To add value to publications and provide information on the review process, The Plant Cell will publish Peer Review Reports, subject to author approval, for all articles by January 2017. Reviewer anonymity will be strictly maintained. The reports which will include the most substantive parts of decision letters, anonymous reviewer comments, and author responses; minor comments for revision and miscellaneous correspondence will not be published. 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 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.

Synergism between Inositol Polyphosphates and TOR Kinase Signaling in Nutrient Sensing, Growth Control, and Lipid Metabolism in *Chlamydomonas*

Inmaculada Couso, Bradley S. Evans, Jia Li, Yu Liu, Fangfang Ma, Spencer Diamond, Doug K. Allen, and James G. Umen

*Plant Cell. Advance Publication Sept. 6, 2016; doi: 10.1105/tpc.16.00351*

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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.)

**TPC2016-00351-BR 1st Editorial decision – declined** June 7, 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 in its present form. We have not made this decision lightly. We have had input from multiple scientists with expertise in this area, have solicited post-review comments from reviewers and discussed the work in the context of the review. Given the highly interesting subject matter and the high quality analytical methodology presented, we would be interested to re-review the work if you and your co-authors are able to address the reviewers’ concern. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments. Thus, our open-ness to re-review is unusual; nevertheless, it would delay publication of this interesting story and we would understand if you wanted to send the work elsewhere.
Reviewers were enthusiastic about the potential connection between inositol phosphate metabolism and TOR signaling, but were less convinced of a clear link by the evidence presented. It seems that two key areas of further experimentation would be warranted to provide a more compelling case. First, a clear demonstration of VIP1 functional activity along with substrate and product characterization. This is important because several species of IPs change in the mutants (not just IP7 and IP8), and further, the LC-MS method cannot distinguish the phosphate isomeric modifications on the inositol. Second, a clear readout of TOR activity would strengthen a connection with IP metabolism and TOR, and perhaps help sort out hierarchy.

In general, although the enthusiasm about your potentially exciting observations was high, considerable additional evidence would be required to substantiate your conclusions. A revision incorporating the two points above and perhaps additional experimental evidence that might address questions about mechanism as raised by the reviewers would be handled by the same editors, and this might be an avenue you wish to consider if results can be obtained within a reasonable time frame; such a "revision" would, however, be considered a new submission. As stated above, this is not a typical decision letter and if you do choose this path, please indicate in the cover letter that a revision had been invited so that we can send it to review without a pre-review consultation and we can direct it to the same editors and reviewers.

Reviewer comments:

Reviewer #1 (Comments for the Author):

This interesting manuscript describes the identification and characterization of a Chlamydomonas gene (VIP1 kinase, a diphosphoinositol pentakisphosphate kinase) mutation of which confers rapamycin hypersensitivity. Rapamycin is a specific inhibitor of the conserved TOR kinase.

Although the TOR protein has previously been implicated in controlling growth and development, there are still many unanswered questions about its functions in algae and plants. The authors have conducted a thorough analysis of the consequences of vip1 mutation in Chlamydomonas.

Therefore this paper is potentially an important step forward in our understanding of the plant TOR regulation and cross-talk with IP (inositol phosphate) that are important signalling molecules. The fact that the rapamycin hypersensitive phenotype is conditional and only observed in acetate medium is particularly interesting even if no clear explanation is provided in this study.

The submitted manuscript is well written and most of the data appear solid and well presented. However, I have several comments on the conclusions drawn by the authors.

1- the link between the TOR signalling pathway and the rapamycin hypersensitive phenotype of the vip mutants is mainly based on the assumption that rapamycin specifically inhibits the TOR kinase. While this is true in yeast and animals, this may not be the case in Chlamydomonas, even if it is unlikely. I think that it would be interesting to test other TOR inhibitors like AZD80550 and to verify that the vip1 mutant also show hypersensitivity to ATP-competitive inhibitors.

