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Peer Review Report for 10.1105/tpc.16.00078

To increase the transparency of the review process, The Plant Cell will publish Peer Review Reports for all articles by January 2017, which will include decision letters, reviewer comments, and author responses. Reviewer anonymity will be strictly maintained. The reports will show only the most substantive parts of letters and comments; minor comments for revision, mundane paragraphs from letters, miscellaneous correspondence, and confidential comments for the editors (including pre- and post-review consultation sessions) will not be published. Any unpublished data submitted confidentially in response to reviewer comments (e.g. figures, tables; data not intended for the manuscript under review but only to support responses to reviewer comments) will also be omitted. The text of reviewer comments and author responses will be unedited except to correct typos and minor grammatical errors (where noticed and easily corrected), and to remove minor and confidential comments.

This report is published as part of a pilot program, including a small set of articles, with the approval of all respective authors and reviewers, to introduce readers and authors to the concept and test the format.

Casein Kinase1-Like Protein2 Regulates Actin Filament Stability and Stomatal Closure via Phosphorylation of Actin Depolymerizing Factor

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TPC2015-00136-RA	Submission received:	Feb. 25, 2015
	1st Decision:	Mar. 12, 2015 *manuscript declined*
TPC2016-00078-RA	Submission received:	Feb. 3, 2016
	1st Decision:	Mar. 2, 2016 *revision requested*
TPC2016-00078-RAR1	1st Revision submitted:	Apr. 12, 2016
	2nd Decision:	Apr. 19, 2016 *accept with minor revisions*
TPC2016-00078-RAR2	2nd Revision submitted:	May 9, 2016
	3rd Decision:	May 11, 2016 *accept with minor revisions*
TPC2016-00078-RAR3	2nd Revision submitted:	May 12, 2016
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REPORT

(Note: The report shows the major requests for revision and author responses to major reviewer comments. Miscellaneous correspondence and confidential comments for the editors 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.

TPC2015-00136-RA 1st Editorial decision – *declined* Mar. 12, 2015

All the reviewers and the reviewing editor agree that this study has great potential impact. However, there are significant concerns with respect to how the in vitro studies were carried out and the subsequent interpretation. These concerns were shared by multiple reviewers in the post-review consultation and have diminished enthusiasm for the current form of the manuscript. As you will see, reviewer 2 has very specific suggestions for how to improve these experiments. If these experiments can be performed as the standard in the field would require, then this study

promises to have high impact. However, in the interest of providing a rapid turn around and not requiring too many extensive experiments, we feel that this work can not be recommended for publication at this point.

Another common theme that arose amongst all reviewers is the lack of evidence supporting specific phosphorylation ADF4. The authors could address this by performing more in vitro studies, and even if phosphorylation is not specific to ADF4, as long as it is specific to ADF, then the work still holds impact for the field.

-- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-00078-RA Submission received	Feb. 3, 2016

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1 (Comments for authors):

Overall, this is a nice manuscript with an abundance of interesting data. Corrections of several issues outlined below will greatly strengthen the manuscript.

Point 1.

-Figure 1 legend change: complemented > rescue. Complementation is a well-established genetic term that refers to a wild-type phenotype in the progeny of a cross. The authors used rescue of a mutant phenotype by genetic transformation, not complementation.

-The authors used Student's t test for significance. This test is only valid when the data are normally distributed. The authors need to perform a test of normality on their data before using the Student's t test, or use the Mann-Whitney U test instead. Furthermore, the authors need to provide the P values, not just (P<0.01). Readers want to know if the p value is 0.0098 or 6x10e-12.

> **RESPONSE: As suggested "complement" in the manuscript has been changed to "rescue". As suggested, before using the paired student's t test, all of the data in the manuscript has been used to perform a test of normality. The analyses show that the data in the manuscript are normally distributed. The P values from corresponding pairs of columns are indicated in the revised manuscript.**

Point 2. The section entitled: CKL2 expression is induced by water-loss and ABA treatment. There are several serious problems with this section. Here the authors used a putative promoter-GUS fusion to examine the expression pattern of CKL2 in transgenic Arabidopsis plants. First, the authors did not sufficiently demonstrate that they had identified the regulatory region(s) necessary for proper regulation of CKL2 gene. There is a well-established standard for promoter-GUS analyses that was adopted many years ago. (see Taylor, C.B. 1997. Promoter Fusion Analysis: An Insufficient Measure of Gene Expression. Plant Cell 9: 273-275, and references therein). Without corroborating in situ hybridization data, or a promoter deletion analysis using the full length gene, there is insufficient evidence that the correct regulatory sequences have been identified. Therefore, the results of their GUS assays cannot be properly interpreted.

