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Peer Review Report for 10.1105/tpc.16.00847

# Protein Phosphatase 2Cs and Microtubule-Associated Stress Protein 1 Control Microtubule Stability, Plant Growth, and Drought Response

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Review timeline:

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| TPC2016-00466-RA | Submission received: | June 6, 2016 |
| | 1st Decision: | July 23, 2016 *manuscript declined* |
| TPC2016-00847-RA | Submission received: | Nov. 8, 2016 |
| | 1st Decision: | Dec. 5 *acceptance pending, sent to science editor* |
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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.)

## TPC2016-00466-RA 1st Editorial decision – *declined* July 23, 2016

Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript.

This decision stems from multiple concerns raised by both reviewers 1 and 3. In particular, the reviewers and the reviewing editor feel that protein-protein interactions assayed by BiFC should be validated using an additional method. Both reviewers were skeptical of the auto-phosphorylation activity as there is no data in the manuscript to support this. In fact reviewer 2, also commented on this. While these issues would be simple enough to address in a revision, there were deeper concerns regarding the quality of the phospho-proteomics data set, the statistics used to analyze these data, and the interpretation of these data since normalization against protein levels was not performed. Reviewer 3 has suggested additional epistasis tests that should help to support the authors' conclusions. Importantly, the authors should consider reviewer 3's point that the observed phenotypes may stem from ethylene insensitivity.

[Reviewer comments shown below along with author responses.]

## TPC2016-00466-RA Submission received June 6, 2016

Reviewer comments on previously declined manuscript and author responses:

Reviewer #1:

The manuscript is written well and the results clearly presented and appears technically sound. Overall the data presented supports the title and the conclusions of the manuscript.

There are several comments that need to be address or clarified by the authors:

Point 1. MASP1 is identified in a quantitative phosphoproteomics approach and the suggestion is made that MASP1 is a direct target of EGR1/2 by the fact that in the *egr1/2* double mutant there is a 1.8 fold ratio of a MASP1 phospho-peptide as compared to wildtype. The ratio observed could be the result of reduced dephosphorylation of MASP1 in the *egr1/2* mutant background, but it could equally represent a 1.8 fold increase in MASP1 protein levels in the *egr1/2* mutant background as a results of indirect regulation of MASP1 protein levels. The inclusion of gene expression data can not substitute for measurement of changes in protein abundance by mass spec and normalisation of the changes

in phosphopeptide abundance to protein levels. The normalisation against protein levels should be provided, either by measuring the changes in protein abundance by mass spec or the authors should more clearly point out that changes in phosphopeptide abundance could also arise by indirect regulation.

RESPONSE: To clarify, it should be pointed out that in the stress treated plants there was an 8.2 fold increase in MASP1 phosphopeptide abundance in *egr1-1 egr2-1* compared to wild type. This difference was a major motivation for further study of MASP1. The 1.8 fold increase is for *egr1-1 egr2-1* versus wild type in the unstressed control. For stress treated *egr1-1egr2-1* versus stress treated wild type, traditional SDS-PAGE and western blotting indicated that MASP1 protein abundance was unaffected while phostag gel-western blotting found increased abundance of phosphorylated MASP1 (albeit at smaller fold change than in the phosphoproteomics data). Thus, based on this combined SDS-PAGE and Phostag data (Fig 2J) we do essentially have normalization of phosphorylated MASP1 versus total MASP1 for *egr1-1egr2-1* versus wild type stress treatment. Conversely, overexpression of EGR1 decreased the amount of phosphorylated MASP1 seen on phostag gels while MASP1 total protein did not change. These new data are presented in Fig 2 and the revised text describing these results is on pages 10-11.

Also, western blot analysis demonstrated that MASP1 protein level does increase in wild type under stress. Thus, the 4-fold increase in MASP1 phosphopeptide abundance in wild type stress versus wild type is influenced by protein abundance change (it could actually indicate a dephosphorylation of MASP1 as the protein abundance increases by more than 4 fold). The revised text describes this in a way we think is clear and straightforward.