2- I think that a central point in this manuscript is the link between IPs and TOR signalling pathways. The authors suggest that TOR is upstream of IPs but, as mentioned in the manuscript, this is far from obvious in other organisms. To my opinion this is also not clear-cut in this study. Indeed one could explain some of the observed phenotypes by a decrease of TOR activity in the vip1 mutants. Consistently rapamycin treatment would thus further decrease TOR activity resulting in higher lipid levels. The simpler explanation for the observed rapamycin hypersensitivity would be that TOR activity is reduced in vip1 mutants.

Therefore the conclusion that vip1 operates independently of TOR is, to my opinion, not fully supported by the data presented here. I think that it would be essential for this paper to try to measure TOR activity in the vip1 mutant. The authors mentioned that they have tried to use the phosphorylation of S6 kinase as readout for TOR activity but without success. I agree that this is not an easy task but S6K phosphospecific antibodies obtained from the plant kinase are now available at Agrisera (http://www.agrisera.com/en/artiklar/ribosomal-s6-kinase-1_2-s6k1_2.html). I
believe that it would be worth trying this antibody! Otherwise the BIP protein has been also identified as a TOR substrate in Chlamydomonas and could be used as readout.

Reviewer #2 (Comments for the Author):

The work presented by Couso et al., is a very elegant and thorough investigation of a Chlamydomonas mutant that is hypersensitive to an inhibitor of an important metabolic regulator, the TOR kinase. The work is highly relevant to readers of the Plant Cell. The work is nicely described and much attention has been paid to the numerous details that come with a topic such as this. One of the major strengths of the work is a new method using LC-MS/MS to detect inositol phosphates (InsPs). This provides a key strength in that the authors can characterize their mutant with regard to levels of the signaling molecules called inositol pyrophosphates. Specifically, they quantify InsP7 and InsP8, the least abundant InsPs. By doing this, it allows them to link inhibition of a major metabolic regulatory kinase to regulation of signaling molecules. In the end, they are well positioned to draw significant conclusions about energy, lipid production, and InsP and Tor signaling pathways. This is probably the finest manuscript I have reviewed in a decade.

The manuscript starts by detailing a screen for mutants with hypersensitivity to rapamycin (Rap). A minor point I have is that the authors need to give the exact name of the inhibitor they used as there are many Rap inhibitors, and work in plants has shown that only certain ones work well (at least in plants). In this screen the authors identify VIP1, a kinase that catalyzes the InsP6 to InsP7 to InsP8 pathway. They show a nice complementation with a western blot indicating the complemented mutants express a tagged VIP1 protein. As well, they show that a double vip1/fkb12 mutant is not sensitive to Rap; this is an important control for specificity. Recent papers on the Arabidopsis VIP enzymes have shown that they can complement the yeast mutants, and so the authors assume that InsP6 is the substrate for their VIP1 enzyme. Mutants in vip1 have elevated TAGs, and changes in certain central carbon metabolites. As I mentioned previously, the authors use LC-MS/MS to show that InsP7 and InsP8 are reduced in their vip1 mutant. Further, when they treat WT cells with Rap, the higher InsPs (6/7/8) decrease. One of the most interesting aspects of the vip1 mutant is that it’s hypersensitivity is dependent on acetate. So it not surprising that growth on +/- acetate also impacts InsP levels in both WT and vip1 mutants.

I feel that the authors have a solid story, but should address the following major and minor points:

Major points (in mostly chronological order):

1. The authors have not illustrated that the Chlamydomonas VIP1 enzyme is active, or whether it can complement a yeast mutant. On the tree, CrVIP1 groups with other uncharacterized VIP enzymes. This begs the question of whether the green algae enzymes are different from the yeast and plant VIPs? The authors show that these mutants have reduced InsP7 and InsP8, and so it seems very likely that they do catalyze the expected reactions. Perhaps a short description of amino acid similarity in the whole ORF and within the ATP grasp domain could address the likelihood that CrVIP1 is as expected.

2. The LC-MS/MS method provides a huge strength to this work. I wondered about the error present in these measurements. In some cases values are almost 2x different, but the error is so large there is not a significant difference. In other cases the error is much smaller and differences less than 2x are significant. Can the authors comment on this?

