THE PLANT CELL

Peer Review Report for 10.1105/tpc.16.00388

Characterization Of Phytochrome Interacting Factors From The Moss *Physcomitrella Patens* Illustrates Conservation Of Phytochrome Signaling Modules In Land Plants

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Review timeline:

TPC2015-00270-RA	Submission received:	April 1, 2015
	1st Decision:	May 1, 2015 *manuscript declined*
TPC2016-00388-RA	Submission received:	May 18, 2016
	1st Decision:	June 20, 2016 *manuscript declined*
	Appeal requested:	Aug. 17, 2016
	Appeal decision:	Aug. 24, 2016 *submit a new substantially revised manuscript*
TPC2016-00388-RAR1-A	Submission received:	Nov. 2, 2016
	1st Decision:	Nov. 24, 2016 *accept with minor revisions*
TPC2016-00388-RAR2	1st Revision received:	Dec. 22, 2016
	2nd Decision:	Jan. 6, 2017 *acceptance pending, sent to science editor*
	Final acceptance:	Jan. 22, 2017
	Advance publication:	Jan. 25, 2017

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.)

TPC2016-00270-RA 1st Editorial decision – *declined* May 1, 2015

The manuscript has been reviewed by three individuals, one with expertise in the general area of evolutionary plant biology, and two others with expertise in light signaling in vascular and non-vascular land plant species. The Editor concurs with the reviewers that this subject is of interest and that experiments are generally solid. However, the experimental evidence presented in support of a common mechanism for phytochromes signaling involving PIFs in mosses and plants is mostly correlative and preliminary at present. Moreover, the overlap of light regulated genes in Arabidopsis and *P. patens* is limited, and therefore, conclusions derived therefrom are somewhat over-stated. Lacking direct genetic data implicating PpPIFs in phytochrome signaling, this work stops short of making a compelling argument for the shared role for PIFs in light signaling in land plants.

---------- Reviewer comments:

Reviewer #1 (Comments for the Author):

This study makes a valuable contribution to our understanding of phytochrome signaling outside of flowering plants, by investigating changes in gene expression in the moss, Physcomitrella patens, induced by red light, by analyzing the results to find which sets of genes are similarly regulated in Arabidopsis, and by studying the structure and function of phytochrome interacting factors (PIFs) in Physcomitrella patens. Using these approaches, the authors find evidence that the involvement of PIFs in phytochrome signaling may be deeply conserved in land plants. Interestingly, the authors discovered that motif-dependent interactions with PHYs differs from those observed in

Arabidopsis. Such differences provide contrasts that can be used to better understand the specificity of PIF-PHY interactions in Arabidopsis, as already hinted at in this paper. Similarly, differences in the nature of the genes that are

differentially expressed in response to red light between Physcomitrella and Arabidopsis are interesting; further study of these differences can be used to better understand how specific components of the networks in Arabidopsis function. All in all, this is a comprehensive study that will be pivotal to further work on light-signaling in Physcomitrella, with implications for light-signaling across land plants.

I have only a few comments to make. These are important, but easily addressed.

Minor Revisions

Point 1. The authors make a distinction only between seed plants and non-seed plants. This is understandable as it simplifies the presentation. The problem with doing so, however, is that it forces them to assume that what we know to be true about PHY and PIF function in Arabidopsis is true for all seed plants. However, few to no data on the function of these proteins exists for the other four major seed plant groups: cycads, conifers, Ginkgo, and gnetophytes. So when the authors say, on line 96 for example, that "Phytochrome downstream signaling in the nucleus has been intensively studied in seed plants ...", it simply is not true. And throughout the paper, "seed plants" are attributed with characteristics that are known only in angiosperms, and in the in-text citations, not a single paper on a conifer, cycad, etc., is cited. I think that there are two ways to handle this. Either make a disclaimer that the phrase "seed plants" is being used to simplify the presentation, even though few to no data exist from the gymnosperm clades to suggest that the assumptions are valid. Or simply use the term "angiosperms" instead. Otherwise, the authors do a disservice to the community by implying that we know many things that we do not know, and that would be valid and productive avenues of research.

Point 2. Much more minor, but along a similar line, on lines 151-154, there is a section about regulation of transcript levels by phytochromes in mosses, liverworts, and green algae, but the Christensen et al. citation refers to a fern.

Point 3. The phylogenetic data were handled very well, but they have limited power. For the most part, the authors have handled this well. But given that the maximum likelihood bootstrap and posterior probability values are both quite low (e.g., in Bayesian trees, PP below 0.95 suggest a large degree of uncertainty about the node), care should be taken not to overinterpret the results. The trees do indeed suggest that the Physco genes evolved within Physco or within mosses, but the distribution of motifs across the proteins in the PIF clades is not actually very informative, particularly given the lack of resolution along the backbone of the tree. Thus, the statement in lines 288-289 is better omitted.