We never claimed that the gene expression data is a substitute for measuring protein abundance. In fact, the second sentence of the section describing the phosphoproeomics data stated "The gene expression data was mainly used as a companion for the phosphoproteomics data to compare proteins with stress-induced increase or decrease in phosphopeptide abundance to genes transcriptionally regulated by low $\psi_w$." In other words, we only used the gene expression data to tell us which parts of the phosphoproteomic data were identifying drought responsive proteins that could not be inferred from transcriptome data. Later in this paragraph (page 8) we have added new text to more explicitly state that the changes in phosphopeptide abundance could be due to either changes in phosphorylation stoichiometry or changes in protein abundance and also mention this in the discussion (page 19). As we state in the text, both are of interest but it will take further experiments to distinguish the mechanism underlying the changes in phosphopeptide abundance. This does not diminish the novelty or usefulness of our phosphoproteomics data set.

For broader normalization across the phosphoproteomics data set, this is technically challenging and rarely attempted, even in studies that focus solely on proteomics. We could find only one example in plant studies , (Roitinger E, Hofer M, Köcher T, Pichler P, Novatchkova M, Yang J, Schlögelhofer P, Mechtler K 2015 Quantitative Phosphoproteomics of the Ataxia Telangiectasia-Mutated (ATM) and Ataxia Telangiectasia-Mutated and Rad3-related (ATR) Dependent DNA Damage Response in Arabidopsis thaliana. Molecular & Cellular Proteomics 14: 556-571). They could not achieve the same coverage of total proteins and phosphoproteins and thus could not normalize much of their phosphopeptide data anyway. As mentioned in Roitinger et al., differences in protein coverage between the phosphopropeptides and non-phosphorylated peptides leave many gaps in the normalization and thus full normalization of phosphoproteomics datasets is not yet feasible. And they seem to have used only one biological replicate and not applied any statistical cutoff for their data, illustrating how various approaches to replications and statistics are used in current phosphoproteomic studies (even those published in MCP).

We also want to note that it is not accurate to state that we made the "suggestion" that MASP1 is a direct EGR target. The text is worded carefully to say that EGRs attenuate MASP1 phosphorylation, which leaves open several possible mechanisms. Direct dephosphorylation is one, and perhaps the simplest explanation for all the data, but we also cannot rule out more indirect mechanisms such as EGR dephosphorylation and inactivation of a kinase that targets MASP1. This is mentioned clearly in the discussion (page 17-18).

Point 2. While the authors use a BiFC approach to show that MASP1 and EGRs can interact, they do not provide conclusive evidence that EGRs dephosphorylate MASP1 in vivo. The Phostag experiments in Fig. 2H is an attempt to suggest that overexpression YFP-EGR1 results is lower amounts of phospho-MASP1, the lack of proper control by including a masp1-2 mutant sample leaves these data open for other interpretations. In other words, at a minimum definitive proof is required that the bands indicated in Fig. 2H are indeed MASP1 and phospho-MASP1.

RESPONSE: As stated above, we did additional replicates of the phostag gel analysis including masp1-1. This, along with treating the samples with calf intestinal phosphatase (C.I.P) demonstrated that the main band we see is

phosphorylated MASP1. We quantified the abundance of this band, along with total MASP1 abundance and HSP70 (a loading control) in the same samples on regular SDS-PAGE. This analysis showed that *egr1-1egr2-1* had greater abundance of phosphorylated MASP1 while MASP1 total protein level was unchanged. Conversely, EGR1 overexpression decreased the abundance of MASP1 without changing MASP1 total protein level. This is consistent with the phosphoproteomic data. What we did find in these experiments was that we could not consistently detect unphosphorylated MASP1 in plant extracts. This is acknowledged in the text and we make no interpretation about the phosphorylation stoichiometry. We think the new experiments and revised text to explain the results answer the reviewer's comment.