3. I think the authors need to point out that their method groups all InsPs containing the same number of phosphates. Thus the really interesting change in InsP3 is not necessarily due to changes in Ins(1,4,5)P3. The correlation of the change in InsP3 with changes in the higher InsPs is really fascinating. I wondered whether the authors had any knowledge on the identity of this molecule? Does it co-elute with the standard InsP3? I also wondered whether there was any indication of the existence of novel PP-InsPs, like PP-InsP4. I point out that PP-InsP4 would probably elute with InsP6. Similarly, the InsP3 peak could be anything with 3 phosphates, even a PP-InsP molecule.

4. Why is there no InsP or InsP2 present in these cells?

5. In growth assays, I am perplexed by the conclusion that viability is only slightly reduced in vip1-1 as compared to WT in TP + rap (Fig. S4D). The viability looks to be 50% in vip1, and closer to 80% in the WT.
6. The authors conclude there is a 6-fold increase in TAGs in the vip1 mutant. Which specific piece of data are they using for this? In 8 B it looks to me that FAMEs increase by about 3 fold, not 6 fold.

7. The authors need to clarify whether they feel their data supports increased lipids in vip1 mutants in TP media, or whether this phenotype is also acetate dependent.

8. Figure 9 needs P value determinations, and the authors need to comment on the interesting increases in metabolites in the mutant under TP conditions.

9. The authors may want to address the Wild et al., paper on SPX domain binding by InsP6 and InsP7. This new paper shows that InsP6 and the inositol pyrophosphates act as sensors by binding to SPX domains contained in proteins in the phosphate sensing pathway.

10. The authors should consider addressing the energy and metabolic alterations in the yeast KCS1 mutant, which has decreased InsP7 levels.

Reviewer #3 (Comments for the Author):

This manuscript describes a new role for inositol polyphosphates (IPs) in TOR (target of rapamycin) signaling in the green alga Chlamydomonas reinhardtii. The TOR complex is a metabolic regulatory instance controlling the allocation of carbon for cellular needs according to developmental or external cues. Despite its obvious importance, there is a significant lack of knowledge about the function of TOR. Therefore, the identification of IPs contributing to TOR functionality is a novel and important finding and of interest to a broad audience studying metabolic adaptation or stress signaling in algae, plants and possibly even other systems.

The authors conduct an insertion-mutagenesis screen for hypersensitivity of C. reinhardtii against the drug, rapamycin, and identify a number of candidate mutants. Only one of these mutants is presented in the present study. The authors perform extensive genetic controls to verify the identity of the mutant and identify the relevant allele as an insertion in a gene encoding an IP kinase resembling VIP-kinases involved in the formation of pyrophosphorylated IPs, such as IP7 or IP8. The mutant is termed vip1-1. Biochemical analysis of the mutant indicates altered patterns of IP formation, including changed levels of IP3, IP6, IP7 and IP8. The CrVIP1 protein is localized in the cytosol of C. reinhardtii cells, according to immunocytochemistry experiments. To elucidate a role for Cr VIP1 in the regulation of carbon allocation, the authors perform a series of experiments and show that the Cr vip1-1 mutant displays altered growth and storage lipid accumulation when grown mixotrophically on media containing acetate. Furthermore, it is shown that IP levels change upon addition of rapamycin to mixotrophically grown cells. The study is further supported by phylogenetic analysis of VIP-related proteins.

The experiments are technically mostly sound, except where noted below. Statistical evaluation has not been included for all graphs. This should be completed.

