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A thermodynamic switch modulates abscisic acid receptor sensitivity

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 20 May 2011

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and support publication here pending relative minor revisions that should not involve too much additional work to address. Given these positive evaluations, I would like to invite you to submit a suitable revised manuscript for our consideration.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Best wishes
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript “A thermodynamic switch modulates abscisic acid receptor sensitivity" by Dupeux et al. provides biochemical evidence for the existence of mono- and dimeric PYR/PYL/RCAR ABA receptors and shows, that compared to receptor monomers the dimeric receptors exhibit lower ABA affinities as consequence of a thermodynamically preferred dimer formation at low ABA concentrations or in the absence of ABA. High ABA concentrations induce rearrangements in the receptors leading to dimer dissociation. Further, the authors confirm that interaction of dimeric receptors with NHAB1 is strictly dependent on ABA, whereas monomeric receptors could interact with NHAB1 independent of ABA, however these complexes are more stable in presence of ABA. The authors report His60 of PYR1 as an important amino acid for the PYR1 dimer stability, which is affected if this His60 is mutated to Pro. Compared to WT PYR1, PYR1 His60Pro exhibits a 1-2 orders of magnitude higher ABA affinity as a consequence of a higher monomeric status of this mutant. A mechanism on how the PYR1 His60Pro mutation breaks the dimer was also provided by structural analyses. Finally, the authors provide in silico predictions on how mono- and dimeric receptors could contribute to the differential inhibition of PP2C phosphatases at different ABA concentrations leading to specific plant responses.

This manuscript provides experimental novelty, is well written and clearly recommended for publication in the EMBO journal. However, prior to publication the authors should make some minor revisions listed below.

1) PYR/PYL/RCARs may not have an activity per se. They inhibit the activity of PP2C phosphatases by forming complexes with these phosphatases and the complex formation is stabilized or enhanced by the presence of ABA. This should be re-worded throughout the text.

2) The authors may comment, why they used NHAB1 instead of HAB1 WT. Is NHAB1 easier to express? Add explanations. Please use NHAB1 instead of HAB1 throughout the text.

3) page 3, define un-activated conditions

4) page 4 middle, for the structure of PYR/PYL/RCAR-PP2C complexes cite also the other relevant papers, for example Myazono et al. (2009), Yin et al. (2009).

5) page7 and later, PAGE-SDS or better SDS-PAGE?

6) For independent HAB1 identification references, include N. Leonhardt et al. (2004) Plant Cell.

7) Figure1, please cite the corresponding references for the structures provided. Please explain the identity of the molecule shown as sticks in B, C and D. ABA? Change HAB1 to NHAB1.

8) Figure 2 and the corresponding text, please provide the PYR/PYL/RCAR: NHAB1 molar ratio and concentration used in the experiment or refer to methods.

9) page 11, change "as shown in Figure 3D, wt PYR1 does not interact with HAB1 in absence of ABA" to "as show in Figure 2 and 3A....".

10) page 11, the kinase assay is not well described, also in the methods. Please mention CABF2 in the main text.

11) In the discussion, especially pages 16-19 only few figures and references were cited. Please cite the relevant figures of this manuscript or papers that support the data discussed.

12) page 20, the references for ABA insensitive mutants are missing.

13) page 20, the reference Fujii & Zhu (2009) is incorrect?

14) page 22, please provide the Data Bank accession code.

15) page 23, " ... ternary complex was prepared by mixing PYR1H60P, ..." .

16) page 34, legend Figure 4, " ... and PYR1H60P (B)..."; " change in the sign of the enthalpy?"

17) page 35, legend Figure 5, " ... and PYR1H60P- NHAB1 (right, yellow)"; please provide also the references and accessions for the PYR1 dimer structure used.

Referee #2 (Remarks to the Author):

Evaluation
manuscript `A thermodynamic switch modulates abscisic acid receptor sensitivity.¥ by Dupeux et al.

The PYR/PYL/RCAR ABA receptor family in Arabidopsis contains 14 genes and several different receptor proteins are often co-expressed in a single cell.
In their present study the authors aim to understand whether functional differentiation among receptors exists and if how this is accomplished.
From their structural biology experiments the authors conclude receptor oligomerization can modulate hormonal responses. This conclusion was based on the finding that:
- dimeric and monomeric receptors, with different intrinsic affinities for ABA exist.
- differential properties are determined by the oligomeric state of their apo forms.
- residue in PYR1, H60 - variable between family members - determines oligomeric state.
- monomeric receptors have a competitive advantage for binding to ABA and PP2Cs.