Second, the authors should make use of the ample mRNA expression data available from the countless microarray datasets on the web (see ArrayExpress http://www.ebi.ac.uk/arrayexpress/, and Genevestigator https://www.genevestigator.com/gv/plant.jsp. Key advantage of the microarray data is that it is quantitative, and much more comprehensive with respect to anatomy and growth conditions. ArrayExpress has 2200 Arabidopsis microarray experiments.

Clearly microarray expression information outweighs a few qualitative figures of plants stained for GUS expression. Since the authors have not provided any evidence that they have identified the full native regulatory sequences for CKL2, the (putative) promoter-GUS fusions experiments should be removed and replaced with a thorough analysis of the existing microarray data.

> **RESPONSE: We thank for this comment and agree that is a problem for most studies using promoter-GUS to**

determine the tissue specific expression pattern. As suggested, we removed the CKL2 promoter-GUS results in the revised manuscript and consulted the microarray datasets on the web including AtGeneExpress (http://jsp.weigelworld.org/expviz/expviz.jsp) and e-FP browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The microarray data show that CKL2 express in various tissues throughout development periods including in guard cells (Supplemental Figure 2A and 4). We also performed real time PCR with CKL2 specific primers using the total RNA extracted from different tissues including root, stem, rossete leaf, cauline leaf, flower and silique (Supplemental Figure 2B). The microarray data also indicate that CKL2 is induced by ABA and drought treatment (Supplemental Figure 1C and 4), which is consistent with the real time PCR results shown in Figure 1F and 1G. There is also a previous study showed that CKL2 expression is induced by ABA treatment (Cui et al., 2012).

Point 3. Lines 94 and 95, the authors state: "However, such kinases have yet been identified in plant cells." This is not entirely true. The papers below show fairly convincingly that there are plant kinases that phosphorylate ADF. The authors should remove their statement, and discuss the previous kinase/ADF work, including the results of Ouellet et al., which the authors omitted.

Allwood, E.G., A.P. Smertenko and P.J. Hussey. 2001. Phosphorylation of plant actin-depolymerising factor by calmodulin-like domain protein kinase. FEBS Lett. 499: 97-100.

Ouellet, F., E. Carpentier, M.J. Cope, A.F. Monroy and F. Sarhan. 2001. Regulation of a wheat actin-depolymerizing factor during cold acclimation. Plant Physiol 125: 360-368.

Smertenko, A.P., C.J. Jiang, N.J. Simmons, A.G. Weeds, D.R. Davies and P.J. Hussey. 1998. Ser6 in the maize actin-depolymerizing factor, ZmADF3, is phosphorylated by a calcium-stimulated protein kinase and is essential for the control of functional activity. Plant J. 14: 187-193.

RESPONSE: As suggested, we have removed the statement "However, such kinases have yet been identified in plant cells." in the revised manuscript. In addition, we discussed the previous kinase/ADF work including the results of the phosphorylation of TaADF is regulated by low temperature (Ouellet et al., 2001). These publications are cited.

Point 4. The authors should quantify the colocalization of CKL2 with actin filaments. See Dunn, K.W., M.M. Kamocka and J.H. McDonald. 2011. A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300: C723-C742 for a practical discussion of using Pearson's correlation coefficient.

RESPONSE: As suggested, the colocalization of CKL2 with actin filaments has been quantified in the revised manuscript (Figure 2G, Dunn et al., 2011; Wu et al., 2012; Mcdonald et al., 2013) with slight modification. The method of colocalization analysis is indicated in the revised manuscript. The data are analyzed by Pearson's correlation test. PCC value is measured as 0.83. P value is calculated as 0.0003 ($P<0.001$). The assays show that there is strong correlation of spatial localization between GFP-CKL2 signal and Rhodamine-phalloidin stained actin filaments.