The plant TOR complex is currently not well understood. This study provides an important new link between IPs and the control machinery for carbon allocation in C. reinhardtii. The genetic evidence is clear and well done. However, the physiological experiments and biochemical analyses leave much room for speculation and the study remains largely descriptive. In particular, it remains totally unclear how IPs might mechanistically influence TOR signaling, or vice versa. While some experiments suggest that VIP1 is involved in TOR function, treatment with rapamycin resulted in changed levels of IPs. At this point it remains open whether IPs might act upstream or downstream of TOR, or both. This point should be addressed in further experiments. Based on the data shown it remains open, even which IP might be relevant. Candidates are all IPs that are found to be altered in the vip1-1 mutant, including IP3, IP5, IP6, IP7 or IP8, all of which have assumed or demonstrated regulatory roles in eukaryotic cells. The effects observed might be related to TOR alone or to a combination with any number of other possible effects of perturbed IP metabolism on physiological regulation, including Ca2+-signaling, mRNA export from the nucleus, phosphate-sensing, the perception of different phytohormones, etc. At this point - and based on the data presented - it appears an open field and premature for publication. Overall, the study makes a very important finding, but remains preliminary in explaining the effects that were observed.

Conceptual comments:
The effects of IPs on carbon allocation are likely also related to the cells’ energy status. Very recently (and likely only after submission of this manuscript), Wild et al. (Science 2016) have reported a role of IPs, including those found in the present study, in the regulation of the adaptation of eukaryotic cells to phosphate starvation, and demonstrate that IPs serve as indicators of the cellular Pi-status. It appears logical that the effects observed in mixotrophic conditions might also involve some of these processes and the link to phosphate-signaling should be experimentally addressed.

The lack of mechanistic understanding of the effects seen is evident from the overly long and unfocused discussion where the authors bring to bear all that can be read on the regulatory roles of IPs in different models.

While the authors present a large volume of literature on possible functions of IPs for different eukaryotic models, they fail to highlight an obvious link that might be discussed in their best interest: that IP5 and IP6 might act in the perception of defense vs. growth hormones and that IPs might thus be involved in switching between growth and defense, likely involving reallocation of carbon, etc. Here, the relevant papers by Mosblech et al. (2008 and 2011) should be mentioned, which demonstrate the influence of altered IP levels on the defense response of Arabidopsis.

Technical and minor issues:

The immuncytochemistry data are not convincing. Why is there no green nuclear fluorescence in the merged image in the top panel (control) even though the label is close to uniform between cytosol and nucleus in the green channel alone? The deconvolution appears to creat particulate signals where there were none in the non-deconvolved samples. Could this be an artefact of the processing? Based on the images presented, to me it seems that no subcellular site can be excluded and that the VIP protein might be in the cytosol, in the nucleus and possibly also in other locations that are not represented.

The phrasing of the description of the lipid analysis on p. 11 is unfortunate and lists Bligh & Dyer, 1959, as a reference for GC-analysis of FAMEs. This is most certainly not correct.

TPC2016-00351-BR   Appeal requested       June 11, 2016

I am following up from our reviews and from a conversation ... about reformatting our manuscript as a Breakthrough Report. Before doing so, I would like to have an email exchange or perhaps phone conversation to make sure that the reformatted manuscript would, in principle, meet your requirements for publication.

I think the idea of a Breakthrough Report fits our work for the following 2 reasons:

1. We identified for the first time a strong connection between the TOR signaling pathway and inositol polyphosphate signaling, both of which are conserved pathways in algae, plants and other eukaryotes. Neither pathway is well characterized in plants or algae so the connection we found is both timely and relevant to a broad audience.

2. We also identified an unusual metabolic phenotype of storage lipid over-accumulation in strains carrying the vip1-1 mutation that reveals a connection (direct or indirect) between inositol polyphosphate signaling and carbon metabolism or partitioning. Prior work in the area of algal lipid metabolism has identified mutants with defects in lipid or starch accumulation, but not a mutant like vip1-1 that affects a signaling pathway and which partially uncouples lipid accumulation from the normal prerequisites of starvation or stress. This phenotype has clear implications for understanding metabolic control and for biotechnology where there is strong interest in understanding how to overcome the constraints on algal storage oil production that is inversely correlated with nutrient availability and growth rates.