Point 3. The suggestion that MASP1 can autophosphorylate on the serine residue 670 is highly likely to be incorrect. The MASP1 protein has no identifiable kinase domain and the only domains that can be found in the protein are several Leucine rich repeats. Also the reference for the autophosphorylation (Nemoto et al, 2011) has this protein annotated as a protein phosphatase regulatory subunit and shows that in a previous study (Sawasaki et al., 2004) this protein showed no autophosphorylation activity. Unless other supportive evidence can be provided for the auto phosphorylation activity of MASP1, these statements should be removed from the discussion and Fig. 7.

RESPONSE: This has been removed as suggested by the reviewer. It was not our intent to suggest that MASP1 autophosphorylates, merely we wanted to thoroughly report and discuss all the relevant literature. However, upon reviewing the manuscript, we realize how the writing of this part may have created the wrong impression. Sorry for the unclarity.

Point 4. In legend of Table I two separate selection criteria are mentioned either 1.5 fold and p=<0.05 or more 2.0 fold and p value of =<0.1 which is highly unusual by current proteomics standards. In the material and method section on page 22 the authors define 'Calculation of Q-values indicated that only slightly more than ten percent of phosphopeptides with p {less than or equal to} 0.05 would be expected to be false positives thus we used this as the main threshold for statistical significance' At the mentioned p value already 10 % false positives are expected (which is already very high by any standard) and thus using a p value over 0.05 would further increase the number of fals positive identified. I understand that the authors need to use these settings to allow their main target MASP1 into table I. If they had run additional replicates they might have been able to get better p values for this particular protein (and the data set in general). The criteria should be set as defined in the methods and materials with the lower p value.

RESPONSE: We have revised Table 1 to only show proteins with p less than or equal to 0.05 and fold change greater than 1.5, as well as MASP1. For MASP1, we point out in the main text and table caption that it is a protein of special interest because of its high fold change (8.2 fold) and p value close to the cutoff (p = 0.07) and our further characterization of it justifies its inclusion in table 1. We mention in the text (page 9) that some other proteins which just missed the statistical cutoff were also are consistent with the putative cytoskeleton-related function of EGRs along with a cautionary note about the p values and relative uncertainty of their phosphopeptide ratios. We think this is appropriate and necessary to fully report our data.

We respectfully disagree that a 10 percent FDR is "already very high by any standard" and have consulted with a statistics expert both before the first submission of the paper and again now (he was and still is listed in the acknowledgements). Both our consultations about statistics and a review of recent literature shows that our strategy to evaluate the phosphoproteomics data is well within the range of current practice and if anything we are being more transparent about the data by reporting the FDR (which a number of studies simply do not mention). To quote directly from advice we received about statistics: "I'd definitely also argue that these cut offs are arbitrary, especially if you think about these as discovery experiments. I like to think of fdr as the proportion of money I might waste following up. Fdr of 0.05 means id waste 5 bucks in a hundred if we follow up on a set of candidates meeting a 5% threshold - clearly very conservative if we are trying to discover new things." The goal of our phosphoproteomics experiment was to discover new targets and regulatory mechanisms related to drought and the EGR phosphatases and our results show that this was successful. I'd gladly sacrifice 10 bucks in a hundred (FDR 0.1) to make such discovery, (indeed we are doing so again in ongoing research). Other recent studies (several reported in MCP for example) use various statistical approaches including uncorrected P values or Z scores. If using P-value, most of the examples we could find simply did not report an FDR. Our analysis of data is appropriate and is as rigourous, or more so, than other recent phosphoproteomic studies.