Questions and comments:
1. Since the interpretation of the ABA mechanism is based on the affinities determined by ITC measurements the experimental details must be indicated. So far neither buffers nor is concentration of any experiment indicated, which provide crucial data for the understanding of the results!
2. The same holds for the gel filtration experiments, the author provide no details at which concentration the proteins were subjected to the SEC. Since the authors state that their approach is more physiological compared to the structure analysis as here protein concentrations are non-physiologically high, these data should be included. In addition the authors should comment on whether the concentration used in their SEC analysis is close to protein concentrations in planta.
3. To determine the ABA receptor dimer dissociation the authors have used ITC dilution heat measurements, Differential scanning calorimetry would be better suited to obtain this data, why hasn't this method be used instead?
4. The ABA affinity determined by ITC is measured as the sum of the Gibbs free energy of the dimer dissociation and the ABA binding, so how did the authors deconvolute the ABA affinity from the dissociation contribution? If this is not possible and I see a problem as both mechanisms are inherently linked the authors cannot state an affinity for ABA binding.
5. The authors use (+)ABA, has the other enantiomer be used in a control? It would be very interesting what affinity (-)ABA has for its receptors.

Minor criticism:
In table 1 the value for the enthalpy should also be indicated for Pyr1 and Pyl2 and not just described by the term endothermic.
The PDB entry code should be indicated, if the data has not yet submitted into the database this should be done during revision.
To update their introduction concerning ABA signaling, the authors may wish to cite recent publications showing that besides using OST1, ABA via RCAR/ABI1 activates the guard cell anion channels SLAC1 and SLAH3 following phosphorylation through protein kinase CPK23 respectively CPK21.

Conclusion:
Given the prime importance of the ABA signaling to overcome stress periods the present characterization of receptor oligomerization and feedback on ABA ligand affinity should be published after minor revision taking the above mention issues into account.

Referee #3 (Remarks to the Author):

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In their manuscript, Dupeux et al. demonstrate that members of the PYR/PYL family of ABA receptors can be divided into monomeric and dimeric receptors. They extend earlier observations from the Yan laboratory to provide further evidence that receptor dimerization competes with ABA binding and therefore reduces basal ABA receptor activation/PP2C interaction. This model is convincingly supported by kinetic, mutational, and structural data linking receptor-ligand affinity and ligand-independent activity to the oligomeric state of receptors. This model also helps to partially explain the high degree of ABA receptor redundancy and the range of different ABA sensitivities of different plant tissues and ABA responses. The manuscript represents a significant advance in the understanding of ABA sensing and signaling and I support its publication in EMBO Journal.

Minor points:
1. Figure 2 (and Suppl. Fig. S10) are at the heart of the paper, yet their presentation is confusing. The Y-axes are labeled as refractive index and mol. mass, both of which seem to refer just to the MALLS signals. The gelfiltration profiles dominate the figure, yet they lack Y-axis labeling (should be A280 or equivalent). Also the calculated mol. weight for the SEC peaks is not indicated. I suggest to just write the values determined by MALLS (together with the ones calculated from SEC) above the SEC peaks. These values contain just as much information as the actual MALLS signals, yet avoid the confusion with multiple (refactive index and mol. mass for MALLS) and missing (A280 for relative receptor amounts for SEC) Y-axes.
2. The oligomerization state of receptors depends on receptor concentration, yet their concentration in the SEC and MALLS experiments are not indicated. Please state concentrations in figure legends or in the Material and Methods section. Also the ABA concentration in these experiments is at least 100 times above physiological levels, which helped to accentuate the observed effects. That leaves the questions of the oligomerization state at physiological ABA concentrations. It should be made clear in the discussion that under physiological conditions the ABA levels are likely to be too low to induce dimer dissociation on their own, but rather only shift the equilibrium to more efficient PP2C-driven dimer dissociation.
3. The principal conclusions from the in silico modeling are consistent with the data and as expected. However, the assumed dimer Kd of 50 nM appears to be very low. While ITC is not suitable to determine the binding constants of tight dimers, the authors should consider alternative assays to validate this parameter for their modeling as well as to provide quantitative data for the analyzed set of receptors.
4. In the discussion and Fig. S4, the authors point out that 4 receptors, including the monomeric PYL8, have a proline at a position equivalent to Pro60 in the dimerization-deficient PYR1 H60P mutant, suggesting that this proline in combination with the adjacent phenylalanine may be the determinant for a monomeric state in these receptors. Can the authors determine the oligomerization state of the other 3 receptors and, if they indeed are monomeric, convert them to predominantly dimeric receptors by changing the proline to a histidine?