Several of the reviewer comments can be addressed in our revision without new experiments and we will do so. The two main concerns raised in the Decision Letter about the activity of VIP1 and the relationship of IP signaling to the TOR signaling pathway cannot completely be addressed without further experimentation, but I do want to respond so that you can consider the impact of not doing these additional experiments.

VIP1 activity: Although relatively recently discovered and characterized, the VIP family of proteins are conserved in domain structure and primary sequence throughout eukaryotes including catalytic residues that are completely
These proteins have a unique and unmistakable sequence/structure that is evident in both similarity searches and which is reflected in phylogenetic analyses that we included in our manuscript. Enzymatic activities of animal, fungal and plant VIPs (three distant phyla) have all been previously tested and shown to catalyze the same reaction of IP6 pyro-phosphorylation to produce IP7 and possibly IP8. Indeed, these are the IP species that are primarily affected in our vip1 mutant in Chlamydomonas. Given these results and the consistency of prior published results where VIP1 enzymes across diverse phyla had been tested, we felt that the value of doing in vitro testing or yeast complementation experiments was not very high. Had the plant VIP1 enzymes not been previously tested then doing these experiments would be more important. It seems extraordinarily unlikely that Chlamydomonas VIP1 separately evolved additional activities (as implied by Reviewer 3) while maintaining conservation of domains and sequences with other VIPs. Asking us to investigate the stereochemistry of Chlamydomonas VIP1 reaction products goes beyond the standard of the field, especially because we made no strong claims about which specific IPs or IP isomers are responsible for the vip1 phenotypes.

Relationship between TOR kinase activity and VIP1: We do show that IP profiles are affected when we inhibit TOR kinase signaling meaning that TOR signaling can formally be placed upstream of VIP1/IPs; but we were unable to test in the other direction because we lack a validated TOR kinase activity readout. Were we able to do this experiment we would find one of two results: Either vip1 mutants show altered TOR signaling activity or they don't. If they do show an alteration, it means that IPs act both upstream and downstream of TOR signaling in a non-linear and possibly complex manner. If there is no change in TOR signaling in vip1 mutant strains compared to wild type strains it would support a linear relationship where TOR acts upstream of VIP1 and IPs. Importantly, the interesting and relevant aspects of our story are unaffected by the outcome of the above experiment, and the outcome would still represent only an incremental step towards figuring out why we see the synergistic interaction between the two pathways. It is also worth considering that the absence of a TOR kinase assay is a problem for the whole plant field and not just Chlamydomonas. Aside from one report in Arabidopsis which we have not seen replicated outside of the laboratory that published it (and which we have been unable to replicate in Chlamydomonas) we are unaware of a validated TOR kinase signaling assay in plants or algae. These are the reasons we felt that it was time to publish and not wait for an advance that essentially the entire plant TOR signaling field is waiting for.
Reviewer #1:

The link between the TOR signalling pathway and the rapamycin hypersensitive phenotype of the vip mutants is mainly based on the assumption that rapamycin specifically inhibits the TOR kinase. While this is true in yeast and animals, this may not be the case in Chlamydomonas, even if it is unlikely. I think that it would be interesting to test other TOR inhibitors like AZD80550 and to verify that the vip1 mutant also show hypersensitivity to ATP-competitive inhibitors.

RESPONSE: Done.

Point 2. I think that a central point in this manuscript is the link between IPs and TOR signalling pathways. The authors suggest that TOR is upstream of IPs but, as mentioned in the manuscript, this is far from obvious in other organisms. To my opinion this is also not clear-cut in this study. Indeed one could explain some of the observed phenotypes, like lipid accumulation, by a decrease of TOR activity in the vip1 mutants. Consistently rapamycin treatment would thus further decrease TOR activity resulting in higher lipid levels. The simpler explanation for the observed rapamycin hypersensitivity would be that TOR activity is reduced in vip1 mutants.