Reviewer #2 (Comments for the Author):

Phytochromes are prominent red and far-red photoreceptors in land plants and they are best studied in seed plants, where they exert their functions both in the nucleus and cytoplasm. In moss Physcomitrella Patens, cytoplasm-localized phytochromes play important roles in mediating phototropism, polarotropism, and chloroplast movement (Kadota, A. et al. Planta 2000 210:932-7; Mittmann F. et al. 2004 PNAS 101:13939-44; Uenaka and Kadota 2007 Plant J 51:1050-61). The authors have recently shown that at least Physcomitrella PHY1 accumulates to the nucleus in response to both red and far-red light (Possart and Hiltbrunner 2013 Plant Cell 25:102-14), suggesting that moss phytochromes might also play a role in the nucleus to regulate gene expression. Consistent with this model, previous characterization of a pubshy2 double mutant, which blocks the biosynthesis of the chromophore of phytochrome, showed that Physcomitrella phytochromes are required for gene regulation by red light (Chen et al. 2012 et al. PNAS 109:8310-5).

The goal of this manuscript was to elucidate mechanisms of nuclear phytochrome signaling that mediate gene regulation by light. First, the authors determined a list of red-light responsive genes and showed that the Arabidopsis orthologs of a large fraction of these genes are also light responsive. Because a key mechanisms by which Arabidopsis phytochromes control gene expression is by regulating a group of master transcriptional regulators called Phytochrome-Interacting Factors (PIFs), the authors investigated PIF orthologs in Physcomitrella and the interaction between Pp-PIF1 and Pp-PHYs. They show that Pp-PIF1 interacts preferentially with photoactivated Pp-PHYs and At-PHYA and the interaction is mediated by the APA motif. Pp-PIF1 and Pp-PIF2 localize to the nucleus in Physcomitrella. Moreover, overexpression of Pp-PIF1 and Pp-PIF2 in Arabidopsis led to alterations in hypocotyl growth.

Overall, this area of research of Physcomitrella phytochromes is certainly very important to broaden our knowledge of the functions of phytochromes in land plants. The characterization of Pp-PIFs and their functions with Pp-PHYs are very interesting. However, the primary concern is that the work, at this stage, has not carried things far enough forward, in terms of sufficiently delineating novel mechanisms of phytochrome signaling in Physcomitrella. With respect to demonstrating the role of PIFs in Physcomitrella, it would be more convincing to include genetic characterization of light-mediated physiological and gene expression responses in various pif mutants in Physcomitrella. It would also be interesting to further investigate the significance of the discrepancies between the PIF-PHY interactions in Arabidopsis and Physcomitrella.

Reviewer #3 (Comments for the Author):

This manuscript reported a functional analysis of PHYTOCHROME INTERACTING FACTORS (PIFs) in Physcomitrella patens, a model system of nonvascular plants. In this study, the authors performed transcriptome profiling to determine differentially expressed genes (DEGs) in response to red light in P. patens. They then compared the list (using potential Arabidopsis homologs of these genes) with AtPIF-dependent genes in Arabidopsis and showed certain degree of overlap. The results suggested the presence of putative PIF-dependent genes in P. patens. The authors found 4 Physcomitrella PIFs (1-4) but selected only PpPIF1 and PpPIF2 for investigating their interactions with phytochromes. Yeast two-hybrid assay for PpPIF1 and in vitro co-immunoprecipitation for PpPIF2 were performed to show these PpPIFs interact with phytochromes in a light-dependent manner and depend on the APA domain. They also found PpPIF1 and PpPIF2 co-localize with AtPHYA in nuclear bodies in <I>Nicotiana benthamiana leaf cells. PpPIF1 and PpPIF2 were expressed in Arabidopsis to further characterize their activity in photomorphogenesis.

Some part of the data and description in this manuscript are reasonably sound. However, the conclusions cannot be adequately supported by the data. Overall, the reviewer does not see the significance and novelty of this study comparing to other reports on PIFs published in The Plant Cell, except the study is the first one to focus on the PIFs in nonvascular plants. The reviewer believe if the authors can provide transcriptomic and physiological data from moss knockout and overexpression lines of PpPIFs, functions of PIFs in nonvascular plants may be further elucidated and the contribution to the field will be significant. Detailed comments are shown as below.

Overall comments

Significance

1. This study is the first one to provide a detailed analysis on the function of light-signaling transcription factors in nonvascular plants. Evolutionary track of light signaling pathway in land plants is revealed.

2. PIFs in Physcomitrella patens were identified and their interactions with phytochromes and subcellular localization were nicely shown.

Weaknesses

1. Comparing large-scale data from different developmental stages, time points, or experimental techniques usually generate large discrepancies. The authors over-interpreted the results and made conclusions based on problematic comparison.

2. Protein-protein interaction and subcellular localization experiments are incomplete.

3. As there is no functional data to show PpPIFs are the light-signaling components in Physcomitrella patens, analyzing their functions in Arabidopsis become less meaningful.