1st Revision - authors' response 14 July 2011

As requested, Please find enclosed a revised version of the Manuscript: "A thermodynamic switch modulates abscisic acid receptor sensitivity"
We have carried out a revision of the MS according to the referees‘ suggestions. Below you will find a detailed description of the modifications as well as a poin-by-point answer to the referees‘ comments. We would like to thank the referees for their constructive comments, which we are convinced have helped improve the quality of the manuscript.
We look forward that the revised manuscript might be considered for publication in the EMBO Journal

Referee #1

The manuscript "A thermodynamic switch modulates abscisic acid receptor sensitivity" by Dupeux et al. provides biochemical evidence for the existence of mono- and dimeric PYR/PYL/RCAR ABA

receptors and shows, that compared to receptor monomers the dimeric receptors exhibit lower ABA affinities as consequence of a thermodynamically preferred dimer formation at low ABA concentrations or in the absence of ABA. High ABA concentrations induce rearrangements in the receptors leading to dimer dissociation. Further, the authors confirm that interaction of dimeric receptors with NHAB1 is strictly dependent on ABA, whereas monomeric receptors could interact with NHAB1 independent of ABA, however these complexes are more stable in presence of ABA. The authors report His60 of PYR1 as an important amino acid for the PYR1 dimer stability, which is affected if this His60 is mutated to Pro. Compared to WT PYR1, PYR1 His60Pro exhibits a 1-2 orders of magnitude higher AB
A
affinity as a consequence of a higher monomeric status of this mutant. A mechanism on how the PYR1 His60Pro mutation breaks the dimer was also provided by structural analyses. Finally, the authors provide in silico predictions on how mono- and dimeric receptors could contribute to the differential inhibition of PP2C phosphatases at different ABA concentrations leading to specific plant responses.

This manuscript provides experimental novelty, is well written and clearly recommended for publication in the EMBO journal. However, prior to publication the authors should make some minor revisions listed below.

1) PYR/PYL/RCARs may not have an activity per se. They inhibit the activity of PP2C phosphatases by forming complexes with these phosphatases and the complex formation is stabilized or enhanced by the presence of ABA. This should be re-worded throughout the text.

In the new version instead of referring to the activity of PYR/PYL/RCAR receptors we refer to the "activation" of PYR/ PYL /RCAR receptors. For example the phrase "the activity of the apo form of some monomeric receptors" has been replaced by "the activation of the apo form of some monomeric receptors" (Page 21 line 19). Similar modifications have been done on page 21 line 24 and page 22 line 2.

2) The authors may comment, why they used NHAB1 instead of HAB1 WT. Is NHAB1 easier to express? Add explanations. Please use NHAB1 instead of HAB1 throughout the text.

The catalytic core of HAB1 (ΔNHAB1, aminoacids 179-511) was used , rather than the full-length protein, which is difficult to produce in recombinant expression systems

In order to clarify this point at page 8 line 1 to 4, the phrase

"We also examined interactions between receptors and the PP2C HAB1 in the absence of ABA, which revealed differences in basal PP2C interactions between monomeric and dimeric receptors."

Has been replaced by
"We also examined interactions between receptors and the PP2C HAB1 in the absence of ABA. For this purpose we used the catalytic core of HAB1 (NHAB1, aminoacids 179-511), rather than the full-length protein, which is difficult to produce in recombinant expression systems. These experiments revealed differences in Ö"

In addition to this, references to HAB1 have been replaced by NHAB1 throughout the text. This includes:

Page 8 line 6, line 13
Page 10 line 8,
Page 11 lines 10, line 11, line 13, line 14, line 23
Page 12 line 2, line 9, Line 13 , line 23
Page 13 line 4
Page 24 ,line 16, line 17,

Page 25, line1, line 11
Page 32 table II header
Page 33 line 28 Figure 3 legend
Page 34 line 21 Figure 4 legend

Also in Page 21 line 2
The phrase
"Finally, our results indicate that monomeric ABA receptors are able to interact with the HAB1 phosphatase"

Has been replaced by

"Finally, our results indicate that monomeric ABA receptors are able to interact with the catalytic core of the HAB1 phosphatase"

3) page 3, define un-activated conditions

The term "un-activated conditions" has been replaced by "under non-stress conditions" (Page 3 line 15)

4) page 4 middle, for the structure of PYR/PYL/RCAR-PP2C complexes cite also the other relevant papers, for example Myazono et al. (2009), Yin et al. (2009).

These two references have been added (page 4, line 16)

5) page7 and later, PAGE-SDS or better SDS-PAGE?

PAGE-SDS has been replaced by SDS-PAGE throughout the text.

6) For independent HAB1 identification references, include N. Leonhardt et al. (2004) Plant Cell.

This reference has been added (page 3 line 12)

7) Figure1, please cite the corresponding references for the structures provided. Please explain the identity of the molecule shown as sticks in B, C and D. ABA? Change HAB1 to NHAB1.

The protein databank codes for the molecules used in these figures have been added to the figure legend.