Point 5. In Figure 2G, the authors should also show that their oryzalin treatment can depolymerize microtubules (using a fluorescent protein microtubule marker), and that their LatA treatment can disrupt the actin cytoskeleton (using an appropriate actin filament marker).

RESPONSE: As suggested, GFP-MBD labeled microtubules was used as a control showing that the oryzalin treatment can effectively depolymerize microtubules but not CKL2 labeled filamentous structure, and GFP-fABD2-GFP labeled actin filaments was used as a control showing that our LatA treatment can effectively disrupt the actin cytoskeleton (Figure 2H and 2I).

Point 6. Figure 3 (and elsewhere): see above for comments on the use of Student's t test and p values.

RESPONSE: Statistical analyses are modified as suggested in the revised manuscript.

Point 7. Figure 3B: The authors did not state how many times the experiment was replicted

RESPONSE: All of the experiments in this study are repeated at least for 3 times, we added the note in the revised manuscript.

Point 8. Figure 4B: The authors state that in the mutant, P-ADF was significantly reduced... The ratio of ADF4 to phosphorylated-ADF4 needs to be determined, quantified, and significance tests need to be performed.

RESPONSE: As suggested, the ratio of phosphorylated-ADF4 was quantified by densitometry and plotted using ImageJ software (http://rsbweb.nih.gov/ij/; version 1.38). The data were used for normality test first and then subjected to student's t test for difference.

Point 9. The authors should examine some of the available proteomic data sets that are specific for guard cells to see if CKL2 and ADF4 are present. This would strengthen their in vivo argument.

RESPONSE: As suggested, we tried to look up the public proteomic data sets, but we did not find such information. However, the expression data on the web (http://jsp.weigelworld.org/expviz/expviz.jsp, Supplemental Figure 4) suggest that both CKL2 and ADF4 express in guard cells.

Reviewer #2 (Comments for authors):

Unfortunately, for a number of reasons listed below, many biochemical experiments are not performed or analyzed correctly in this manuscript, rendering the understanding of the effects of CKL2 very difficult.

Point 1. Figure 3F-G and Supplemental Movies tracking actin filaments in cells. Could the authors provide better movies and detailed time series of images? It is very hard from these examples to understand how the authors measured all the parameters quantified in Figure 3H.

RESPONSE: As suggested we have provided better movies and detailed time series of images (Figure 4A and Supplemental Movie 1 and Movie 2) in the revised manuscript. The representative individual actin filaments are highlighted with blue dots. The severing events are indicated with yellow arrows.

Point 2. Figure 4: Kinase assays are not performed convincingly. The authors should provide autoradiographs in the presence and in the absence of kinase as negative controls. Also, the major problem in these experiments is the concentration of kinase that is used. Usually, several nanograms of kinase are sufficient to phosphorylate specifically proteins in vitro, and excess of kinase in solution can trigger non-specific phosphorylation. I would also recommend the authors to provide phosphorylation data with other purified actin binding proteins or cofilin isoforms, in order to demonstrate the specificity (or not) of ADF4's phosphorylation.

RESPONSE: As suggested, we used actin binding protein SCAB1 (Zhao et al., 2011) as a negative control. In addition, we also provided autoradiographs of ADF4 in the absence of kinase as a negative control (Figure 5C). The results demonstrate that CKL2 phosphorylated ADF4 but not SCAB1.

We further determined whether CKL2 specifically phosphorylates ADF4 among other ADF isovariants. To do this, we selected ADF proteins from subclass I, including ADF1, ADF2, and ADF3, to determine whether they can act as substrates for CKL2. Our results showed that CKL2 could phosphorylate all of those ADFs, which to some extent suggests that CKL2 does not have preference for ADF isovariants.

The activities of kinases vary greatly while being assayed in vitro compared to that in vivo. Several kinases working on our hands are good examples. For examples, SOS2 shows a very low kinase activity in vitro, though its in vivo activity is induced by salt stress (Guo et al., 2001; Guo et al., 2004; Quan et al., 2007; Lin et al., 2009; Zhou et al., 2014); another kinase PKS5 (Fuglsang, et al., 2007; Yang et al., 2010; Du et al., 2011; Lin et al., 2014) has a very strong auto kinase activity in vitro, however, it is very hard to detect its kinase activity in vivo. This is similar to CKL2 that has a strong auto- but weak trans- activity in vitro. Nonetheless, it remains to be determined how the CKL2 kinase activity is precisely regulated by ABA/drought.