TPC2016-00388-RA 1st Editorial decision – *declined* **June 20, 2016**

All three reviewers concur that this manuscript contains interesting new data with regard to the roles of two of four PIFs in phytochrome signaling in the moss *Physcomitrella patens*. The editors also concur that the manuscript is greatly improved from the version submitted earlier. In the intervening period, another manuscript on the phytochrome-PIF system in the liverwort *Marchantia polymorpha* has been published that sheds new light on the

evolutionary interpretations that you have drawn from your new data. Since the single *M. polymorpha* phytochrome interacts with a single (more 'angiosperm-like') PIF, it is therefore more likely that signaling functions would have diverged, rather than have been conserved, amongst the more complex phytochrome-PIF system in P. patens. To address these concerns will require extensive restructuring of the present manuscript and additional experiments to understand the differences as well as similarities of phytochrome-PIF functions amongst different extant plant lineages.

---------- Reviewer comments:

Reviewer #1:

This is a data-rich paper that provides functional evidence of the role of PIFs in light signaling in Physcomitrella patens. The P. patens genome has four PIFs and four phytochromes, and the authors explore the interactions between PIFs and PHY, the translocation of PIFs to the nucleus, and the regulation of gene expression by red light in dark-adapted gametophores. Their data suggest that with light activation 1) Pp-PIF1 interacts with all four PHY via the APA motif; 2) Pp-PIF2 interacts with Pp-PHY4 via the APA; 3) Pp-PIF3 interacts with Pp-PHY2 through an undetermined motif; 4) Pp-PIF4 interacts with PpPHY1 via an undetermined motif. The data demonstrate that all four PIFs show light-activated transfer to the nucleus and localization in nuclear bodies. Complementation and over-expression experiments with Pp-PIF1 and Pp-PIF2 constructs are consistent with roles for these genes that are similar to those of Arabidopsis PIFs. Overall, the functional data were incomplete in that sequence motifs in Pp-PIF3 and Pp-PIF4 were not determined and the complementation and over-expression experiments did not include Pp-PIF3 and Pp-PIF4 constructs. Not including the available Marchantia sequences in the alignment and analyses is a major oversight, even in the absence of the newly published data on Marchantia PIF, since the PIF sequence appears to have both APA and APB.

There are some major concerns over the evolutionary analyses and/or the interpretation of the phylogenetic results, which are included in the numbered comments below.

Point 1. Throughout the paper, the authors make inappropriate references to "seed plants" and "non-seed plants". This leads to many inaccuracies in the background information that they present because in fact, in a very high proportion of the cases, we don't have data from "seed plants" and "non-seed plants". Rather, we have data from Arabidopsis, sometimes rice, and Physcomitrella. A good rule of thumb before using these labels might be to ask oneself whether "seed plant" references can be included as citations for the statement. That is, when "seed plant" is used, can references to data from conifers, cycads, Ginkgo, and gnetophytes be provided? Similarly, when "non-seed plant" is used, can to the statement be backed up by citations from the literature on liverworts, hornworts, mosses, ferns, and lycophytes? In the case of seed plants, not only do the five clades represent 100's of millions of years of independent evolution, during which they became genomically VERY distinct, but they represent just a small fraction of the seed plant clades that once existed! Doesn't this make any conservation we find in these important functions that much more significant? This is not just a phylogeneticist being sticky. Brushing over all this diversity does not serve the field of photobiology because it misleads readers (who might become spectacular evo-devo experimentalists) to think that major unanswered questions have been answered. It is critical, then, that statements in the manuscript appropriately reflect the underlying data.

Point 2. Another general comment pertains to the use of "ortholog". I appreciate that in most cases, the authors have used "homolog" rather than "ortholog". It is appropriate to use "homolog" in the case of the Pp-PIFs because PIF paralogs have evolved independently in Physcomitrella, based on the tree in Figure 2. I question whether it is appropriate in the Abstract (line 46) to use "ortholog" when referring to light-regulated genes in Physcomitrella. Were trees inferred for all these genes such that the authors could determine if "ortholog", or the more general term "homolog", were appropriate? Please check the manuscript for appropriate use of ortholog/homolog. For example, on line 522, "ortholog" is incorrectly used.

Point 3. Page 3, line 62. It is best to stay away from referring to plants as "rudimentary" just because they belong to a lineage that diverged from the rest of land plants before the evolution of flowering plants, or even because they lack a particular innovation.