Therefore the conclusion that vip1 operates independently of TOR is, to my opinion, not fully supported by the data presented here. I think that it would be essential for this paper to try to measure TOR activity in the vip1 mutant. The authors mentioned that they have tried to use the phosphorylation of S6 kinase as readout for TOR activity but without success. I agree that this is not an easy task but S6K phosphospecific antibodies obtained from the plant kinase are now available at Agrisera (http://www.agrisera.com/en/artiklar/ribosomal-s6-kinase-1_2-s6k1_2.html). I believe that it would be worth trying this antibody! Otherwise the BIP protein has been also identified as a TOR substrate in Chlamydomonas and could be used as readout.

RESPONSE: We agree with Reviewer 1# that a TOR kinase assay will required to fully understand the relationship between TOR and InsP signaling. Although we followed Reviewer 1# suggestion of using the above mentioned anti-phospho S6K we have been unable to replicate in Chlamydomonas the results obtained in Arabidopsis, and know of no other validated TOR substrate for Chlamydomonas that can be tested.

Minor points addressed.

Reviewer #2:

Point 1. The authors have not illustrated that the Chlamydomonas VIP1 enzyme is active, or whether it can complement a yeast mutant. On the tree, CrVIP1 groups with other uncharacterized VIP enzymes. This begs the question of whether the green algae enzymes are different from the yeast and plant VIPs? The authors show that these mutants have reduced InsP7 and InsP8, and so it seems very likely that they do catalyze the expected reactions. Perhaps a short description of amino acid similarity in the whole ORF and within the ATP grasp domain could address the likelihood that CrVIP1 is as expected.

RESPONSE: We understand the reviewer's critique, but felt that our in vivo results of reduced InsP7 and InsP8 in Chlamydomonas vip1-1 mutants combined with the already documented conservation of VIP biochemical activity across a huge phylogenetic spectrum (plants, animals, fungi) is compelling. Were data from plant VIP proteins not already published the reviewer's point would be stronger. Plant and Chlamydomonas VIPs are much closer to each other than either is to animal and fungal VIPs (Fig. 2), so lack of conserved activity or altered activity arising in a Chlamydomonas VIP homolog would be extraordinary. We do show the conserved catalytic regions of CrVIP1 as the reviewer suggested in the alignment of panel 2A.

Point 2. The LC-MS/MS method provides a huge strength to this work. I wondered about the error present in these measurements. In some cases values are almost 2x different, but the error is so large there is not a significant difference. In other cases the error is much smaller and differences less than 2x are significant. Can the authors comment on this?

RESPONSE: InsPs are highly labile so even though we took care to handle replicate samples as similarly as possible, we are not surprised by variability in the error term in some measurements. We used multiple technical and biological replicates for each sample so that we could report significant and reproducible results.
Point 3: I think the authors need to point out that their method groups all InsPs containing the same number of phosphates. Thus the really interesting change in InsP3 is not necessarily due to changes in Ins(1,4,5)P3. The correlation of the change in InsP3 with changes in the higher InsPs is really fascinating. I wondered whether the authors had any knowledge on the identity of this molecule? Does it co-elute with the standard InsP3? I also wondered whether there was any indication of the existence of novel PP-InsPs, like PP-InsP4. I point out that PP-InsP4 would probably elute with InsP6. Similarly, the InsP3 peak could be anything with 3 phosphates, even a PP-InsP molecule.

RESPONSE: We agree with the reviewer and have added a note in our Methods and Results section about the limitations of our analytical method regarding stereoisomers. Identification and differentiation of InsP stereoisomers are a general issue for this field and are difficult in the context of in vivo InsP profiling (e.g. with 3H-myoinositol labeling that is the current standard) where there is limited ability to discriminate isomers and no standards available for some theoretically possible isomers.

Point 4. Why is there no InsP or InsP2 present in these cells?

RESPONSE: We did not try to purify or monitor InsP1 and InsP2 since they have the same mass and retention time as other common hexose mono- and di-phosphates. Doing so would have required a major additional validation effort. Most other studies of InsP signaling also omit these precursors.

Point 5. The authors conclude there is a 6-fold increase in TAGs in the vip1 mutant. Which specific piece of data are they using for this? In 8 B it looks to me that FAMEs increase by about 3 fold, not 6 fold.