Point 3. Figure 5A and 5B: High-speed co-sedimentation assays do not demonstrate any **depolymerizing/disassembly activity. At a 2 µM concentration, ADF/cofilin** decorates and stabilizes actin filaments (see Andrianantoandro et al., Mol Cell, 2006), but does not modify significantly the critical concentration of actin at steady-state (see for example Ressad et al., JBC, 1998). Therefore, differences observed in this experiment are likely to be artifacts. I refer the authors to other papers (for exemple Blanchoin et al., JBC, 1999 or Andrianantoandro et al., Mol Cell, 2006) to find appropriate methods to characterize these differences.

RESPONSE: To yield a quantitative view about the effect of CKL2 phosphorylation on ADF4's actin disassembling activity, we initially re-performed the high-speed F-actin cosedimentation assay via incubating 4 µM preassembled actin filaments with different concentrations of ADF4 after phosphorylated by CKL2 or not, as well as incubating with different concentrations of ADF4's phosphormimic protein (ADF4 S6D) or ADF4's unphosphorylatable protein

(ADF4 S6A). Our results showed that ADF4 disassembles actin filaments in a dose-dependent manner, and phosphorylation of ADF4 by CKL2 decreased its actin disassembling activity (Figure 6A and B). Our results also showed that Serine 6 of ADF4 might be the major phosphorylation site for CKL2 (Figure 6A and B).

To complement the high-speed F-actin cosedimentation assay, we further performed the kinetic actin depolymerization assay, fluorescence light microscopy, and TIRFM assay to determine the actin disassembling activity and severing activity of ADF4 as well as the effect of CKL2-mediated phosphorylation of ADF4 on the F-actin disassembling and severing activities of ADF4. Please find the results in the revised manuscript (Figure 6E, 6F and 6G).

Point 4. Supplemental Figure 3B-3C and Figure 5C-5D: Because ADF/cofilin does not modify significantly the critical concentration of actin at steady-state (see above), one would expect a similar amount of actin polymer in all these images. Differences on the length of the filaments are also hard to interpret given the fact that actin filaments re-anneal easily in solution.

More convincing experiments should include pyrene actin assembly/disassembly assays in the presence of CKL2 +/- a low concentration of cofilin (WT or mutant). Such experiments would indicate unambiguously whether CKL2 affects the kinetics of actin assembly/disassembly.

RESPONSE: As suggested we have improved our experiments to determine the effects of CKL2 phosphorylation on ADF4 activity to actin.

Fist, considering ADF/cofilin does not modify significantly the critical concentration of actin at steady-state (Hawkins et al., 1993; Carlier et al., 1997; Maciver et al., 1998; Ressad et al., 1998; Blanchoin and Pollard, 1999), we performed high speed cosedimentation assays using eluding 1:1 and 1:2 molar ratios of ADF4 with 4 μM F-actin (Figure 6A and 6B).

Second, we carried out actin filaments fluorescence microscopy assays using 1:1 molar ratio of ADF4 with 4 μM F-actin according to (Okada et al., 2002; Andrianantoandro et al., 2006) in Figure 6C and 6D.

As suggested, to further determine whether CKL2 phosphorylation affects the effects of ADF4 on the kinetics of actin assemble/disassembly we performed pyrene actin disassembly assays as previously described (Andrianatoandro et al., 2006; Jansen et al., 2014) using low concentration of ADF4 after phosphorylated by CKL2 or not and found that ADF4 promotes actin disassembly whereas CKL2 phosphorylation inhibits its actin disassembling activity (Figure 6E).

To further determine the effect of ADF4 on the dynamics of single filaments, we directly visualized the behavior of single filaments by total internal reflection fluorescence (TIRF) microscopy and showed that ADF4 generates breaks along actin filaments whereas CKL2 phosphorylation inhibits this activity (Figure 6F and 6G). Therefore, our results demonstrate that ADF4 affects the kinetics of assemble/disassemble in vitro.