Reviewer #2:

This manuscript adds a significant insight into light signaling mechanisms in the cryptogamic plants. Phytochrome Interacting Factor (PIF) is thought to be one of the most important phytochrome signaling components in higher plants and there have been little information about cryptogams (non-seed plant) PIF. This paper characterized molecular properties of the non-seed plant PIFs of the moss Physcomitrella. However, the paper entitled "Phytochrome signaling is mediated by PHYTOCHROME INTERACTING FACTOR in the liverwort Marchantia polymorpha" (Inoue et al.) was published in Plant Cell Advance Publication on June 1. This Marchantia paper demonstrated non-seed plant PIF functions, and thus it contains closely related findings to this manuscript and main conclusions mentioned are almost overlapped. In addition, there are some discrepancies between these two reports. On the other hand, authors performed light dependent gene expression profiling using microarray assay and functional complementation experiment using Arabidopsis pifq mutant background. These unique data provide intriguing insights into the cryptogam PIF function and evolutionary conservation of PIF-PHY signaling among the land plants. Thus, authors should refer this newly published Marchantia paper and focus on the original findings different from the Marchantia paper. I have some suggestions for improvement.

Point 1. In PIF-PHY colocalization experiments, coexpresion of PpPHYs:CFP (PpPHYs-NLS:CFP) with PpPIFs:YFP will strengthen authors' hypothesis of PpPIF-PpPHY interaction and nuclear body formation in Physcomitrella.

Point 2. Authors noted the identification of putative target genes that are orthologs of PIF-controlled genes in Arabidopsis. Investigation for light-dependent gene expression change of such candidate genes in transgenic Arabidopsis (pifq background) would support authors' speculation about PpPIFs involvement in transcriptional regulation.

Reviewer #3:

In this work, Possart and co-authors perform an extensive phylogenetic analysis to identify and clone the light signaling PIF transcription factor orthologs of the moss Physcomitrella patens. Through a series of extensive and carefully performed analyses, they identify certain structural and functional similarities between Arabidopsis and P. patens PIFs, suggesting that the phy-PIF signaling module has been conserved in land plants. Importantly, and in agreement with this hypothesis, ectopic expression of Pp-PIFs complements the constitutive photomorphogenic phenotype of Arabidopsis pifq mutants lacking PIFs 1, 3, 4 and 5. The manuscript is well written and clearly presented. Although the question is interesting and novel, and, overall, experiments appear well-performed and data well-analyzed, some aspects of this manuscript appear incomplete.

Point 1. The authors claim that Pp-PIFs function is in part conserved between Arabidopsis and *P. patens*, and they provide evidence that Pp-PIFs are functional in Arabidopsis. However, one main concern of this study is that authors do not provide evidence of Pp-PIFs function in Physcomitrella patens, and therefore, it is not possible to conclude that Pp-PIFs act as transducers of light signaling downstream of PHY in the moss.

Point 2. In the microarray study authors relate red light regulation of gene expression in dark-adapted moss with PIF-regulated gene expression in response to light during Arabidopsis de-etiolation. However, no evidence is provided that Pp-PIFs act as transcriptional regulators of gene expression in response to light. In the absence of such evidence, the microarray analysis is not relevant. To strengthen this analysis, at the very least authors should determine the expression of described PIF-regulated target genes in the pifq lines expressing Pp-PIFs (Figure 7), to test whether the pifq phenotype is also rescued at the gene expression/molecular level. To be able to make a direct connection with the microarray analysis in the moss, authors should also include the gene expression analysis of the Arabidopsis homologs responding to red light in the moss. In the current version of the manuscript, authors should avoid sentences like "We conclude that PIFs from P. patens and A. thaliana may act similarly as nuclear regulators of light-dependent gene expression. (line 415)", as they are an overinterpretation of the data.

One intriguing observation that authors can comment on is the fact that among the red-light regulated genes in the moss, only the R-induced subset shows overlap with PIF-regulated genes during Arabidopsis de-etiolation. This is somehow inconsistent, as it is well known that R-repressed genes are much enriched in PIF-target genes (reviewed in Leivar et al. (2014). Plant Cell, 26:56-78; Pfeiffer et al. (2014). Molecular Plant, 7:1598-618).

Point 3. The authors show that Pp-PIFs accumulate in the nucleus, where they colocalize with Pp-PHY or At-PHYA in nuclear speckles. However, authors surprisingly ignore in their analysis one of the main molecular events in PHY signaling: the phy-induced proteolytic degradation of the PIFs. Authors should test whether red or far-red light induces proteolytic degradation of Pp-PIFs. Otherwise, the analysis of the phytochrome signaling module is incomplete.

TPC2016-00388-RA Appeal requested **Aug. 17, 2016**

We have of course been disappointed by the outcome regarding our manuscript. Given the extent of criticism raised by the reviewers, we do not wish to argue against your decision not to publish our work in The Plant Cell. After having discussed your arguments and the reviews with my colleagues and collaborators, we feel, however, that we should express our opinion on some aspects regarding the process. We found it very surprising that while our manuscript was under review at TPC, the same journal published the Marchantia paper, which then in turn served as an argument against the publication of our work. Besides this being an unusual process in our view, we do not understand the conflicting dichotomy that you and the reviewers describe when comparing these two studies. Both studies conclude that PIF-phytochrome signaling nodes have been conserved in land plant evolution. The divergence of this signaling system within Physcomitrella, where the situation is more complex than in Marchantia and admittedly would require more experimental data, was not the focus of our manuscript. Moreover, after carefully reading the Inoue et al. paper, it remains unclear to us on what basis the Mp-PIF should be more "angiosperm-like" than Pp-PIFs.