RESPONSE: We thank the reviewer for catching this error and have corrected the text to match the data in the bar graph.

Point 6. The authors need to clarify whether they feel their data supports increased lipids in vip1 mutants in TP media, or whether this phenotype is also acetate dependent.

RESPONSE: Although the effects in TP media are more difficult to validate statistically, the trend in our data are for increased lipids under all conditions (Fig. 5B in revised manuscript), and this increase is further corroborated by our qualitative observations with Nile Red staining (Fig. 5A in revised manuscript).

Point 7. Figure 9 needs P value determinations, and the authors need to comment on the interesting increases in metabolites in the mutant under TP conditions.

RESPONSE: Done

Point 8. The authors may want to address the Wild et al., paper on SPX domain binding by InsP6 and InsP7. This new paper shows that InsP6 and the inositol pyrophosphates act as sensors by binding to SPX domains contained in proteins in the phosphate sensing pathway. The authors should consider addressing the energy and metabolic alterations in the yeast KCS1 mutant, which has decreased InsP7 levels.

RESPONSE: We thank the reviewer for bringing this paper to our attention. We have included the Wild et al citation in our revision. We also noted that our culture media has excess phosphate and we think it unlikely that defects in phosphate metabolism underlie the vip1-1 phenotype. The possibility of SPX domain proteins being the sensors for InsPs is very exciting but beyond the scope of this report to examine.

Point 9. Describe the exact Rap used in the work.

RESPONSE: There is only a single rapamycin drug that we know of. The vendor from which we purchased rapamycin and other inhibitors is in Methods.

Point 10. In Figure 4. The authors should use abbreviations used previously in the literature such as MINPP, and IPK1. I am a fan of the JBC abbreviations for the InsPs as well. Also in this figure: the authors have speculated that the CrVIPs make a 1PP and a 3PP-InsP5, and then a 1,3PP-InsP4. I want to point out that Stephen Shears’s group showed that the mammalian VIP enzyme adds at the 1-position. Shears feels strongly that in the absence of structure information it is best to conclude that VIPs make 1-PP-InsP5. I think the authors should include an asterisk next to the InsP7 and InsP8 molecules and define that the enantiomer designation is a speculation.

RESPONSE: Done.
Reviewer #3 (Comments for author):

Point 1. Statistical evaluation has not been included for all graphs. This should be completed.

**RESPONSE:** Done.

Point 2. The effects of IPs on carbon allocation are likely also related to the cells’ energy status. Very recently (and likely only after submission of this manuscript), Wild et al. (Science 2016) have reported a role of IPs, including those found in the present study, in the regulation of the adaptation of eukaryotic cells to phosphate starvation, and demonstrate that IPs serve as indicators of the cellular Pi-status. It appears logical that the effects observed in mixotrophic conditions might also involve some of these processes and the link to phosphate-signaling should be experimentally addressed.

**RESPONSE:** See response to Reviewer #2 above who also brought the Wild et al paper to our attention.

Point 3. The lack of mechanistic understanding of the effects seen is evident from the overly long and unfocused discussion where the authors bring to bear all that can be read on the regulatory roles of IPs in different models.

**RESPONSE:** We have shortened the Discussion to be more concise and focused. We also note as described above that our work brings together several disparate areas of research and we wanted to address the implications for each of them.

Point 4. The relevant papers by Mosblech et al. (2008 and 2011) should be mentioned, which demonstrate the influence of altered IP levels on the defense response of Arabidopsis.

**RESPONSE:** We have included these citations in the revision.

Point 5. The immunocytochemistry data are not convincing. Why is there no green nuclear fluorescence in the merged image in the top panel (control) even though the label is close to uniform between cytosol and nucleus in the green channel alone? The deconvolution appears to create particulate signals where there were none in the non-deconvolved samples. Could this be an artefact of the processing?