Point 5. The previous point also leads onto an additional concern about the quality of the proteomics data set. With the provided supplemental data it is not possible to get a sense of the quality of the data, especially for the phosphopeptide data for the proteins in Table I. Although the authors reportedly submitted the data in PhosPhAt, the data is not publically available and for reviewing purposes the data set needs to be submitted in a public repository such as Pride, where also to raw data can be inspected. Please provide log in details and password for reviewing access once data has been deposited in Pride.

RESPONSE: We have now uploaded all the data to PRIDE and it is available with the dataset identifier PXD00486. This information is now listed in the Accession numbers section of the manuscript at the end of the discussion as per Plant Cell guidelines. PhosPhat will also post our data but it seems their policy is to only do so after publication.

Reviewer #2:

I have evaluated the paper by Bhaskara and co-workers on the role of EGR type phosphatases and MASP1 in microtubule stability during drought in Arabidopsis.

The authors identify EGR1 and EGR2 as negative regulators of growth during both control and stress conditions and apply quantitative phospho-proteomics to identify their targets. One of the identified targets concerns a novel protein MASP1 and the authors show that its function in protecting the plant from drought depends on its phosphorylation status. The enhanced resistance against drought was furthermore correlated to the stability of the microtubule cytoskeleton by showing that *egr1/egr2* mutants as well as plants expressing the phosphorylated form of MASP1 showed increased stability of their microtubule cytoskeleton upon drought and oryzalin.

This paper contains a wealth of data and travels great lengths to enhance our understanding of how plants cope with mild drought stress. I nevertheless have some remarks which are outlined below.

Point 1. It might be worthwhile to mention more clearly that the reasoning to generate the egr1/egr2 double mutant is because of the fact that these two members (in contrast to EGR3) showed the highest upregulation upon drought stress.

RESPONSE: The *egr1-1egr2-1* double mutant was constructed both because EGR1 and EGR2 are both highly expressed under stress and also because they are more closely related to each other than to EGR3. This is now pointed out in the text (page 6). Thanks for the careful reading of the manuscript.

Point 2. As the *egr1/egr2* mutant shows both increased growth both during control and stress conditions, they do not specifically confer resistance to drought, but rather are negative regulators of growth in general. I would therefore not specifically conclude that they restrict growth during stress.

RESPONSE: We agree and have modified the text in several places to say that EGRs are negative regulators of growth with an especially prominent effect on growth during drought stress. We think that this is an accurate description of the data.

Point 3. I do not think a function in phosphatidylinositol dephosphorylation is adequate proof to warrant the claim of a plasma membrane localization, please rephrase or provide additional arguments to support this conclusion.

RESPONSE: This sentence is not essential to the main conclusion of this section and has been deleted.

Point 4. The failure to observe functional MASP1 on microtubules is unexpected, given the fact that all other data, including transient expression to achieve higher levels points to the fact that it is a MT-binding protein. As the authors have antisera against this protein, would it be possible to perform immunofluorescence analysis to localize endogenous MASP1 (under mild stress conditions) to microtubules in Arabidopsis?

RESPONSE: We do not think immunolocalization would reveal any new information about MASP1 MT association, only show that it is diffusely localized in the cell cortex as the FM4-64 colocalization data added to Fig 3 demonstrate more clearly. Also, we have insufficient amount of MASP1 antisera to perform this experiment (the antisera was obtained from AbMart and the quantity supplied was limited).

Point 5. Page 14 line 384: the localization of MASP1 along the cell periphery and membrane recruitment of structural analogs is a rather poor argumentation to show membrane recruitment of MASP1. Co-localization analysis with a membrane marker or some dye like FM4-64 to distinguish between the cytoplasm underlying the plasma membrane and the actual plasma membrane would be more convincing.

RESPONSE: Thanks for this good suggestion. We did the FM4-colocalization experiment for EGR1, EGR2 and MASP1. We found that EGR1 and EGR2 were nearly completely co-localized with FM4-64 along the plasma membrane while MASP1 was along the plasma membrane but also more diffusely localized in the cell cortex beneath the plasma membrane. These new data are discussed and the final model we present for EGR-MASP1 function reflects the finding that MASP1 is in the cell cortex and may have limited interaction with the plasma membrane.