We are not raising these points in an attempt to revert your decision. In the light of the publication of the Marchantia paper, however, the novelty and impact of our manuscript has considerably decreased. We would also like to emphasize that, after the first round of reviews at TPC in 2015, we added extensive additional experiments and restructured the manuscript to comply with the reviewers' concerns and suggestions. Now we are faced with yet another set of requests, all of which were not brought up at the time. Given that in its current state the manuscript already contains an extensive amount of data, we do not believe that it would benefit from the addition of the entirety of the requested experiments. For instance, the circumstance that Physcomitrella has 4 PIF genes should not demand for the full description of all of them to the same level of detail (similarly, in A. thaliana studies, it is rare that a full analysis of complete gene families is published). We agree, however, with some of the reviewers' requests, explained in more detail below.

In the light of all of the above, we still believe that our manuscript has the potential to be published in an ASPB journal. We would therefore like to ask for your support to have our manuscript and the reviews forwarded to your colleagues at *Plant Physiology* and to get the opportunity to submit a revised version to them, improved in particular on the following aspects:

- As requested by reviewers 2 and 3, we will include a gene expression analysis of PIF-regulated genes in the rescued *A. thaliana pifq* mutant.

- In response to reviewer 3, we will investigate PIF degradation in the same lines.

- We will adjust and rephrase our discussion of the phylogenetic analysis to comply with the criticisms raised by reviewer 1.

- We will of course include a discussion of and comparison to the *Marchantia* data from Inoue et al.

We would highly appreciate if you considered our arguments and hope that you will judge this a viable option. We are looking forward to hearing from you.

TPC2016-00388-RA Appeal decision - *submit a new substantially revised manuscript* **Aug. 24, 2016**

We have decided to reconsider your work for publication in The Plant Cell. Despite the problems noted by the reviewers, all three made many positive comments about the work. We think that ultimately it will be a good

companion paper to that of Inoue et al. on Marchantia, and the work will have more impact if it is published sooner rather than later.

The points for revision include:

-adding gene expression analysis of PIF-regulated genes in the rescued *A. thaliana pifq* mutant

-adding analysis of PIF degradation in the same lines

-discussion of and comparison to the Marchantia data from Inoue et al.

-revising the language and conclusions carefully to adequately address all of the major (numbered) concerns of Reviewer 1 with respect to the evolutionary analyses and/or the interpretation of the phylogenetic results (this is critical as the journal maintains high standards with respect to phylogenetics and discussions of evolutionary relationships)

-In addition, please ensure that the phylogenetic analysis meets or exceeds the standards of the journal as summarized in the Instructions for Authors.

---------- Reviewer comments:

[Provided below along with author responses]

TPC2016-00388-RAR-1A Submission received Nov. 2, 2016

Reviewer comments and **author responses**:

Reviewer #1:

This is a data-rich paper that provides functional evidence of the role of PIFs in light signaling in Physcomitrella patens. The P. patens genome has four PIFs and four phytochromes, and the authors explore the interactions between PIFs and PHY, the translocation of PIFs to the nucleus, and the regulation of gene expression by red light in dark-adapted gametophores. Their data suggest that with light activation 1) Pp-PIF1 interacts with all four PHY via the APA motif; 2) Pp-PIF2 interacts with Pp-PHY4 via the APA; 3) Pp-PIF3 interacts with Pp-PHY2 through an undetermined motif; 4) Pp-PIF4 interacts with PpPHY1 via an undetermined motif. The data demonstrate that all four PIFs show light-activated transfer to the nucleus and localization in nuclear bodies. Complementation and over-expression experiments with Pp-PIF1 and Pp-PIF2 constructs are consistent with roles for these genes that are similar to those of Arabidopsis PIFs. Overall, the functional data were incomplete in that sequence motifs in Pp-PIF3 and Pp-PIF4 were not determined and the complementation and over-expression experiments did not include Pp-PIF3 and Pp-PIF4 constructs. Not including the available Marchantia sequences in the alignment and analyses is a major oversight, even in the absence of the newly published data on Marchantia PIF (see comment 9), since the PIF sequence appears to have both APA and APB. There are some major concerns over the evolutionary analyses and/or the interpretation of the phylogenetic results, which are included in the numbered comments below.