Two phrases have been introduced in the text of figure legend 1 to clarify the identity of the molecules shown as sticks and CPK.

Figure legend 1
Line 3: "The abscisic acid molecule is shown in CPK representation."
Line 6: "The side chains of the Leu 87, from the ABA-free subunit of the PYR1 dimer (C) and the side chain of Trp 385 from NHAB1 (D) that occupy similar positions near the PYR1 gating loops are indicated as stick models."

8) Figure 2 and the corresponding text, please provide the PYR/PYL/RCAR:NHAB1 molar ratio and concentration used in the experiment or refer to methods.

For the receptor-HAB1 interaction experiments presented in figure 2 equimolar concentrations of receptor and phosphates were used. As requested this is now specified in the legend of figure 2.

"SEC-MALLS analysis of PYR1 (top) and PYL5 (bottom) alone (left) and in the presence of

equimolar concentrations of NHAB1 (right) as described in the Materials and Methods section."

The protein concentrations used for these experiments are now clearly specified in main text (page 7 line 7)…

"Additionally, with this technique it is possible to work at moderate protein concentrations. For all the MALLS experiments described below proteins were injected at a concentration of 80 M (see the Materials and Methods section). "

…and the methods section. Page 25, lines 9 to 12

"Receptor proteins were injected at a concentration of 80 µM. For the analysis of receptor-PP2C complexes receptor molecules were mixed prior to injection with equimolecular amounts of NHAB1 (at a final concentration of 80 µM each protein) in the presence or absence of 1mM (+)ABA"

Further details on the protocols used for SEC-MALLS analysis have been added to the methods section. page 25 line 6 to 18.

"Size-exclusion chromatography (SEC) was performed using a S200 Superdex column (GE Healthcare) equilibrated with 20mM Tris pH7.5, 150mM NaCl and 1mM mercaptoethanol. For the experiments with ABA, either 1 or 5mM ABA was included in the equilibration buffer. Receptor proteins were injected at a concentration of 80 µM. For the analysis of receptor-PP2C complexes receptor molecules were mixed prior to injection with equimolecular amounts of NHAB1 (at a final concentration of 80 µM each protein) in the presence or absence of 1mM (+)ABA. All separations were performed at 20∞C with a flow rate of 0.5 mL.min-1. On-line Multi-Angle Laser Light Scattering (MALLS) detection was performed with a DAWN-EOS detector (Wyatt Technology Corp., Santa Barbara, CA) using a laser emitting at 690 nm. Data were analyzed and weight-averaged molar masses (Mw) were calculated using the ASTRA software (Wyatt Technology Corp., Santa Barbara, CA) as described previously {Gerard, 2007 #21}."

9) page 11, change "as shown in Figure 3D, wt PYR1 does not interact with HAB1 in absence of ABA" to "as show in Figure 2 and 3A....".

This change has been introduced. Page 11. Line 12

10) page 11, the kinase assay is not well described, also in the methods. Please mention CABF2 in the main text.

A specific reference to ABF2 has been included in the introduction: page 3 line 22:

"…, including ABF2, required for transcriptional activation of stress responsive genes (Fujii et al, 2009; Fujita et al, 2009; Geiger et al, 2009; Lee et al, 2009) "

A more detailed description of the in vitro kinase assay has been included in the main text. Page 11 line 22 to page 12 line 6:

"For this purpose wt PYR1, or PYR1^{H60P} were combined in a test tube with NHAB1, SnRK2.6/OST1 and a C-terminal deletion of the ABF2 transcription factor, (ΔCABF2, amino acids 1-173) as described in the Materials and Methods section. In this assay, OST1 activity is measured as auto-phosphorylation as well as trans-phosphorylation of its natural substrate, CABF2. In the absence of ABA, OST1 activity is inhibited by Δ NHAB1-mediated dephosphorylation, while addition of ABA leads to the receptor-mediated inactivation of the phosphatase and increased levels of OST1 activity (Dupeux et al, 2011; Fujii et al, 2009). As can be observed in Figure 4 C, PYR^{1H60P} was able to recover OST1 activity at lower ABA concentrations than wt PYR1."

A more detailed description of the in vitro kinase assay has been included in the methods section. Page 27 line 10 to 23 :

"OST1 phosphorylation assays were done as described previously (Belin et al, 2006; Vlad et al,

2009). Inactivation of OST1 was achieved by incubation with the protein phosphatase NHAB1. Assays to test recovery of OST1 activity were done by previous incubation during 10 minutes of NHAB1 with either wt PYR1 or PYR1H60P proteins in 30 l of kinase buffer: 20 mM Tris-HCl pH 7.8, 20 mM MgCl2, 2 mM MnCl2) in the presence of the indicated concentration of (+)-ABA. Then CABF2 recombinant protein (100 ng) and 3.5 µCi of -32ATP (3000 Ci/mmol) were added to the mix and the reaction was incubated for 50 minutes. The reaction was stopped by adding Laemmli buffer. After the reaction, proteins were separated by SDS-PAGE using an 8% acrylamide gel and transferred to an Immobilon-P membrane (Millipore)Radioactivity was detected using a Phosphoimage system (FLA5100, Fujifilm). Quantification of activity based on relative intensity of phosphorylated bands was performed using a phosphoimager Image Gauge V.4.0. The data presented are averages of at least three independent experiments."