Point 6. The authors state that they could not recover an egr triple mutant, yet they do not show whether this was caused by gametophytic or embryo lethality. Stating that the triple mutant could not be recovered requires some experimental data describing how this conclusion was reached.

RESPONSE: This is not a major point of the study and mention of egr triple mutant has now been deleted.

Point 7. The statement that MASP1 autophosphorylates is not supported by the description of the presence of a kinase domain in MASP1. The authors mention and favor the autophosphorylation hypothesis compared to an upstream kinase which would be responsible for MASP1 phosphorylation, but the explanation for this (by referring to the Nemoto paper) is mentioned only much later in the discussion. For clarity, this should be adapted.

RESPONSE: As noted above, we do not favor the autophosphorylation hypothesis, we were merely trying to be thorough and present and incorporate all relevant reports from the literature. The revised manuscript does not mention MASP1 autophosphorylation.

Point 8. Figure 1: For clarity, it could be mentioned in the methods section why one-sided T-tests were chosen in the statistical analysis.

RESPONSE: One sided T-test were chosen as an appropriate method to see if our relative growth data differed from 100 percent of the wild type level.

Point 8. Levels of phosphorylated MASP1 appear quite similar between WT and the egr1/egr2 mutant, which is not what you would expect based on the quantitative phosphoproteomics. Also, although the band of phosphorylated MASP1 disappears upon over expression of EGR1 or CIP treatment, the levels of non-phosphorylated MASP1 do not visibly increase, which is unlike what would be expected. Please clarify.

RESPONSE: We did several more replicates of phostag gels and, as mentioned above in response to reviewer 1, quantification of band intensities does show that there is more phosphorylated MASP1 in *egr1-1egr2-1* (albeit not an 8-fold increase). This is consistent with the phosphoproteomics data. In these new replicates of the phostag gels it is also clear that we cannot reproducibly detect unphosphorylated MASP1 in plant extracts (reasons for this are unclear); but this does not affect interpretation of the phosphorylated MASP1 band intensity which was reproducible.

Reviewer #3:

Drought and high osmolarity induce growth inhibition and adaptive responses in plants. The molecular mechanisms by which these responses are mediated are not fully elucidated. It is known that inhibition of root growth by drought and high osmolarity involves the phytohormones abscisic acid (ABA) and ethylene. ABA acts via ethylene to inhibit root growth.

Bhaskara et al. have characterized now three protein phosphatases 2C of clade E (EGRs) as regulators of the drought response in Arabidopsis. The authors could show that EGR-deficiency provide osmotic stress-insensitive root growth of seedlings. A phospho-proteomic analysis identified MASP1 as a putative target of EGRs. MASP1 was associated with microtubules (MT). Expression of MASP1 mutated at the phosphorylation site S670 modified the sensitivity of roots towards osmotic stress consistent with the notion that the phosphorylated S670 of MASP1 mediates insensitivity to growth inhibition by high osmolarity, similar to EGR-deficiency.

Bhaskara et al. present in this study a large number of experiments to support the conclusions. The findings shed light on a poorly understood growth response by showing a function of PP2Cs in regulating cytoskeleton dynamics via the largely uncharacterized protein MASP1. While the novel insights are exciting the rigorousness of the conclusion is not fully convincing.

Point 1. The physical interaction of EGR with MASP1 is supported by BIFC analysis. Independent protein-protein interaction analyses are missing to corroborate this important finding.

RESPONSE: We now show co-immunoprecipitation of EGR1 and EGR2 with MASP1 (Fig. 2G).

Point 2. The EGR/MASP1 interaction, the phospho-proteomic analysis of EGR-deficient lines, and MASP1-S670 analysis imply a function of EGRs in dephosphorylating MASP1-pS670. No data are presented which examined that important issue. MASP1 is annotated as a functional (autophosphorylating?) protein kinase (TAIR). The authors purified MASP1 and mutant forms but did not present data on its presumed kinase activity.