Point 1. Throughout the paper, the authors make inappropriate references to "seed plants" and "non-seed plants". This leads to many inaccuracies in the background information that they present because in fact, in a very high proportion of the cases, we don't have data from "seed plants" and "non-seed plants". Rather, we have data from Arabidopsis, sometimes rice, and Physcomitrella. A good rule of thumb before using these labels might be to ask oneself whether "seed plant" references can be included as citations for the statement. That is, when "seed plant" is used, can references to data from conifers, cycads, Ginkgo, and gnetophytes be provided? Similarly, when "non-seed plant" is used, can to the statement be backed up by citations from the literature on liverworts, hornworts, mosses, ferns, and lycophytes? In the case of seed plants, not only do the five clades represent 100's of millions of years of independent evolution, during which they became genomically VERY distinct, but they represent just a small fraction of the seed plant clades that once existed! Doesn't this make any conservation we find in these important functions that much more significant? This is not just a phylogeneticist being sticky. Brushing over all this diversity does not serve the field of photobiology because it misleads readers (who might become spectacular evo-devo

experimentalists) to think that major unanswered questions have been answered. It is critical, then, that statements in the manuscript appropriately reflect the underlying data.

RESPONSE: We agree with the reviewer and have amended the manuscript in many places to reflect the species tackled. In most places we now refer to *A. thaliana* and *P. patens* only.

Point 2. Another general comment pertains to the use of "ortholog". I appreciate that in most cases, the authors have used "homolog" rather than "ortholog". It is appropriate to use "homolog" in the case of the Pp-PIFs because PIF paralogs have evolved independently in Physcomitrella, based on the tree in Figure 2. I question whether it is appropriate in the Abstract (line 46) to use "ortholog" when referring to light-regulated genes in Physcomitrella. Were trees inferred for all these genes such that the authors could determine if "ortholog", or the more general term "homolog", were appropriate? Please check the manuscript for appropriate use of ortholog/homolog. For example, on line 522, "ortholog" is incorrectly used.

RESPONSE: We agree with the reviewer that the term ortholog should be used with caution. We have checked all occurrences of ortholog and altered the corresponding terms or phrases where necessary. In the abstract, we are talking not about the PIFs but about their target genes. Since no phylogenies have been carried out for them (we rely on reciprocal best hits) we changed the phrase to "putative orthologs".

At the end of the introduction, we talk of "potential functional PIF orthologs", since indeed one-on-one orthology as per the definition by Walter Fitch cannot be assigned, due to in-paralogs being present. However, the term functional ortholog has been coined for cases where paralogs can be shown to perform the orthologous function. The term putative functional ortholog appears to us to carry the necessary caution.

Point 3. Page 3, line 62. It is best to stay away from referring to plants as "rudimentary" just because they belong to a lineage that diverged from the rest of land plants before the evolution of flowering plants, or even because they lack a particular innovation.

RESPONSE: We agree and have modified the text accordingly.

Reviewer #2:

This manuscript adds a significant insight into light signaling mechanisms in the cryptogamic plants. Phytochrome Interacting Factor (PIF) is thought to be one of the most important phytochrome signaling components in higher plants and there have been little information about cryptogams (non-seed plant) PIF. This paper characterized molecular properties of the non-seed plant PIFs of the moss Physcomitrella. However, the paper entitled "Phytochrome signaling is mediated by PHYTOCHROME INTERACTING FACTOR in the liverwort Marchantia polymorpha" (Inoue et al.) was published in Plant Cell Advance Publication on June 1. This Marchantia paper demonstrated non-seed plant PIF functions, and thus it contains closely related findings to this manuscript and main conclusions mentioned are almost overlapped. In addition, there are some discrepancies between these two reports. On the other hand, authors performed light dependent gene expression profiling using microarray assay and functional complementation experiment using Arabidopsis pifq mutant background. These unique data provide intriguing insights into the cryptogam PIF function and evolutionary conservation of PIF-PHY signaling among the land plants. Thus, authors should refer this newly published Marchantia paper and focus on the original findings different from the Marchantia paper.

RESPONSE: Thank you for the overall positive evaluation. As the reviewer has pointed out, the Marchantia paper by Inoue *et al.* was published while our manuscript was under review. We therefore did not have the chance to compare our data to that from Inoue and colleagues. We have done so extensively in the revised version of our manuscript.

I have some suggestions for improvement.

Point 1. In PIF-PHY colocalization experiments, coexpresion of PpPHYs:CFP (PpPHYs-NLS:CFP) with PpPIFs:YFP will strengthen authors' hypothesis of PpPIF-PpPHY interaction and nuclear body formation in Physcomitrella.