11) In the discussion, especially pages 16-19 only few figures and references were cited. Please cite the relevant figures of this manuscript or papers that support the data discussed.

References to the manuscript figures have been added to the discussion

Page 17 line 12: (Table 1, Figure 2 and Supplementary Figure 1)
Page 17 line 16: (Figure 1)
Page 17 line 22: (|Figure 3C)
Page 17, line 24: (Figure 4A)
Page 18, Line 3: (Figure 2 and Supplementary Figure 1S)

Additional bibliographic references have been included in the discussion section:

Page 16 line 22:
"Recent structural studies have contributed significantly to the understanding of the process of ABA perception and the activation the plant ABA signaling pathway (Dupeux et al, 2011; Melcher et al, 2009; Melcher et al, 2010a; Miyazono et al, 2009; Nishimura et al, 2009; Peterson et al, 2010; Santiago et al, 2009a; Yin et al, 2009)."

Page 18, Line 7;
"Even though their ABA binding pockets and PP2C interaction regions are virtually identical (Dupeux et al, 2011; Melcher et al, 2009; Melcher et al, 2010a; Miyazono et al, 2009; Nishimura et al, 2009; Peterson et al, 2010; Santiago et al, 2009a; Yin et al, 2009). and this work)"

Page 18, line 14
"The analysis of the ABA binding regions in the PYR/PYL/RCAR family indicates that, for dimeric receptors like PYR1, PYL1 and PYL2, the residues involved in dimerization are surrounded or adjacent to residues involved in ABA binding (Dupeux et al, 2011; Melcher et al, 2009; Melcher et al, 2010a; Miyazono et al, 2009; Nishimura et al, 2009; Peterson et al, 2010; Santiago et al, 2009a; Yin et al, 2009) (see Supplementary Figure S4)."

Page 19, line 1
"all of which results in a reduction in the number of Van der Waals contacts and hydrogen bonds between the two subunits and a reduction of the dimer interaction surface (Yin et al, 2009)."

Page 19, Line 8
"which explains why both monomeric and dimeric receptors show increased affinities for ABA in the presence of clade A PP2Cs (Ma et al, 2009; Melcher et al, 2009; Miyazono et al, 2009; Santiago et al, 2009b; Yin et al, 2009)"

Page 20 Line 7
"The PYR/PYL/RCAR family contains 14 members in Arabidopsis (Ma et al, 2009; Nishimura et al, 2010; Park et al, 2009)"

12) page 20, the references for ABA insensitive mutants are missing.

The corresponding reference hass been added, Page 21 line 19:

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Fujii H, Zhu JK (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc Natl Acad Sci U S A 106: 8380-8385

See Page 29 Line 32 and bibliography section

13) page 20, the reference Fujii & Zhu (2009) is incorrect?

This reference has been corrected; See above and Page 29 Line 32

14) page 22, please provide the Data Bank accession code.

The coordinates for the PYRH60P-ABA- NHAB1 complex will be deposited in the Protein databank and the accession code will be included in the final version of the Manuscript.

15) page 23, " ... ternary complex was prepared by mixing PYR1H60P, ..." .

Indeed "H60P" had been omitted. This error has been corrected .Page 24, line 16

16) page 34, legend Figure 4, " ... and PYR1H60P (B)..."; " change in the sign of the enthalpy?"

The number "1", which was missing in PYR1H60P has been added; page 37, line 22

Concerning the "change in the sing of the enthalpy". Indeed, the enthalpy does not only change in sign but also in module. This phrase has been replaced by "Ö and the change in enthalpy": Page 37, line 24

17) page 35, legend Figure 5, " ... and PYR1H60P-NHAB1 (right, yellow)"; please provide also the references and accessions for the PYR1 dimer structure used.

"(right, yellow)" has been added. Page 38 line 8

PDB codes have been added to the figure legend (Page 38 lines 5 & 7)
The PDB code for the structure of the PYR1H60P- NHAB1 complex presented here will be included in the final version of the manuscript.

