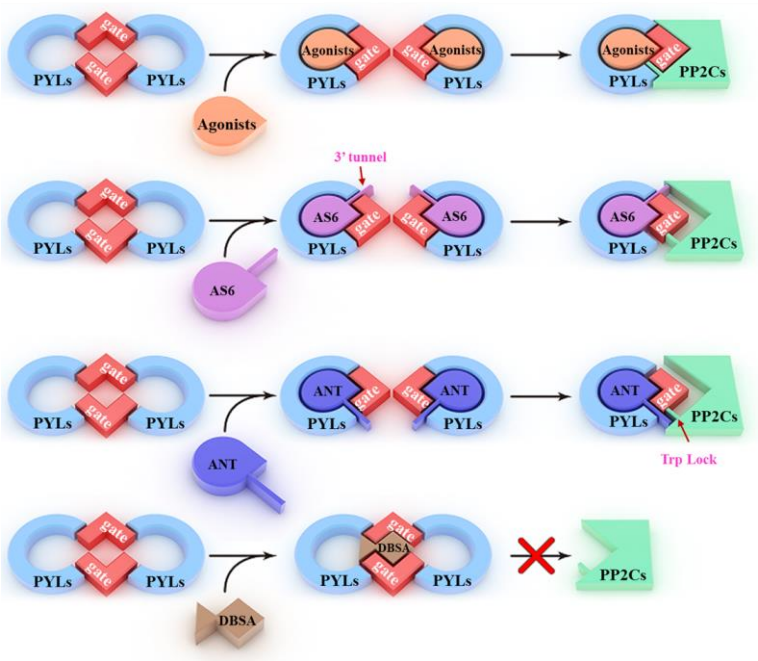


Figure 5. (B) Mode of action of ABA receptor agonists, ABA-mimic receptor antagonists AS6, antabactin and DBSA. ABA receptor agonists cause gate-closed conformations and PYL dimer dissociation, which inhibits PP2Cs. ABA-mimic receptor antagonists AS6 and antabactin also cause gate-closed conformations and PYL dimer dissociation, but AS6 obstructs the interaction between PP2C and PYLs by occupying the 3' tunnel, and antabactin blocks the conserved Trp lock of HAB1 to PYL. DBSA stabilizes the PYL dimer, which results in PP2C activation.

Comment 13: To properly evaluate the experimental approach, the methods section should provide protocols for the purification of all the receptors used for the PPase activity assays. In addition, it should include the protocol for ITC, including buffer composition for ligand and protein samples.

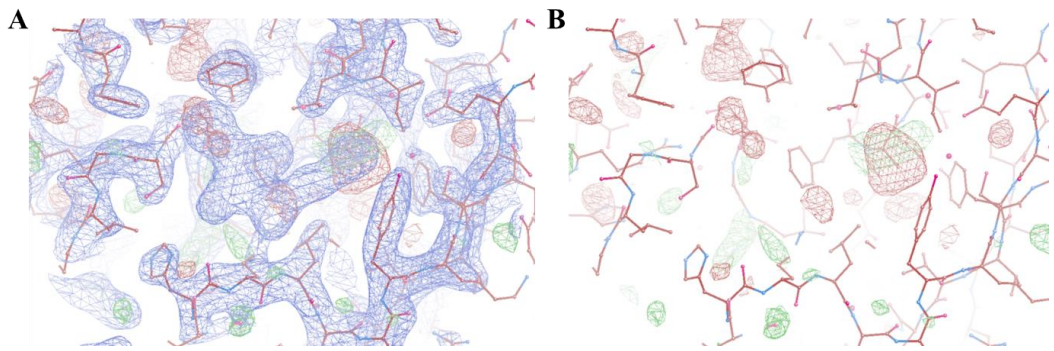
Answer: We apologize for our mistake. We have added the detailed experimental protocols in the Methods part, which contained the purification of all the receptors used for the PPase activity assays and the ITC protocol (including buffer composition for ligand and protein samples). Please see the following modifications.

