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# Negative control of BAK1 by Protein Phosphatase 2A during plant innate immunity

Cécile Segonzac, Alberto P. Macho, Maite Sanmartín, Vardis Ntoukakis, José Juan Sánchez-Serrano and Cyril Zipfel

*Corresponding author: Cyril Zipfel, The Sainsbury Laboratory*

**Review timeline:**

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| Submission date: | 10 April 2014 |
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## Transaction Report:

(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.)

*Editor: Andrea Leibfried*

1st Editorial Decision 14 May 2014

Thank you for submitting your manuscript entitled 'Negative control of BAK1 by Protein Phosphatase 2A during plant innate immunity'. I have now received the reports from all referees.

As you can see below, all referees appreciate your findings very much and suggest only few additional amendments to better support your data and clarify some aspects. The issues are all clearly outlined in the reports and I suspect that you should be able to address them. Given the comments provided, I would like to invite you to submit a revised version of the manuscript. Please contact me in case of any questions regarding the revision.

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

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REFEREE COMMENTS

Referee #1:

The ms by Segonzac et al. describes precise biochemical and functional experiments that support a role of the phosphatase PP2A in controlling activity of the Arabidopsis PPR complex. In particular I like the identification through genetic means of the precise specific subunit composition that

 

generates specificity. The experiments are mostly complete, well presented and support the main conclusions.

There are however a few clarifications required.
1. On p. 6, in describing the results shown in Figure 2, the authors claim that the fact that PP2A associates primarily with BAK1 is consistent with BAK1 acting upstream of BIK1. This I think is not certain without evidence that only BAK1 activates PP2A activity and therefore the statement should be modified.
2. Phosphatases are themselves activated via trans phosphorylation by the associated kinase. Therefore, to complete the picture, trans phosphorylation assays of PP2A by BAK1, FLS2 or BIK1 before and after ligand application should be included. If, as could be predicted, only BAK1 trans phosphorylates PP2A (in a bik1 mutant), this would confirm that indeed both BAK1 and PP2A act upstream of BIK1. This would also serve to complete the present model of BAK1 stability and activity as shown in Figure 6 and should be an extension of Figures 2 and S6.
3. A minor point is that cantharidin will not be specific for the PP2A family member described here. Thus, it would be good to include a series of images of the treated Col-0 plants as part of for instance Figure S4 to show there are no general effects.

Referee #2:

This paper reports a negative regulatory role of a PP2A holoenzyme on flagellin-dependent Arabidopsis PTI. In particular, PP2A negatively regulates the phosphorylation status of the FLS2 PRR co-receptor BAK1 in a quantitative manner. Importantly, PP2A does not seem to affect the ligand-induced assembly of the FLS2 PRR-Co-receptor complex as such, but affects the extent of BAK1 phosphorylation only. Experiments performed here are state-of-the-art and fully support the conclusions drawn. There are a few issues though that the authors need to clarify prior to acceptance.

Phosphatase-dependent negative regulation of PRR activity is n to a new phenomenon. In particular, FLS2 activity has been associated with the activity of a phosphatase called KAPP. I am surprised to find no mention of this regulatory mechanism throughout the text.

The model shown in Figure 6 implies that BIK1 stays associated with FL2 as well as BAK1 after ligand-induced complex formation. Is there any experimental evidence to that? This is important as a previous study by the He lab has shown that BIK1 likely dissociates after stimulation. And how about the interaction of BIK1 with BAK1/FLS2 in the pp2a background? It is further surprising to see in this figure that the number of BIK1 molecules seems to get reduced upon stimulation of FLS2 BAK1 complex formation. Is there any experimental evidence for this?

Minor point: Does cantharidin actually block BAK1-associated PP2A activity?