Referee #2 (Remarks to the Author):

The PYR/PYL/RCAR ABA receptor family in Arabidopsis contains 14 genes and several different receptor proteins are often co-expressed in a single cell.
In their present study the authors aim to understand whether functional differentiation among receptors exists and if how this is accomplished.
>From their structural biology experiments the authors conclude receptor oligomerization can modulate hormonal responses. This conclusion was based on the finding that:
- dimeric and monomeric receptors, with different intrinsic affinities for ABA exist.
- differential properties are determined by the oligomeric state of their apo forms.
- residue in PYR1, H60 - variable between family members - determines oligomeric state.
- monomeric receptors have a competitive advantage for binding to ABA and PP2Cs.

Questions and comments:
1. Since the interpretation of the ABA mechanism is based on the affinities determined by ITC measurements the experimental details must be indicated. So far neither buffers nor is concentration of any experiment indicated, which provide crucial data for the understanding of the results!

The methods section concerning the ITC experiments has been thoroughly revised. Details on buffers and protein concentrations used in the assays are specified in the new version. Page 25 line

21 to page 26 line 22:

2. The same holds for the gel filtration experiments, the author provide no details at which concentration the proteins were subjected to the SEC. Since the authors state that their approach is more physiological compared to the structure analysis as here protein concentrations are non-physiologically high, these data should be included. In addition the authors should comment on whether the concentration used in their SEC analysis is close to protein concentrations in planta.

The methods section concerning the MALLS-SEC analysis has been revised. In the new version a more detailed description of the protocols used is included (Page25, Lines 6 to 18) . Protein concentrations used for these experiments are now included in the methods section (page 25 lines 9 and 11) and in the main text (page 7, line 7)

Concerning the question whether the protein concentrations used in our SEC-MALLS experiments are close to physiological in planta concentrations; To the best of my knowledge, no data is available on physiological concentrations of the protein components of the ABA pathway. The fact that some responses require ABA concentrations in the low micromolar range would indicate that at least in some cases the concentration of the components of the ABA signaling pathway could be in this range. The protein concentrations used in our SEC-MALLS experiments are slightly higher (80 μM, lower concentrations lead to weak MALLS signals making determination of molecular weights unreliable. As we mention in the discussion, constitutive interactions (in the absence of exogenously added ABA) between some receptors and PP2Cs had been previously reported by other groups in yeast two hybrid systems and through proteomic methods in Arabidopsis plants (Ma et al., 2009, Park et al., 2009, Nishimura et al., 2010). However, these previous observations left open the question on whether the reported interactions were really ABA -independent or were promoted by small amounts of endogenously produced ABA (whose presence could not be ruled out in these experimental systems). The SEC-MALLS experiments we provide here allow us to rule out the presence of ABA and confirm that at least at these concentrations constitutive interactions can be observed. This lends weight to the previous observations mentioned above.

A phrase has been introduced in the discussion to clarify this issue; page 21 line 9:

"The protein concentrations used in our SEC-MALLS assays are probably higher than those found under physiological conditions, but they allow to completely rule out the presence of ABA, lending weight to previous observations of constitutive interactions (Ma et al, 2009; Nishimura et al, 2010; Park et al, 2009; Santiago et al, 2009b)."

3. To determine the ABA receptor dimer dissociation the authors have used ITC dilution heat measurements, Differential scanning calorimetry would be better suited to obtain this data, why hasn't this method be used instead?

ITC is the most direct approach to assess binding constants of protein-ligand or protein-protein interactions, and the dilution technique used here is a well established technique to measure protein dissociation . See for example M.Lovatt, A.Cooper & P.Camilleri (1996) Eur.Biophys.J. 24, 354-357; and A.Cooper (1997). Microcalorimetry of protein-protein interactions. Methods in Molecular Biology Vol.88). DSC could produce similar results. However, its interpretation may be complicated by protein aggregation and precipitation. Moreover, this type of instrument is not available in our laboratory.

4. The ABA affinity determined by ITC is measured as the sum of the Gibbs free energy of the dimer dissociation and the ABA binding, so how did the authors deconvolute the ABA affinity from the dissociation contribution? If this is not possible and I see a problem as both mechanisms are inherently linked the authors cannot state an affinity for ABA binding.

Indeed for dimeric receptors, ABA binding and dimer dissociation are inherently linked. To clarify this point in the text we now use the term "apparent ABA binding affinity" when referring to dimeric receptors rather than the term "ABA binding affinitiy" (see page 8 line 18 and 21; page 9 line2 and line 8; page 9 line 15; page 10 line 8 etc). Now Table I refers to "Apparent ABA binding affinities and oligomeric state ofÖ" (Page 34 line3)

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The relative energetic contribution to the receptor activation process can be deconvoluted through the comparison of ABA binding properties in wt PYR1 and the monomeric PYR1H60P mutant protein (see table 1). The H60P mutation destabilizes the PYR1 dimer without affecting the ABA binding cavity. This mutant shows ABA binding affinities and enthalpies similar to those of monomeric receptors. This indicates that both monomeric and dimeric receptors bind ABA in a similar manner, and demonstrates that the decreased apparent affinity is directly linked to dimerization.