Added text
Protein expression and purification. The full-length PYR1, PYL1, PYL6 and PYL10 were cloned into pET15b vector, while PYL2, PYL3 and PYL5 were cloned into pET28a vector, and HAB1 was cloned into pET15s vector to generate N-terminal His6-tagged recombinant proteins. The plasmid was transformed into *E. coli* BL21 (DE3). One litre of lysogeny broth medium supplemented with 100 mg ml^{-1} ampicillin was inoculated with a transformed bacterial preculture and shaken at 37 °C until the cell density reached an OD_{600} of 1.0-1.2. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactoside at 20°C for 12-16 h. The cells were collected by centrifugation, homogenized in buffer A (25 mM Tris, pH 8.0, 150 mM NaCl), and lysed by a lysozyme (Sigma-Aldrich). Cell debris was removed by centrifugation at 14,000 rpm and 4 °C for 1 h, and the supernatant was loaded onto a column equipped with Ni^{2+} affinity resin (Ni-NTA, Qiagen), washed with buffer B (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10 mM imidazole), and eluted with buffer C (25 mM Tris-HCl, pH 8.0, and 250 mM imidazole). The protein was then separated by cation exchange chromatography (Source 15Q, GE Healthcare) using a linear NaCl gradient in buffer A. The N-terminal His tag was removed by dRICE. The purified protein was subjected to gel filtration chromatography (Superdex-200 Increase 10/300 GL, GE Healthcare) in a buffer containing 25 mM Tris, pH 8.0, 150 mM NaCl. The buffer used for HAB1 added extra 2 mM $MgCl_2$. The peak fractions were pooled for crystallization immediately or stored at -80 °C. **ITC of PYR1 and PYL1 with ABA or DBSA.** ITC experiments were performed using an iTC200 microcalorimeter (MicroCal) in ITC buffer (25 mM HEPES, 100 mM NaCl, pH 7.5) at 25 ℃. ABA or DBSA were dissolved in ITC buffer, adjusted to pH 7.5 and used directly in titration experiments. Both protein and ligand solutions were degassed extensively and their concentrations were determined precisely using a UV/Vis spectrophotometer. In the Isothermal titration calorimetry (ITC) assay, the final concentrations of PYR1 and PYL1 were in the range of 50 to 100 μM and ABA or DBSA were in the range of 500 to 1000 μM, respectively. Each titration consisted of 19

injections, and the K_d value was fitted using a one-site binding model by MicroCal ITC200 analysis software Origin 7.0 (Malvern).

Comment 14: Please include figures showing a section of the final 2Fo-FC map and an unbiased Fo-Fc map obtained after omitting the ligand from the model in the supplementary material.

Answer: Thank you for your kind suggestions. We have included a final 2Fo-FC map and an unbiased Fo-Fc map obtained after omitting the ligand from the model in the supplementary material as Figure S2. Please see the following modifications.

Added Figures
 **Figure S6.** The final 2Fo-FC map (A) and an unbiased Fo-Fc map (B) obtained after omitting the ligand from the X-ray crystal structure.

Comment 15: Line 32 (abstract) and Line 439 (discussion). What are the genetic approaches used in this study to discover DBSA?

Answer: Thank you for your kind comments. According to the definition, chemical genetics is the study of gene-product function in a cellular or organismal context using exogenous ligands. In this approach, small molecules that bind directly to proteins are used to alter protein function, enabling an analysis of the in vivo consequences of these changes (*Plant Physiol.* 2003, 133, 448-455; *Curr. Opin. Chem. Biol.* 2001, 5, 360-367.). Indeed, chemical genetics might include a series of different approaches involving several areas. In this study, chemical design, chemical synthesis, molecular modeling, biochemistry, structural biology and botany were used to identify DBSA and reveal the stabilizing PYL dimer could antagonize ABA signaling pathway. But due to the word limit, these approaches were not added in the abstract. Please see the following modifications.

Previous text	**Modified text**

In this study, using the chemical genetic approach, we revealed that stabilizing PYL dimer can relieve the inhibition of seed germination induced by ABA.	In this study, using the chemical genetic approach, including chemical design, chemical synthesis, molecular modeling, biochemistry, structural biology and botany, we revealed that stabilizing PYL dimer can relieve the inhibition of seed germination induced by ABA.

We have modified our manuscript according to the comments of reviewers. And a labeled version of modifications is upload for peer review. We thank for the hard working for all reviewers!