RESPONSE: The TAIR annotation of MASP1 was confusing for a number of reasons (it was also listed as a dynein light chain protein, which it clearly is not). As pointed out by reviewers 1 and 2, the putative autophosphorylation of MASP1 reported in one study is odd because MASP1 has no recognizable kinase domain (and our analysis of structural analogs using I-Tasser and ModWeb did not turn up anything related to kinases). We have now deleted any mention of MASP1 kinase activity as the published data on this are not convincing and it is beyond to scope of this study to address further.

The phostag gel analysis (now improved) and data of EGR-MASP1 interaction indicate that EGRs act to attenuate the levels of phosphorylated MASP1. Note that we did not (and do not in this new submission) make any statement that EGRs dephosphorylate MASP1. The term "attenuate" is used deliberately as it leaves open several possible mechanisms. In this new submission, we now more clearly explain that EGRs may directly dephosphorylate MASP1 but we cannot rule out more indirect mechanism such as EGR regulation of a kinase that dephosphorylates MASP1 (page 18, this is also shown in the model in Fig 8).

Despite several attempts, we have been unable to purify active EGRs for in vitro dephosphorylation assays (we tried both full length and truncated EGRs as well as several variations of induction and extraction of the recombinant protein from *E. coli*.) Whether or not EGRs directly dephosphorylate MASP1 is still an open question. As there was little or no function information on EGRs or MASP1 prior to this study, we report much new information and there are inevitably additional questions for future study.

Point 3. The study suggests that EGR and MASP1 function in a common response pathway. The epistatic analysis of EGR and MASP1 (mutant forms) should corroborate this conclusion.

RESPONSE: We agree with this point and had been working on it. Analysis of *egr2-1masp1-1* double mutant is now presented in Figure 5. The *egr2-1masp1-1* double mutant is similar to *masp1-1* in its growth response to stress, consistent with MASP1 acting downstream of EGRs. Other phenotypes (proline accumulation in particular) behaved differently and we also noted that *egr2-1masp1-1* had a partial recovery of microtubule stability. This indicates that EGRs regulate additional targets related to cytoskeleton and stress signaling, possibly including the proteins listed in Table 1 or others yet to be identified.

Point 4. The contribution did not consider that the phenotypes observed might be attributable to an ethylene insensitivity of the *egr* lines. The root growth of egr lines, the larger pavement cells and leaf organs, and the different growth of leaf rosettes under water deficit might reflect altered ethylene responsiveness.

RESPONSE: We tested the root growth response of EGR and MASP1 mutant and overexpression lines to treatment with ACC to increase ethylene and AVG to inhibit ethylene. This new data is presented in Supplemental Figures 8. Neither ACC nor AVG could restore growth of *masp1-1* to wild type level nor suppress *egr1-1egr2-1* to the wild type level under stress (AVG did have some effect on growth of unstressed *masp1-1*). We also examined the root swelling phenotype (Supplemental Figure 9) and found that *masp1-1* (and to lesser extent EGR1 overexpression) were more sensitive to root swelling induced by ACC while *egr1-1egr2-1* and MASP1 overexpression lines were less sensitive. AVG could alleviate stress-induced root swelling but not completely restore wild type root morphology and not restore growth. All these data indicated that any differences in response to ACC and AVG were a consequence of altered microtubule stability and do not indicate a direct involvement of EGRs or MASP1 in ethylene signaling. These new results are described on page 14-15.

TPC2016-00847-RA 1st Editorial decision – *acceptance pending* Dec. 5, 2016

We are pleased to inform you that your paper entitled "Protein phosphatase 2Cs and Microtubule-Associated Stress Protein 1 control microtubule stability, growth, and drought response" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor Dec. 22, 2016