CALCIUM-DEPENDENT PROTEIN KINASE5 Associates with the Truncated NLR protein TIR-NBS2 to Contribute to *exo70B1*-mediated Immunity

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Review timeline:

TPC2016-00822-RA	Submission received:	Oct. 31, 2016
	1st Decision:	Dec. 13, 2016 *revision requested*
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00822-RA 1st Editorial decision – *revision requested* Dec. 13, 2016

We have received reviews of your manuscript entitled "CALCIUM-DEPENDENT PROTEIN KINASE5 associates with the truncated NLR protein TIR-NBS2 to contribute to *exo70B1*-mediated immunity." Thank you for submitting your best work to *The Plant Cell*. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision.

(1) Improve the writing in order to clarify the ambiguous points detected by the reviewers and also to provide alternative explanations of the data.

(2) Clarify whether the fusion protein is functional and provide data on more alleles of *cpk5* and genetic complementation.

(3) Measure *CPK5* transcript level in the *exo70B1* mutant to determine whether an increase in *CPK5* expression could be the reason for the defense phenotype in *exo70B1*.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

-- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-00822-RAR1 1st Revision received Mar. 2, 2017

Editor and reviewer comments and **author responses**:

Editor:

(1) Improve the writing in order to clarify the ambiguous points detected by the reviewers and also to provide alternative explanations of the data.

RESPONSE: We have made changes to clarify the ambiguous points, and provided alternative explanations of the data. Please see Responses to the comments by Reviewers below.

(2) Clarify whether the fusion protein is functional and provide data on more alleles of cpk5 and genetic complementation.

RESPONSE: We have clarified that the CPK5-GFP fusion protein is functional and able to complement a *cpk5-2* mutant phenotype (Fig 4A). We provided data on more alleles of *cpk5* (NEW Fig. S5) and on the genetic complementation of *cpk5-2* by a genomic fragment of *CPK5* under its native promoter (NEW Fig. S6). Please also note that a CPK5-YFP translational fusion protein has already been shown to be biochemically active in a previous publication by Dubiella *et al*., (2013), in which CPK5 kinase activity was required for enhanced pathogen resistance of *CPK5-YFP* transgenic lines.

(3) Measure *CPK5* transcript level in the *exo70B1* mutant to determine whether an increase in *CPK5* expression could be the reason for the defense phenotype in *exo70B1*.

RESPONSE: We measured the *CPK5* transcript level in the *exo70B1* mutant, and *CPK5* expression was not increased in *exo70B1* (NEW Fig. S8).

Reviewer #1:

1. The authors claimed that they identified five *cpk5* alleles that suppressed *exo70B1*-mediated defense. The readers will appreciate more of the work if the phenotypes of these mutants (single and double) are shown in suppl. data.

RESPONSE: We provided data on all alleles of cpk5 (NEW Fig. S5).

2. For *cpk5-2*, the mutant happens in the second intron. It is not clear how a mutation in the intron causes a premature stop codon. Please show the position of the *cpk5-2* mutation in Fig. S4C. Does it still carry kinase activity?

RESPONSE: We indicated the position of the *cpk5-2* mutation in Fig. S4C, which results in a premature stop codon. The deduced truncated protein would contain an incomplete kinase domain, which unlikely carries kinase activity.

Biochemical assessment of recombinant CPK5 alleles in protein kinase assays reveals that these identified missense mutations lead either to full inactivity or at least to a reduction in V_{max}. Because CPK5 is regulated by calcium (and TN2), thus detailed analysis would have to include a thorough characterization of the calcium-dependency on one hand, and the respective interaction capacity with TN2 on the other hand, of each single allele. We consider this as very interesting with respect to CPK5 reaction mechanism, but we think this is beyond the key message of this manuscript.

3. In Fig. 2, the authors included *cpk5-1*, a T-DNA KO here, which is very nice. They should emphasize this in the text (they even did not mention this mutant in the result part). The same phenotype of T-DNA KO with their mutants may provide evidence that the mutants they identified are not gain-of-function or constitutively active mutants, for which they did not perform this type of assay.

RESPONSE: We emphasized this in the text as suggested. It reads "In addition, *cpk5-1*, a T-DNA knockout mutation suppressed *exo70B1* mutant phenotypes (Figure 2), indicating that the *cpk5* alleles identified here are not gain-of-function or constitutively active mutants."

4. They showed that "Overexpressing *CPK5* results in TN2-dependent autoimmunity and enhanced disease resistance, reminiscent of the *exo70B1* phenotypes". It seems that both *CPK5* expression and kinase activity are related to *exo70B1*-mediated defense. Does *exo70b1* mutant have increased *CPK5* transcript?

RESPONSE: As mentioned above (Point 3 to the Editor), we measured the *CPK5* transcript level in the *exo70B1* mutant, and *CPK5* expression was not increased in *exo70B1* (NEW Fig. S8).