Sincerely,

Ge-Fei Hao, Ph.D. & Professor

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Wuhan, Hubei, P. R. China

Email: gefei_hao@foxmail.com

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have revised their manuscript in response to each of my comments carefully and sincerely, so I do not request any further revisions.

Reviewer #2 (Remarks to the Author):

The manuscript by Wang et al. has been substantially improved from the previous version. The authors have successfully addressed most of my concerns.

However, there is an issue with their responses to comments 2 and 10 that need to be corrected before I can approve the manuscript. The crystal structure 3JRS (ABA-bound PYL1 (3JRS), consist of three protomers (Chains A, B and C) in the asymmetric unit. Of them, the chains B and C are forming a dimer. Thus, it is incorrect to state that there is no structural information on the of dimeric ABA-bound PYL1 and should be corrected. That said, in the present version of the manuscript, the authors make an additional argument for their selection of the structures of apoPYL1 and ABA-bound PYR1, as they share an almost identical ABA-binding pocket.

Dear Reviewers,

Thank you for your reviewing of our manuscript entitled "**Stabilization of dimeric PYR/PYL/RCAR family members relieves abscisic acid-induced inhibition of seed germination**", which we previously submitted for consideration of publication in *Nature Communications*. We appreciate you for the helpful comments and suggestions, which may help us to improve our manuscript. The manuscript has been revised in response to your suggestions. Please notice below our responses to your comments. Here is a summary of our changes and responses point by point:

RE Comments from Reviewer #1:

Comments 1: The authors have revised their manuscript in response to each of my comments carefully and sincerely, so I do not request any further revisions.

Answer: Thank you for your kind comments on our manuscript to improve the quality of our work. We are pleased that we have answered your concerns.

RE Comments from Reviewer #2:

Comment 1: The manuscript by Wang et al. has been substantially improved from the previous version. The authors have successfully addressed most of my concerns.

However, there is an issue with their responses to comments 2 and 10 that need to be corrected before I can approve the manuscript. The crystal structure 3JRS (ABA-bound PYL1 (3JRS), consist of three protomers (Chains A, B and C) in the asymmetric unit. Of them, the chains B and C are forming a dimer. Thus, it is incorrect to state that there is no structural information on the of dimeric ABA-bound PYL1 and should be corrected. That said, in the present version of the manuscript, the authors make an additional argument for their selection of the structures of apoPYL1 and ABA-bound PYR1, as they share an almost identical ABA-binding pocket.

Answer: We sincerely thank you for kind comments on our manuscript to improve the quality of our work. And we apologize for our mistakes. For the crystal structure 3JRS (ABA-bound PYL1), it contained three monomers in the asymmetric unit and each monomer contained an ABA. But in this study, we want to design a PYL dimer stabilizer to antagonize ABA signaling pathway. In 3K3K, the two monomers of PYL1 dimer were both in the gate close conformation, which was much different

with the apo-PYL1 dimer. And the stabilizer was need to bind at the dimer interface, and dimer might only bind with one stabilizer. Under this condition, it was more important to compare the apo-PYL dimer and PYL dimer with single ABA. But there was no PYL1 dimer with only one ABA, while PYR1 dimer bind with single ABA could be obtained (Science, 2009, 326, 1373-1379; Nature, 2009, 462, 665-668). Therefore, as you say, because they share an almost identical ABA-binding pocket, the PYR1-ABA dimer (PDB code 3K3K) was employed for the analysis of binding pocket for stabilizer discovery. We sincerely thank you for your kind suggestions. Please see the following modifications.

Previous Text	**Modified Text**
But due to the lack of dimeric ABA-bound PYL1 or dimeric apo-PYR1 structures, the binding pockets in ABA-bound PYR1 and apo-PYL1 were detected as they shared highly conserved ligand binding pockets among dimeric PYL members.	It was generally thought that one compound is sufficient to stabilize PYL dimer, but single ABA-bound PYL1 dimer or dimeric apo-PYR1 structures were lacking for the comparison of surface conformation change. Therefore, the binding pockets in single ABA-bound PYR1 dimer and apo-PYL1 dimer were used as they shared highly conserved ligand binding pockets among dimeric PYL members.

Sincerely,

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