As suggested we also improved our experiments of biochemical assays to determine the effect of CKL2 on actin including spontaneous actin nucleation assay to detect whether CKL2 has effect on actin assembly (Ressad et al., 1998; Blanchoin and Pollard, 1999; Andrianatoandro et al., 2006; Jansen et al., 2014).

Reviewer #3 (Comments for authors):

Most of the experimental data is solid and presented clearly. I have several suggestions about specific experiments and their presentation to further improve the clarity of the manuscript and several suggestions for additional experiments that will make the story more complete and textual changes that will improve the clarity of the concept that is presented. The importance of the comments has been prioritised.

Point 1. Figures are very small, particularly figure 3, 5 and 6. It is impossible to read the information in the graphs and the images are too small to interpret them. I would suggest to increase the size of the figures to the whole width of the page..

RESPONSE: As suggested the size of the figures are increased in the revised manuscript.

Point 2. Single channel fluorescence images will be much clearer when they are presented in black and white rather than green and black.

RESPONSE: As suggested GFP channel fluorescence images in green and black have been changed that in black and white.

3. Although the language of the paper is comprehensible and as non-native English speakers the authors have done a great job in writing the manuscript, there are many small grammatical mistakes that should be corrected.

RESPONSE: As suggested, we read the manuscript more carefully, and corrected more grammatical mistakes in this revised manuscript.

4. The discussion is weak. Rather than focusing on what is unknown, the authors should position their novel concept in the wider fields of the control of actin dynamics and regulation of stomatal apperture.

RESPONSE: As suggested, we discussed more about how CKL2 phosphorylation on ADF4 is involved in regulation stomatal aperture through control actin dynamics.

5. Although I am not a specialist in stomata, the term 'stomatal movement' sounds confusing since it suggests that the stomata migrate. I Googled the term and only 9 hits were returned. Apparently, it is not a term that is used often. I would suggest to use stomatal aperture or stomatal opening and closing rather than stomatal movement.

RESPONSE: As suggested, we changed the "stomatal movement" used in this manuscript to more precise description as "stomatal opening" or "stomatal closure".

6. It is surprising that CKL2 localises to the actin cytoskeleton. Under most circumstances, ADF is a cytoplasmic protein and although LIM-kinases that phosphorylate mammalian ADF preferentially localizes to regions with high actin turnover, as far as I know, no (putative) ADF kinases localize to actin filaments. A logical hypothesis is that the interaction between CKL2 that does not bind to actin by itself and the actin cytoskeleton is mediated by the actin binding protein ADF that is capable of binding both actin and CKL2. This hypothesis can be tested by studying the localization of ADF4 in stomata in closed and opened state (since the localization of ADF4 may differ between these states). The authors have studied co-localization of CKL2 and actin in many different cell types, but they forget to study the co-localization of CKL2 with the actin cytoskeleton in guard cells of stomata in closed and opened state. Together, these two experiments will (dis)prove my hypothesis and increase the impact of the paper.

RESPONSE: GFP-CKL2 collocates with actin filaments during stomatal closure or opening in both Col-0 and adf4 mutant. We do not find the translocation of CKL2 from actin filaments to cytosol during stomatal movement. CKL2 interacts with subfamily I ADFs, therefore we cannot exclude that ADFs play a role to mediate the actin filament localization of CKL2. However our results suggest that regulation of CKL2 kinase activity may be more important for actin dynamics during stomatal movement.

We transformed the mCherry-ADF4 plasmid to both Col-0 and ckl2 mutant, and we are not able to obtain a transgenic line in which the mCherry-ADF4 locates at actin filament in guard cells.

In terms of the intracellular location of ADF, there are different results out in the literatures. Indeed, it was reported that ADF is cytosolic in roots hairs using immunostaining approach probed with anti-ZmADF3 antibody (Smertenko et al., 1998). Meanwhile, there are several recent reports showed that ADF proteins decorate actin filaments in cells. For instance, it was shown that ADF7 and ADF10 mainly decorate actin filaments in pollen tubes (Bou Daher et al., 2011, 2012; Zheng et al., 2013). In the case of ADF4, it was shown that ADF4 colocalizes with actin filaments in plants expressing 35S:: mCherry-ADF4 (Tian et al., 2009; Porter et al., 2012), suggesting that ADF4 may also decorate actin filaments in guard cells. Further, we found that, similar to that in other cells, CKL2 forms filamentous structures in guard cells.