5. The authors use (+)ABA, has the other enantiomer be used in a control? It would be very interesting what affinity (-)ABA has for its receptors.

This is an interesting question. However it is not in the scope of this work, and has already been treated in previous publications. See for example Park et al., 2009; Ma et al., 2009; Santiago et al., (2009) for the comparison of the differential activities and binding affinities of (+)ABA and (-)ABA. The structural aspects determining stereoselectivity have been also discussed extensively in the numerous structural works on PYR/PYL/RCAR receptors.

Minor criticism:
In table 1 the value for the enthalpy should also be indicated for Pyr1 and Pyl2 and not just described by the term endothermic. #

Quantitative estimations of binding enthalpies for PYR1 and PYL2 are not available. As stated in the text, the apparent ABA binding affinity of PYR1 for ABA was too low to obtain a quantitative determination of thermodynamic parameters through ITC (see Page 9 line 2) . However the thermogram indicates clearly an endothermic binding curve (Figure 4A). The PYR1 ABA binding affinity was estimated through NMR experiments (see page 9 line 4). However this method does not allow determination of the enthalpy. The Parameters reported for PYL2 in this paper are those reported by Yin et al. (Nat Str Biol 2009) (this protein is not available in our lab). Unfortunately the enthalpy for PYL2 was not reported in that work although again a clear endothermic binding curve could be observed in their expeiments.

The PDB entry code should be indicated, if the data has not yet submitted into the database this should be done during revision.

The PDB code for the structure of the PYR1H60P- NHAB1 complex presented here will be included in the final version of the Manuscript.

To update their introduction concerning ABA signaling, the authors may wish to cite recent publications showing that besides using OST1, ABA via RCAR/ABI1 activates the guard cell anion channels SLAC1 and SLAH3 following phosphorylation through protein kinase CPK23 respectively CPK21.

A reference to this work has been introduced in the introduction (Page 4 line 2)

Conclusion:
Given the prime importance of the ABA signaling to overcome stress periods the present characterization of receptor oligomerization and feedback on ABA ligand affinity should be published after minor revision taking the above mention issues into account.

Referee #3 (Remarks to the Author):

In their manuscript, Dupeux et al. demonstrate that members of the PYR/PYL family of ABA receptors can be divided into monomeric and dimeric receptors. They extend earlier observations from the Yan laboratory to provide further evidence that receptor dimerization competes with ABA binding and therefore reduces basal ABA receptor activation/PP2C interaction. This model is convincingly supported by kinetic, mutational, and structural data linking receptor-ligand affinity and ligand-independent activity to the oligomeric state of receptors. This model also helps to

partially explain the high degree of ABA receptor redundancy and the range of different ABA sensitivities of different plant tissues and ABA responses. The manuscript represents a significant advance in the understanding of ABA sensing and signaling and I support its publication in EMBO Journal.

Minor points:
1. Figure 2 (and Suppl. Fig. S10) are at the heart of the paper, yet their presentation is confusing. The Y-axes are labeled as refractive index and mol. mass, both of which seem to refer just to the MALLS signals. The gelfiltration profiles dominate the figure, yet they lack Y-axis labeling (should be A280 or equivalent). Also the calculated mol. weight for the SEC peaks is not indicated. I suggest to just write the values determined by MALLS (together with the ones calculated from SEC) above the SEC peaks. These values contain just as much information as the actual MALLS signals, yet avoid the confusion with multiple (refactive index and mol. mass for MALLS) and missing (A280 for relative receptor amounts for SEC) Y-axes.

This is not exact. The SEC-MALLS technique is currently very frequently used for the precise determination of molecular weights and we have chosen a standard representation for this type of experiments. See for example Gerard, et al., (2007). Biochemistry 46: 10328-10338 or Dupeux et al., (2009) Nature 462, 665-668. Indeed the molecualr weights calculated by MALLS are indicated on top of the peaks. SEC elution volumes tend to produce very poor estimations of molecular weight and have not been used here.

The MALLS analysis uses the excess refractive index (which is proportional to the protein concentration) and the static light scattering signals to determine the molecular weight of the eluting species (see Materials and Methods section). It is important to show both refractive index and MALLS signals (as we do in our figures) as it demonstrates that the signals are appropriate for the calculation. Showing the values of absorption at 280nm in addition to the excess refractive index, both of which are proportional to the protein concentration would be redundant leading to a very confusing figure. However, we have modified the legend of figure 4 to facilitate its interpretation in the following way; Page 36, line 18;

" Both the SEC elution profiles (monitored through the excess refractive index, which is proportional to the protein concentration) and the molecular size calculated by MALLS (shown above the peaks for each species) indicate dissociation ofÖ"

2. The oligomerization state of receptors depends on receptor concentration, yet their concentration in the SEC and MALLS experiments are not indicated. Please state concentrations in figure legends or in the Material and Methods section. Also the ABA concentration in these experiments is at least 100 times above physiological levels, which helped to accentuate the observed effects. That leaves the questions of the oligomerization state at physiological ABA concentrations.