**RESPONSE:** We have removed the deconvolution images as they are indeed prone to some distortion effects. We added some annotation to new wide-field images to make the localization easier to interpret. These images make it clear that VIP1 is cytoplasmic, and they also show a decreased signal in the nuclear region, though we do not emphasize the latter point as it is not central to our study or its main conclusions.

Point 6. The phrasing of the description of the lipid analysis on p. 11 is unfortunate and lists Bligh & Dyer, 1959, as a reference for GC-analysis of FAMEs. This is most certainly not correct.

**RESPONSE:** The misleading wording has been corrected in the revised version.

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TPC2016-00315-BRR1 2nd Editorial decision – accept with minor revision Aug. 17, 2016

Several members of the Editorial Board have reviewed of your revised, ‘breakthrough report” entitled “Synergism between inositol polyphosphates and TOR kinase signaling in nutrient sensing, growth control and lipid metabolism in Chlamydomonas.” On the basis of our review, 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 the following minor, but important issues:

1.) TPC requirements for the phylogenetic analysis require bootstrap values from a minimum of 1000 trials, and you report 200 (Fig. 2). Please update and revise accordingly.

2.) In addition, you indicate in your point-by-point response that you performed the suggested S6K1 experiment (R#1), but that you “have been unable to replicate in Chlamydomonas the results obtained in Arabidopsis”. What does this mean exactly? Please explain briefly what you have tried and share with the readers your preliminary conclusion as to why it hasn’t worked or led to conclusive results. For example, if the antibody doesn’t work in Chlamydomonas, this would be an important piece of information for the community, in addition to justifying why no TOR activity data are provided. Also, in addition to S6 kinase, another reasonably well-characterized substrate of
TOR is the eukaryotic translation initiation factor 4E binding protein1 (4E-BP1). Could 4E-BP have been used instead of S6K? If not, why not? Please discuss these concerns in 2 or 3 sentences in the manuscript.

**TPC2016-00315-BRR2  2nd Revision received**

Editor comments and author responses.

Point 1. TPC requirements for the phylogenetic analysis require bootstrap values from a minimum of 1000 trials, and you report 200 (Fig. 2). Please update and revise accordingly.

**RESPONSE: Done.**

Point 2.1. In addition, you indicate in your point-by-point response that you performed the suggested S6K1 experiment (R#1), but that you "have been unable to replicate in Chlamydomonas the results obtained in Arabidopsis". What does this mean exactly? Please explain briefly what you have tried and share with the readers your preliminary conclusion as to why it hasn't worked or led to conclusive results. For example, if the antibody doesn't work in Chlamydomonas, this would be an important piece of information for the community, in addition to justifying why no TOR activity data are provided.

**RESPONSE: We include in our revision (Lines 487-494) two sentences about the phospho-S6K antibody we tested (referenced in Xiong, 2013) that was reported to work in Arabidopsis, but failed to give a signal in Chlamydomonas. We noted that there are some non-conserved positions nearby the phospho-site that differ between Chlamydomonas/Arabidopsis/human S6K that might be the underlying reason for the absence of a signal in Chlamydomonas.**

Point 2.2 Also, in addition to S6 kinase, another reasonably well-characterized substrate of TOR is the eukaryotic translation initiation factor 4E binding protein1 (4E-BP1). Could 4E-BP have been used instead of S6K? If not, why not? Please discuss these concerns in 2 or 3 sentences in the manuscript.

**RESPONSE: 4E-BP1 is not found in Chlamydomonas (or any green lineage organism) so could not be tested. We did our own BLAST search to confirm that no identifiable homologs were present in Chlamydomonas (or plants). We did not include these findings in the revision because researchers interested in TOR kinase signaling in green organisms are generally aware of the conserved/non-conserved aspects of the pathway.**

**TPC2016-00351-BRR2  3rd Editorial decision – acceptance pending**

We are pleased to inform you that your paper entitled "Synergism between inositol polyphosphates and TOR kinase signaling in nutrient sensing, growth control and lipid metabolism in Chlamydomonas" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

**Final acceptance from Science Editor**

Sept. 2, 2016