RESPONSE: Thank you for this suggestion. Unfortunately, PpPHYs:CFP lines do not exist; their generation would take about one year. In the meantime, we have tried to transiently transform the available PpPHY1:YFP line (Possart and Hiltbrunner, 2013). However, as ballistic transformation is very inefficient in *P. patens*, with very few cells

expressing Pp-PIF:CFP, we were not able to record the co-localization due to the light-dependent decrease of Pp-PHY1:YFP levels. The alternative of doing co-transformation and localization studies using transient transformation would also be very inefficient in *P. patens*, with very few cells expressing both constructs. We would argue that showing interaction with two independent methods, yeast-two-hybrid assays and co-immunoprecipitation, as we have done in our manuscript, allows high confidence in the interaction of these two proteins. Furthermore, we would like to point out that specific mutations in the APA motif abolish the interaction of PIF1 with all PHYs that have been tested, suggesting a specific interaction between PIF1 and PHYs.

Point 2. Authors noted the identification of putative target genes that are orthologs of PIF-controlled genes in Arabidopsis. Investigation for light-dependent gene expression change of such candidate genes in transgenic Arabidopsis (pifq background) would support authors' speculation about PpPIFs involvement in transcriptional regulation.

RESPONSE: Thank you for this very helpful suggestion. In the revised version of the manuscript, we now provide qPCR results that show reconstitution or even over-compensation of transcriptional levels through expression of PpPIFs in the Arabidopsis *pifq* mutant. We picked 5 genes known to be PIF-regulated and clearly down-regulated in the *pifq* mutant; all 5 showed rescued or over-compensated levels of expression upon PpPIF1 or PpPIF2. Results are shown in Figure 7 and Supplemental Figure 11.

Reviewer #3:

In this work, Possart and co-authors perform an extensive phylogenetic analysis to identify and clone the light signaling PIF transcription factor orthologs of the moss Physcomitrella patens. Through a series of extensive and carefully performed analyses, they identify certain structural and functional similarities between Arabidopsis and P. patens PIFs, suggesting that the phy-PIF signaling module has been conserved in land plants. Importantly, and in agreement with this hypothesis, ectopic expression of Pp-PIFs complements the constitutive photomorphogenic phenotype of Arabidopsis pifq mutants lacking PIFs 1, 3, 4 and 5. The manuscript is well written and clearly presented. Although the question is interesting and novel, and, overall, experiments appear well-performed and data well-analyzed, some aspects of this manuscript appear incomplete.

Point 1. The authors claim that Pp-PIFs function is in part conserved between Arabidopsis and *P. patens*, and they provide evidence that Pp-PIFs are functional in Arabidopsis. However, one main concern of this study is that authors do not provide evidence of Pp-PIFs function in Physcomitrella patens, and therefore, it is not possible to conclude that Pp-PIFs act as transducers of light signaling downstream of PHY in the moss.

RESPONSE: We agree that providing evidence for PIF function in *P. patens* would be the ideal case. However, we would like to ask the reviewer to compare the situation to that in *A. thaliana*, where single *pif* mutants generally do not show a distinct phenotype that would allow conclusions on PIF function. There are 4 PIFs encoded in the *P. patens* genome, therefore we hypothesize that the situation in moss would be similar and that multiple-gene-knockouts would be necessary. Despite homologous recombination being possible in moss, the generation of multi-gene-knockouts is very inefficient, and the generation of such lines would take months or even years to accomplish. In our opinion, such an attempt surpasses the scope of our manuscript. Moreover, convincing support of the PIF-like function of Pp-PIFs is now provided by complementation of gene expression in Arabidopsis *pifq* mutants (see below).

Point 2. In the microarray study authors relate red light regulation of gene expression in dark-adapted moss with PIF-regulated gene expression in response to light during Arabidopsis de-etiolation. However, no evidence is provided that Pp-PIFs act as transcriptional regulators of gene expression in response to light. In the absence of such evidence, the microarray analysis is not relevant. To strengthen this analysis, at the very least authors should determine the expression of described PIF-regulated target genes in the pifq lines expressing Pp-PIFs (Figure 7), to test whether the pifq phenotype is also rescued at the gene expression/molecular level. To be able to make a direct connection with the microarray analysis in the moss, authors should also include the gene expression analysis of the Arabidopsis homologs responding to red light in the moss. In the current version of the manuscript, authors should avoid sentences like "We conclude that PIFs from P. patens and A. thaliana may act similarly as nuclear regulators of light-dependent gene expression. (line 415)", as they are an overinterpretation of the data.

RESPONSE: Reviewer 2 has made the same helpful suggestion. We provide this data in the revised manuscript; please see our response to reviewer 2 for details.

One intriguing observation that authors can comment on is the fact that among the red-light regulated genes in the moss, only the R-induced subset shows overlap with PIF-regulated genes during Arabidopsis de-etiolation. This is somehow inconsistent, as it is well known that R-repressed genes are much enriched in PIF-target genes (reviewed in Leivar et al. (2014). Plant Cell, 26:56-78; Pfeiffer et al. (2014). Molecular Plant, 7:1598-618).

RESPONSE: We agree that this is an interesting observation, although we would not necessarily consider it inconsistent, keeping in mind that the species and the time points analyzed by Leivar et al. (2009) and by us are different. However, we now emphasize the discrepancy in the discussion, referring to the papers also mentioned by the reviewer.