7. The authors give a weak motivation (similar expression patterns) for selecting ADF4, and later they indicate that there may be other ADFs that are targeted by CKL2. The text in the results section suggests that the interaction of CKL2 and ADF4 is specific. I would suggest to tone this down by clearly indicating that ADF4 was selected for further experimentation, but that this does not mean that CKL2 specifically interacts with ADF4.

RESPONSE: As suggested, we toned down our conclusion in the revised manuscript. We also performed new kinase assays to show that CKL2 phosphorylates other ADFs in subclass I.

8. The authors should discuss the relevance of actin reorganization on the regulation of stomatal aperture. Their results offer the possibility to uncouple actin dynamics from other ABA induced processes. What does this tell us about the role of the actin cytoskeleton in the opening and closing?

> **RESPONSE: As suggested, we have revised the discussion part and discussed more about actin reorganization on the regulation of stomatal aperture during drought response. It is generally accepted and relatively well-characterized that actin filaments undergo reorganization during stomatal closure and opening, and actin dynamics have been implicated in mediating intracellular Ca^{2+} fluxes, regulation of K^+ channel activity as well as maintenance of vacuole shape (Hwang et al., 1997; Gao et al., 2009; Zhao et al., 2013), though the cause and consequence relationship between actin dynamics and these physiological cellular processes remains to be established. In particular, however, how diverse signals were perceived and subsequently integrate into actin dynamics remains to be characterized. Our study points out a new pathway of regulating actin dynamics mediated by CKL2 through phosphorylating the central regulator of actin dynamics, ADF, during stomatal closure. How exactly CKL2 perceives the upstream stress signals as well as the relationship of CKL2-mediated regulation of actin dynamics pathway with other actin dynamics regulatory pathways remains to be characterized. Nonetheless, our study suggests that reorganization of actin filaments is an essential step during stomatal closure and opening, which allows stomatal closure and opening to be achieved finally via perceiving different signals.**

9. It should be better explained what serine 3 in mammalian ADF and serine 6 in plant ADFs have in common. The reader has to guess that these serines are the same main phosphorylation targets.

> **RESPONSE: As suggested, we have explained in the Introduction part about what serine 3 in mammalian ADF and serine 6 in plant ADFs have in common, and cited relative publications.**

10. The paragraph about ADF/cofilin in the introduction is confusing (p4, line 82 and further) because data from plants and animals are mixed and generalized. For example, plant ADFs are not phosphorylated at serine 3 (see comment 8).

> **RESPONSE: As suggested, in the revised manuscript, we re-wrote this part in the Introduction and removed the sentence such as "Plant ADFs are not phosphorylated at serine 3".**

11. I am surprised that FABD2-GFP rather than GFP-FABD2 is used. Can the authors give a reference for this construct, or is it a mistake?

> **RESPONSE: It is a mistake. We actually used GFP-fABD2-GFP to label actin filaments in our manuscript (Wang et al., 2007). We fixed this problem throughout the revised manuscript. Thanks for pointing out this problem.**

12. The authors mention that they use suspension cells generated from 35Sp:EGFP-CKL2 plants, but they fail to mention how they did this and what cell type was used to generate these cultures.

> **RESPONSE: The suspension cells we used were generated from leaves of GFP-CKL2 transgenic plants. This information has been added in the revised manuscript.**

TPC2016-00078-RA 1st Editorial decision – *revision requested* **Mar. 2, 2016**

We ask you to address the reviewers' concerns and pay particular attention to the following points in preparing your revision.

Please be sure to address all of reviewer #1's concerns. In addition, reviewers #2 and #3, as well as myself, are concerned with the localization experiments carried out with the 35S-GFP-CKL2 construct. While this construct does rescue, which is excellent, the localization along actin filaments might still result from overexpression. Thus, we recommend that you complement the mutant with a construct that drives expression of GFP-CKL2 with the native CKL2 promoter and then analyze the localization.