As mentioned above, the methods section concerning the MALLS-SEC experiments has been revised. In the new version a more detailed description of the protocols is included (Page25, Lines 11 to 22) . The protein concentrations used for these experiments are mentioned in the methods section (page 25 line 14) and in the main text (page 7, line 8)

It should be made clear in the discussion that under physiological conditions the ABA levels are likely to be too low to induce dimer dissociation on their own, but rather only shift the equilibrium to more efficient PP2C-driven dimer dissociation.

I find this statement ambiguous, as it is not completely clear to me what the referee means by "Ö more efficient PP2C-driven dissociation". The sentence seems to suggest that physiological concentrations of ABA would not be sufficient to produce dimer dissociation and that PP2C binding to partially saturated dimeric receptors would be needed to facilitate their dissociation under physiological conditions. I do not agree with this interpretation and I think there is no experimental basis to support it. We have shown experimentally that dimeric receptors do not interact with a PP2C. Indeed their PP2C interaction surface is occluded (figure 1, figure 2 and supplemental figure 1). Moreover, using the reaction scheme of figure 6, that does not involve this type of interaction, and the in silico modeling approach, we show how both dimeric and monomeric receptors would actually be capable of responding under non-saturating concentrations of ABA in the relevant

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physiological range (page 15 line 19 to page 16 line 12 and figure 6 B), which counters the notion advance by the reviewer. Indeed, increased cellular levels of ABA, even if they are not fully saturating, would be sufficient to shift the dimerization equilibirum of the receptor producing an increased amount of monomeric ABA-bound receptors which would in turn favor the formation of stable complexes with PP2Cs. I find the idea that additional interactions between PP2Cs and dimeric receptors should exist is rather speculative and the experimental data does not support it.

3. The principal conclusions from the in silico modeling are consistent with the data and as expected. However, the assumed dimer Kd of 50 nM appears to be very low. While ITC is not suitable to determine the binding constants of tight dimers, the authors should consider alternative assays to validate this parameter for their modeling as well as to provide quantitative data for the analyzed set of receptors.

We have attempted to measure the dimer dissociation constant of wt PYR1 through analytical ultracentrifugation experiments, but no dissociation was detected at protein concentrations required to produce an adequate signal in these experiments. The influence of the value of the dimerization constant on the assays is clearly discussed in the text.

See page 15 lines 4 to 22:
"The equilibrium constant for dimer dissociation (reaction 2) is an important parameter in the model, since it determines the proportion of monomeric and dimeric forms of the receptor. Due to the stability of the PYR1 dimers it was not possible to determine this value experimentally. A series of models were generated with values of this dissociation constant in a range between 0.3 M and 0.05µM. Values above this range would have been easily detected by the ITC dissociation experiment with wt PYR1, while values much lower than 0.05 M would result in a very stable receptor dimer, which would prevent formation of the ternary complex"

And page 16 lines 14 to 19:
"The competitive advantage of monomeric receptors decreases with increasing values of the dimerization constant and at lower receptor concentrations, both conditions that would promote the dissociation of dimeric receptors. However, an advantage towards the activation of monomeric receptors is always observed for all the balanced models, even when protein concentrations are below the value of the dimer dissociation constant."

4. In the discussion and Fig. S4, the authors point out that 4 receptors, including the monomeric PYL8, have a proline at a position equivalent to Pro60 in the dimerization-deficient PYR1 H60P mutant, suggesting that this proline in combination with the adjacent phenylalanine may be the determinant for a monomeric state in these receptors. Can the authors determine the oligomerization state of the other 3 receptors and, if they indeed are monomeric, convert them to predominantly dimeric receptors by changing the proline to a histidine?

We have analysed all the receptors that were available and could be expressed as soluble proteins in our laboratory. The experiments proposed by the referee could represent an additional confirmation of the results presented here. However, the amount of work required to accomplish these tasks, would result, in the best of cases, in a significant publication delay. All the reviewers seem to agree that this is an important contribution to the field of ABA signalling, and we are convinced we have provided very solid experimental evidence to support the conclusions presented in this manuscript. Indeed referee 3 raises the present suggestion as a minor point. Being this a very competitive field I would like to ask the editors to consider publication of the MS in its present form without further delay.

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