Point 3. The authors show that Pp-PIFs accumulate in the nucleus, where they colocalize with Pp-PHY or At-PHYA in nuclear speckles. However, authors surprisingly ignore in their analysis one of the main molecular events in PHY signaling: the phy-induced proteolytic degradation of the PIFs. Authors should test whether red or far-red light induces proteolytic degradation of Pp-PIFs. Otherwise, the analysis of the phytochrome signaling module is incomplete.

RESPONSE: Thank you for this suggestion. We now provide immunoblot analyses of PpPIF proteins expressed in the Arabidopsis *pifq* mutant background. Unlike AtPIF3, which gets degraded after 10 min of red light, PpPIFs remain more stable and were detected even after 24h of red light. This situation is reminiscent of AtPIF7, which has also been reported to be light-stable (Leivar et al., 2008).

TPC2016-00388-RAR1-A 1st Editorial decision – *accept with minor revisions* Nov. 24, 2016

We have received a review from one of the original reviewers and consulted with members of the editorial board concerning your manuscript entitled "Characterization of PIFs from the moss Physcomitrella patens illustrates conservation of phytochrome signaling modules in land plants". On the basis of the advice received, we would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on minor revisions based on the comments below. In particular, please consider the following:

[Reviewer comments provided below along with author responses]

TPC2016-00388-RAR2 1st Revision submitted Dec. 22, 2016

Point 1. Please address reviewer 1 comments, which call for minor (but important) editing in a number of places in the text.

Point 2. Note that all primary data (supporting main conclusions) should be shown in main manuscript if possible, and all supplemental figures should provide direct support for a main figure. In this respect, it is somewhat incongruous that the first section of the results refers only to supplemental data and draws no clear conclusion (for example, in the section title). Perhaps Supplemental Figure 1A (or panels A, B, and C) might be moved to the main manuscript (panel D as supporting supplemental) - or another figure created that illustrates the main conclusion of this section? With respect to Supplemental Figure 1: the Venn diagram in panel A should be drawn with ovals proportional to number of elements in each, and the value of panels B and C is not clear. What is the take-home message from this data? Also please present and refer to the location (in a Supplemental Data Set) of the underlying data. This figure should also make better (more consistent) use of color- panels B&C have same color scheme but panel A uses same colors in different manner, which can be confusing.

RESPONSE: We applied the following changes: We altered the section heading to make the conclusion evident. We altered Fig. S1A as suggested and present it as a main text Figure now, supported by the remainder (previous panels B-D) of Fig. S1. The take-home message of panel B and C (now A and B) has been added to the results. The dataset as analysed in Supplemental Figure 1 is available from ArrayExpress, which has been added to the same section. The DEG and cluster data are available as Supplemental Dataset 1, which is referred to in the first results

section. The colour scheme of Fig. 1 and Supplemental Figure A and B has been unified in order to be better comprehensible.

Point 3. Figure 2 is oversized. All important elements and text in figures must be clearly visible and legible at the printed size (authors are encouraged to print their figures to check on the appearance). In this figure, the value (with respect to the main conclusions) of the entire lower unshaded portion relative to the upper shaded portion is unclear; might the unshaded portion be presented in supplemental data (or else as a separate figure)? This would enable better visualization of the upper portion (which of primary importance)?

RESPONSE: We have followed your advice and have transferred the lower part of the phylogeny to a new Supplemental Figure (6). The remaining phylogeny has been enlarged, such that fonts are well readable.

Point 4. For Figure 3, Panels B, C, and D might be moved to supplemental data - they show basically same result as panel A, w/ Pp-PIF1 interacting with the other Pp-PHYs. Panel E/F should stay in main manuscript (this would also solve the problem of poor use of space with this figure). The legend might offer a better description of samples (what constitutes N), and of gel images to far right of panels A-D (protein abundances - Supp Fig 5 is cited - it is actually Supp Fig 6?). It is somewhat difficult (for the general reader) to figure out the difference between Figure 3 and Supplemental Figures 7, 8, and 9. It may be mentioned in the text, but it should also be clearer in the figure legends - they all have similar titles and it is not immediately clear what is being shown that is different.

RESPONSE: We have moved panels B-D from Figure 3 (which is now Figure 4) to a supplemental figure (7), and we have adjusted the size and arrangement of the remaining panels. The legend now contains more information on the experimental design. We have also emphasized the differences between the main and the supplemental figures.

TPC2016-00388-RAR2 2nd Editorial decision – *acceptance pending* **Jan. 6, 2017**

We are pleased to inform you that your paper entitled “Characterization Of Phytochrome Interacting Factors From The Moss *Physcomitrella Patens* Illustrates Conservation Of Phytochrome Signaling Modules In Land Plants” has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **Jan. 22, 2017**