-- Reviewer comments:

[Provided below along with author responses]

TPC2016-00078-RAR1 1st Revision received **Apr. 12, 2016**

Reviewer comments on previous submission and **author responses**:

Reviewer #1

The authors have performed a large number of experiments to address the issues of the manuscript. I would recommend the publication of the manuscript provided that the following points are corrected:

Major revisions:

As I explained previously, high speed centrifugation assays (Fig 6A and 6B) and micrographs of actin filaments stabilized by phalloidin (Fig 6C and 6D) are not relevant methods to measure the activity of ADF/cofilin in vitro.

In addition, I am highly suspicious about the results of these experiments. For example, curves of Fig 6D should have an inversed bell shape, as fragmentation reaches an optimal efficiency at low concentrations of ADF/cofilin.

In conclusion, I would remove the four panels 6A to 6D from the manuscript.

Instead, pyrene actin disassembly assays (Fig 6E) and TIRF microscopy (Fig 6F and 6G) are relevant assays.

However, the pyrene actin disassembly assay is not interpreted correctly. The relevant parameter to characterize the disassembling efficiency is the slope of the curves at time 0, which appears to be identical for both ADF4 and ADF4+CKL2 conditions. This is in clear contradiction with the conclusion of the authors.

> **RESPONSE: We thank the reviewer for these great comments and suggestions. As suggested, we have removed the Figures 6A to 6D in the revised manuscripts. We agree with this reviewer that the kinetic pyrene actin disassembling assay is an effective way to probe the activity of ADF/cofilin. In this assay, ADF/cofilin contributes to actin disassembly mainly via its filament severing activity to generate more ready depolymerizable filament ends at the initial stage. In this regard, we agree with this reviewer that the slope of the curves at time 0 is the relevant parameter to judge the disassembling effect of ADF/cofilin. The small difference between the curves of ADF4 and ADF4+CKL2 in terms of their initial slope is very likely due to the short incubation time of ADF4 with CKL2 (0.5 h), in which only partial ADF4 proteins are subject to phosphorylation by CKL2. In support of this notion, we found that longer incubation time (1.5 h, blue curve in the revised figure) causes dramatic decrease in the slope of the curve at time 0. We actually have taken this reviewer's criticism seriously, and have repeated this experiment several times recently and obtained the similar results.**

Reviewer #2:

The authors made all the previously suggested changes. There are only a few additional items that should be addressed to further strengthen this manuscript.

[Editor's note: minor comments were largely addressed].

Reviewer #3:

The revised manuscript by Zhao et al. has been greatly improved. The data are more clearly presented and additional experiments make the story more convincing. There are still several issues that should be addressed:

Point 1. The CKL2 localization studies have been performed by a construct driven by the 35S promoter. Why did the authors use the 35S and not the native promoter of CKL2 to complement the mutant phenotype? I realize that I did not comment on this in the previous version of the manuscript and I apologize for this, but although unlikely, CKL2 localization with actin filaments may be an overexpression artifact and the authors should exclude this.

> **RESPONSE: We knew that the 35S promoter driven expression of the GFP-CKL2 would be an important issue and have been working on the CKL2 native promoter:GFP-CKL2 transgenic lines for long time. We added the results that the GFP-CKL2 driven by the CKL2 native promoter locates at the actin filaments and rescues the stomatal closure phenotypes in the ckl2 mutant. These results are shown in Supplemental Figure 3B in the revised manuscript.**

2. The discussion still needs some work. The phrasing is not very scientific and occasionally somewhat naive. Although many papers describe the importance of actin reorganization in opening and closing of guard cells, the

forces that can be generated by actin filament polymerization are orders of magnitude lower than the force generated by turgor pressure. Thus, the link between actin reorganization and regulation of stomatal aperture is vague and not understood. If the authors would like to say anything about this (I am not sure if they should...), I would suggest a possible function in the regulation of membrane recycling, similar to the sequential polymerization and depolymerisation of actin filaments in the apex of pollen tubes during tip growth. This may be the reason for changes in cytosolic ion concentrations. If they wish to remain silent, I would suggest shortening the discussion; the first half of the discussion mainly repeats the statements made in the results.

The authors could consider clarifying the pathway they identified by adding a scheme to the discussion that graphically displays their findings.

RESPONSE: As suggested, we shortened and modified the discussion in the revised manuscript.