Referee #3:

This is an exceptionally well-written manuscript with very little that is unclear. Segonzac et al. address the important question how plant immune receptors are negatively regulated. Several modes of negative regulation, such as gene expression, protein stability or inhibitory complexes have been demonstrated for the branch of the immune system controlled by resistance proteins. To my knowledge the manuscript by Segonzac et al. is the first to directly address the negative regulation of PAMP-triggered immunity on the level of the enzymatic function of pattern recognition receptors. These receptors are often receptor-like kinases, or associate with kinases. Here, the authors show that a PP2A protein phosphatase negatively regulates the RLK BAK1, which associates with the PRRs FLS2 and EFR, most likely by counteracting BAK1-mediated phosphorylation.

With the PP2A-type phosphatase-specific inhibitor cantharidin as a starting point, the authors demonstrate using several read-outs that treatment of plants with cantharidin leads to induction of defenses that is comparable to pretreatment with the FLS2 agonist flg22. This effect is shown to occur at a very early stage of the FLS2 signaling pathway, since BIK1, the kinase immediately

 

downstream of FLS2/BAK1, is phosphorylated in the presence of cantharidin. Consistent with this, a PP2A activity is immunoprecipitated with BAK1. Knock-out lines of individual PP2A subunits further show that A1, C4 and B' subunits eta and zeta mainly constitute the PP2A activity that regulates this process. Consistent with BAK1 being the regulatory target, these PP2A subunits are shown to play a similar role in responses to the EFR agonist elf18 and brassinolide, but not chitin, and that BAK1 is hypo- and hyperphosphorylated in a C4 overexpressing and c4 knock-out line, respectively. Together, these results allow the authors to propose a coherent picture of PP2A regulation of FLS2-type PTI signaling.

Essential to be addressed
The only major concern that needs to be addressed is the interpretation of results with B' eta and zeta knock-out lines in Figures 3C and 4B. In results, the authors state that these lines are significantly more resistant to bacteria (3C) or hypersensitive to elf18 (4B). However, while values are clearly intermediate for eta and zeta, the columns are labeled as not significantly different from wild-type ("ac" and "a"). Either the labels are wrong, or the statements need to be qualified.

Minor comment
In all figures showing bacterial growth, the period between cfu and cm-2 in the y-axis labels should be removed. Perhaps this occurred during pdf conversion.

Suggestions
Among the PP2A subunit knock-out lines, the B' eta and zeta lines clearly show the weakest effect, and the obvious question is what the double eta/zeta knock-out line would look like. The authors might have those data and should include them if they do or at least comment on this. This is not essential since a negative result (no marked effect in the double compared to the singles) might just indicate that additional B subunits can substitute for eta and zeta. Since this is the largest PP2A subunit family, the authors may want to comment whether eta and zeta are more closely related to each other than to other B subunits.

1st Revision - authors' response 25 June 2014

**Referee #1**

*The ms by Segonzac et al. describes precise biochemical and functional experiments that support a role of the phosphatase PP2A in controlling activity of the Arabidopsis PPR complex. In particular I like the identification through genetic means of the precise specific subunit composition that generates specificity. The experiments are mostly complete, well presented and support the main conclusions.*
*There are however a few clarifications required.*

*1. On p. 6, in describing the results shown in Figure 2, the authors claim that the fact that PP2A associates primarily with BAK1 is consistent with BAK1 acting upstream of BIK1. This I think is not certain without evidence that only BAK1 activates PP2A activity and therefore the statement should be modified.*
>> Our data in Figure 2C show that PP2A activity can only be detected in the extract enriched for BAK1, indicating that PP2A is mainly in complex with BAK1 but not with BIK1 or FLS2. It is however true that this is not sufficient to conclude that only BAK1 could regulate PP2A activity and to make any specific claims about the hierarchical relationship between BAK1 and BIK1. We have thus revised the text accordingly.