5. From genetic studies, *CPK5* functions in between *EXO70B1* and *TN2*. I do not understand the sentence "TN2-activated immunity and increased immunity in *exo70B1* depend on *CPK5*". I did not find the evidence that TN2

activated defense depends on *CPK5*. From kinase assay, CPK5 also phosphorylates EXO70B1. What is the biological function of this phosphorylation? Similarly, the sentence "Thus, the autoimmune responses in *exo70B1* appear to be due to the constitutive activation of CPK5 mediated via TN2, which, in the absence of downstream substrate EXO70B1, lacks a component of the control circuit." is not clear to me.

RESPONSE: We modified these two sentences, now they read: "increased immunity in *exo70B1* depends on CPK5 and TN2", and "Thus, the autoimmune responses in *exo70B1* appear to be due to the constitutive activation of CPK5 mediated via TN2". We agree with the Reviewer that it is not clear what the biological function of the EXO70B1 phosphorylation by CPK5 is. We will further address this very interesting aspect in future, but we prefer not to speculate on it at this point.

6. In the Discussion, line 440, the sentence "Overexpression of *CPK5* leads to hypersensitive response-like cell death and enhanced pathogen resistance (Dubiella et al., 2013) (Supplemental Figure 9)" is contradictory to line 444 " Therefore, overexpression of *CPK5* alone is not sufficient for the activation of defense responses."

RESPONSE: We modified the second sentence to read: "Therefore, overexpression of *CPK5* is not sufficient for the activation of defense responses when TN2 is absent."

Reviewer #2:

There are two possible scenarios in activation of immunity in the *exo70B1* mutant. In the first scenario, TN2 is activated when Exo70B1 is absent. CPK5 is subsequently activated by TN2 and transduces defense signals to downstream components. In the second scenario, CPK5 is guarded by TN2. Loss of Exo70B1 leads to perturbation of CPK5 (either protein level or activity). TN2 senses the change in CPK5 protein and activates downstream defense signaling. In the manuscript, only the first possibility is presented. Unfortunately, the data presented cannot differentiate these two possibilities. Since the close homologs of CPK5 previously shown to function redundantly in PTI do not contribute to *exo70B1*-mediated immunity and CPK5 is not required for *edr2*, *pmr4-1*, or *acd5*-mediated resistance to powdery mildew, CPK5 appears to have a very specific role in TN2-mediated immunity. It is likely that CPK5 is a guardee of TN2. This should be discussed in the manuscript.

RESPONSE: We added the alternative explanation as suggested. It reads, "Alternatively, the activation of immunity in the *exo70B1* mutant could be explained by a guard model. In this model, CPK5 is guarded by TN2, and loss of EXO70B1 leads to perturbation of CPK5. TN2 senses the change in CPK5 protein and activates downstream defense signaling. Consistent with this model, CPK5 appears to have a very specific role in TN2-mediated immunity, as CPK5 is not required for *edr2*, *pmr4-1*, or *acd5*-mediated resistance to powdery mildew."

Minor comments:

1. Page 9, line 194-195, it is unclear how the *cpk5-2* mutation (G1522A) in the second intron of the *CPK5* gene leads to a premature stop codon (Supplemental Figure 4B). Please explain.

RESPONSE: As mentioned above (Reviewer 1, Point 2), we showed the position of *cpk5-2* mutation in NEW Fig. S4C. And we explained how the *cpk5-2* mutation leads to a premature stop codon in the text.

2. Page 11, line 245-247, "Interestingly, CPK5-G2A-GFP occurred in both a higher molecular mass form (indicating phosphorylation) and a wild-type CPK5-GFP form (Figure 4C)", a phosphatase-treated control is needed to show that the mobility shift is caused by phosphorylation.

RESPONSE: We deleted this sentence, as the point we are making here is that CPK5-G2A-GFP has defects in membrane localization, whereas CPK5-126M-GFP is kinase-inactive. None of these is able to functionally complement the *cpk5-2* mutation (Fig. 4A).

It has already previously been demonstrated that a G2A mutation yields an active CDPK protein kinase, which is still able to (auto-) phosphorylate (Witte et al., JBC, 2010), but which due to the lack of membrane localization loses its biological functionality (e.g. lack of target protein accessibility). The kinase inactive protein is unable to (auto-) phosphorylate and has likewise been demonstrated to be non-functional (Witte et al., JBC, 2010).

TPC2016-00822-RAR1 2nd Editorial decision – *acceptance pending* **Mar. 17, 2017**

We are pleased to inform you that your paper entitled "CALCIUM-DEPENDENT PROTEIN KINASE5 associates with the truncated NLR protein TIR-NBS2 to contribute to *exo70B1*-mediated immunity" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **Mar. 24, 2017**