3. The GFP in some panels of Figure 2, 3, 6 and 7 is still displayed in black/green. In addition there is a problem with Figure 4a, where black/green images have been directly converted to greyscale images. The authors should separate the RGB channels before converting the images. The contrast between black and white is much higher then between black and green and black/white images will be much clearer. Colour images only make sense when showing co-localisations, for example in Figure 2f.

RESPONSE: As suggested, we separate the RGB channels before converting the images. GFP channel fluorescence images in green and black have been changed that in black and white in Figure 2, 3, 4, 6 and 7.

4. I can hardly discriminate microtubules and actin filaments in Figure 2h and i. This low magnification hardly supports the statement by the authors that microtubules or actin filaments have been fully polymerized.

RESPONSE: As suggested we have corrected Figure 2H and 2I with high magnification.

TPC2016-00078-RAR1 2nd Editorial decision – *accept with minor revisions* Apr. 19, 2016

On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on editorial review. In particular, please consider the following:

Improve the quality of the images and breadth of tissues analyzed with the native promoter-driven construct. Having done so, this should be included in the main text and the 35S promoter-driven construct should replace supplemental figure 3. Finally, please consider working with a native English speaking professional science writer to improve the manuscript text. Your work will have much greater impact if you choose to do so.

TPC2016-00078-RAR2 2nd Revision received May 9, 2016

1. The manuscript was edited by [a professional editing service].

2. We moved the localization and complementation results of the native promoter-driven construct to Figure 2, and moved the results of 35S promoter-driven construct to supplemental figures.

3. We added better and new images with different tissues for the native promoter-driven construct in the revised manuscript. The GFP signal is weak using the native promoter-driven construct. The reason of quality problem of the images in the last version is mainly due to the PDF conversion.

We believe that the added data and revisions further strengthen our conclusions. We hope that you will agree that all of the additions and changes have allowed us to substantially improve the clarity of our manuscript for the readers of The Plant Cell.

TPC2016-00078-RAR2 3rd Editorial decision – *accept with minor revision* May 11, 2016

Contingent on a few details that need to be specified (see below), the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. In particular, please address the following:

1. Please specify what the complementation lines are in supplemental figure 1B. Are these the same as the complemented lines that are in the new Figure 2?

2. Did you use native promoter-GFP fusion in the background of wild type or the null mutant? The figure legend states that it is in wild type. Please add a brief rationale for why the imaging was done in this line as opposed to the complemented line that is described in that figure.

TPC2016-00078-RAR3 3rd Revision received May 12, 2016

The complemented lines in the new Figure 2 are the CKL2 native promoter-GFP-CKL2 fusion in the ckl2 mutant. To avoid confusion, we removed the wording "supplemental figure 1B" in the revised manuscript text in page 9. I try to tell that the CKL2 promoter used in the CKL2 native promoter-GFP-CKL2 fusion is the same as that used in the genomic complementation assay in the figure 1 and the supplemental figure 1B. I am sorry that it is a mistake to write only "supplemental figure 1B". To clarify this, we add the RT-PCR result in the new supplemental figure 2C of the revised manuscript to show that the expression of GFP-CKL2 driven by the CKL2 native promoter is similar to that in wild type.

We transformed the CKL2 native promoter-CKL2-GFP fusion in both of the backgrounds of wild type and the ckl2 mutant, and used the wild-type transgenic lines for the GFP-CKL2 localization assay in this manuscript. When we used the native promoter-GFP-CKL2 lines in the ckl2 mutant to detect GFP signal, it forms filamentous structures similar to that observed in the Col-0 transgenic plants. However it is very weak, and it is difficult to obtain high-quality images. We have been working on isolation of the CKL2 native promoter-GFP-CKL2 fusion transgenic line for long time that expresses CKL2 similar to wild-type level and shows stronger GFP signal (to have high-quality image), however we have only got few lines in wild-type background.

These changes are highlighted in the revised manuscript.

TPC2016-00078-RAR3 4th Editorial decision – *acceptance pending* May 12, 2016

We are pleased to inform you that your paper entitled "Casein Kinase1-Like Protein2 Regulates Actin Filament Stability and Stomatal Closure via Phosphorylation of Actin Depolymerizing Factor" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor June 6, 2016