*2. Phosphatases are themselves activated via trans phosphorylation by the associated kinase. Therefore, to complete the picture, trans phosphorylation assays of PP2A by BAK1, FLS2 or BIK1 before and after ligand application should be included. If, as could be predicted, only BAK1 trans phosphorylates PP2A (in a bik1 mutant), this would confirm that indeed both BAK1 and PP2A act upstream of BIK1. This would also serve to complete the present model of BAK1 stability and*

 

*activity as shown in Figure 6 and should be an extension of Figures 2 and S6.*
>> Our observation that PP2A activity is reduced upon elf18 treatment (current Figure S7B) suggests that PP2A are inhibited, rather than activated in response to PAMP perception. Also, it is important to note that our finding that PP2A associates with BAK1 and regulates negatively its basal phosphorylation status does not necessarily implies that it is BAK1 itself that plays a role in PP2A inhibition upon PAMP perception. This regulation may actually be mediated by EFR/FLS2, BIK1, or potentially any other protein that get activated upon PAMP perception. Also, it is still unclear whether PP2A inhibition in response to PAMP perception necessarily involves phosphorylation, or another type of post-translational modification. As stated in the Discussion, we are very interested to decipher the exact *in vivo* mechanisms underlying the inhibition of PP2A activity in response to PAMP perception.

*3. A minor point is that cantharidin will not be specific for the PP2A family member described here. Thus, it would be good to include a series of images of the treated Col-0 plants as part of for instance Figure S4 to show there are no general effects.*
>> We have now added pictures of Col-0 leaves infiltrated with cantharidin (new Figure S1A), which shows that cantharidin treatment does not trigger any visible symptoms by itself under the same conditions used for the pre-treatment experiments.

**Referee #2**
*This paper reports a negative regulatory role of a PP2A holoenzyme on flagellin-dependent Arabidopsis PTI. In particular, PP2A negatively regulates the phosphorylation status of the FLS2 PRR co-receptor BAK1 in a quantitative manner. Importantly, PP2A does not seem to affect the ligand-induced assembly of the FLS2 PRR-Co-receptor complex as such, but affects the extent of BAK1 phosphorylation only. Experiments performed here are state-of-the-art and fully support the conclusions drawn. There are a few issues though that the authors need to clarify prior to acceptance.*

*Phosphatase-dependent negative regulation of PRR activity is not a new phenomenon. In particular, FLS2 activity has been associated with the activity of a phosphatase called KAPP. I am surprised to find no mention of this regulatory mechanism throughout the text.*
>> Indeed, a previous study has shown that the KAPP FHA domain interacts with the FLS2 cytoplasmic domain in yeast two-hybrid, and that *KAPP* overexpression leads to flg22-insensitivity (Gomez Gomez et al., Plant Cell 2001). However, the effect of KAPP on FLS2 activity has never been proven, and the specificity of KAPP is questionable since it was also reported to interact with many RKs (Ding et al., Biochemistry 2007). We have nevertheless amended the text to add mention of KAPP.

*The model shown in Figure 6 implies that BIK1 stays associated with FL2 as well as BAK1 after ligand-induced complex formation. Is there any experimental evidence to that? This is important as a previous study by the He lab has shown that BIK1 likely dissociates after stimulation. And how about the interaction of BIK1 with BAK1/FLS2 in the pp2a background? It is further surprising to see in this figure that the number of BIK1 molecules seems to get reduced upon stimulation of FLS2 BAK1 complex formation. Is there any experimental evidence for this?*
>> We apologize that our simplified model created confusion. We never meant to suggest that BIK1 stays associated with FLS2 and BAK1 after PAMP perception. We have accordingly modified Figure 6 to fit the current model of activation for the FLS2/BAK1/BIK1 complex. Similarly, the presence of a single symbol representing BIK1 after PAMP perception is for simplicity and should not be interpreted as an indication of protein stoichiometry.

Minor point: Does cantharidin actually block BAK1-associated PP2A activity?

 

>> We have now added data showing that cantharidin inhibits BAK1-associated PP2A activity (new figure S1B).

**Referee #3**

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*With the PP2A-type phosphatase-specific inhibitor cantharidin as a starting point, the authors demonstrate using several read-outs that treatment of plants with cantharidin leads to induction of defenses that is comparable to pretreatment with the FLS2 agonist flg22. This effect is shown to occur at a very early stage of the FLS2 signaling pathway, since BIK1, the kinase immediately downstream of FLS2/BAK1, is phosphorylated in the presence of cantharidin. Consistent with this, a PP2A activity is immunoprecipitated with BAK1. Knock-out lines of individual PP2A subunits further show that A1, C4 and B' subunits eta and zeta mainly constitute the PP2A activity that regulates this process. Consistent with BAK1 being the regulatory target, these PP2A subunits are shown to play a similar role in responses to the EFR agonist elf18 and brassinolide, but not chitin, and that BAK1 is hypo- and hyperphosphorylated in a C4 overexpressing and c4 knock-out line, respectively. Together, these results allow the authors to propose a coherent picture of PP2A regulation of FLS2-type PTI signaling.*

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*The only major concern that needs to be addressed is the interpretation of results with B' eta and zeta knock-out lines in Figures 3C and 4B. In results, the authors state that these lines are significantly more resistant to bacteria (3C) or hypersensitive to elf18 (4B). However, while values are clearly intermediate for eta and zeta, the columns are labeled as not significantly different from wild-type ("ac" and "a"). Either the labels are wrong, or the statements need to be qualified.*

>> This reviewer is correct to point out that our statistical analysis (one-way Anova followed by Dunnett's test) does not allow us to distinguish *pp2a-b'* response from either WT or *pp2a-a1* and *pp2a-c4*. Statements in the result section concerning *pp2a-b'* mutants in Figures 3C and 4B have been corrected accordingly. However, it is noteworthy that flg22-induced resistance to *Pto* DC3000 is significantly enhanced in *pp2a-b'eta* and that BAK1-associated PP2A activity is significantly reduced in *pp2a-b'eta* and *pp2a-b'zeta*.

*Minor comment*

*In all figures showing bacterial growth, the period between cfu and cm-2 in the y-axis labels should be removed. Perhaps this occurred during pdf conversion.*

>> We now have checked all concerned figures after PDF conversion and they correctly show 'cfu.cm$^{-2}$' (which is equivalent to cfu/cm$^2$) within the y-axis label.

*Suggestions*

*Among the PP2A subunit knock-out lines, the B' eta and zeta lines clearly show the weakest effect, and the obvious question is what the double eta/zeta knock-out line would look like. The authors might have those data and should include them if they do or at least comment on this. This is not essential since a negative result (no marked effect in the double compared to the singles) might just*

 

*indicate that additional B subunits can substitute for eta and zeta. Since this is the largest PP2A subunit family, the authors may want to comment whether eta and zeta are more closely related to each other than to other B subunits.*
>> We now show a phylogenetic tree of PP2A-B' subunits that has been obtained by neighbor-joining method following MUSCLE alignment of the protein sequences (Figure S2B). Although B'eta and B'zeta are not the closest subunits of this subfamily, they do form a small sub-tree with B'delta, B'theta and B'gamma. A double *pp2a-b'eta pp2a-b'zeta* mutant is currently not available, and may also be challenging to obtain given that both genes lie close to each other on the top arm of chromosome 3. Of note, expression of B'epsilon, which lies at the other extremity of the B' subunit tree, is negatively regulated by biotic stresses. Unfortunately, no insertion line is available for this gene.

2nd Editorial Decision 08 July 2014

I have now received comments from two of the original referees of your manuscript who are both satisfied with the amount of revisions. I attach their comments below. Please update once more your figures making sure that the multiplication sign is correctly depicted for all graphs (see comment of referee #3). You can send the updated figures by email to me. After this little correction, I am pleased to accept your manuscript for publication here.

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Referee #1:

My concerns have all been met by the authors in this revised manuscript. I agree that the experiment I suggested to determine the mode of activation of the PP2A protein by either one of the kinases involved is part of a future study. I have no further suggestions to make and congratulate the authors with a very nice manuscript!

Referee #3:

The authors have addressed most of the reviewers' comments very well, and have added interesting information. As for the minor point regarding the expression "cfu.cm-2", the math is trivial and needs no explanation. My point was that a period is not a mathematical operator, and that the authors should use a centered multiplication point, an "x" or simply a space. I did not think they would mistype a period on purpose.

 