# SPAs promote thermomorphogenesis by regulating the phyB-PIF4 module in *Arabidopsis*

Sanghwa Lee, Inyup Paik and Enamul Huq
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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/189233

MS TITLE: SPAs promote thermomorphogenesis via regulating the phyB-PIF4 module in Arabidopsis

AUTHORS: Sanghwa Lee, Inyup Paik, and Enamul Huq

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

Temperature is an important signal affecting multiple plant developmental processes collectively referred to as thermomorphogenesis. A key signalling module consisting of the light and temperature sensor phytochrome B (phyB) and the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) is known to control these responses in Arabidopsis. Here, Lee et al. report that SUPPRESSOR OF PHYA-105 (SPA) proteins, core components of the Arabidopsis light

signalling pathway, are also essential for thermomorphogenic responses, particularly hypocotyl elongation. This phenotypic response is underpinned by temperature-induced changes in gene expression, and part of the warm temperature transcriptome is dependent on the presence of SPA proteins. The authors go on to show that SPAs regulate these changes by affecting protein levels of the core phyB-PIF4 module, negatively affecting phyB accumulation while stabilising PIF4 protein. The latter appears to be mediated by direct phosphorylation of PIF4 by SPA1 suggesting that the kinase function of SPA proteins is essential for thermomorphogenesis.

While a potential role of SPAs in thermomorphogenesis has been hinted at (Delker et al., 2014), this is the first report showing that SPAs are essential for thermomorphogenic responses at the molecular level and providing mechanistic insight into this process.
Research on how temperature signals are perceived and transmitted, and how they are integrated with other environmental cues, has gained strong momentum over the last decade in the context of climate change and crop productivity. In this regard, the findings presented by Lee et al. should be highly relevant for the temperature and light signalling field, but also the wider plant and developmental science community.

*Comments for the author*

The authors employ appropriate methodology and analyses and the data are well presented. I have a few suggestions to improve the manuscript and further strengthen the conclusions drawn by Lee et al.

Major points

(1) The authors conclude from their phenotypic analyses that SPAs are required for thermomorphogenesis, yet they focus entirely on heat-induced hypocotyl elongation. To strengthen the conclusion that SPAs are generally involved in thermomorphogenic responses, the authors might want to analyse some of the additional phenotypic responses they mention in the introduction in higher order spa mutants.
(2) A key observation by Lee et al. is that phosphorylation of PIF4 by SPA1 underlies the function of SPAs in thermomorphogenesis. The mSPA1 construct that displays reduced kinase activity is an excellent tool employed for in vivo analyses, but should also be included in the in vitro studies (in vitro kinase assays in Fig. 4A and interaction studies in Fig. S3). Showing that mSPA1 reduces PIF4 phosphorylation, but does not have a major effect on SPA1-PIF4 interaction would lend further strength to the notion that it is SPA1 kinase activity per se that is required for PIF4 stabilisation.
(3) Related to point (2): Destabilisation of PIF proteins, including PIF4, usually involves proteasomal degradation. Can the reduction in PIF4 accumulation in spaQ/mSPA1 be prevented by treatment with proteasome inhibitors (MG132/bortezomib)?
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Minor points

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5. The previous works showed that phyB interacted with SPA1 to promote the formation of PHYB-SPA1-PIF1 trimolecular complex and enhance the phosphorylation of PIF1(Paik et al., 2019). Whether phyB, SPA1 and PIF4 form a complex in regulating thermomorphogenesis?
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**First revision**

Author response to reviewers' comments

Response to Reviewers' comments
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Author response: This is an excellent suggestion. We have now added petiole length and flowering time data using higher order spa mutants (Fig. S1). The results show that the petiole length is significantly shorter in spa single mutants compared to wild type control. In addition, the double, triple and quadruple mutants displayed progressively shorter petiole length compared to wild type under high ambient temperature. However, the flowering time did not show any difference in spa mutants compared to wild type between the two temperatures. Although our experiments were performed at 22 vs 28C, similar observations were reported recently (Chung et al., Nature Comm., 2020) showing that flowering time did not change at 27 compared to 17 using Col-0.

(2) A key observation by Lee et al. is that phosphorylation of PIF4 by SPA1 underlies the function of SPAs in thermomorphogenesis. The mSPA1 construct that displays reduced kinase activity is an excellent tool employed for in vivo analyses, but should also be included in the in vitro studies (in vitro kinase assays in Fig. 4A and interaction studies in Fig. S3). Showing that mSPA1 reduces PIF4 phosphorylation, but does not have a major effect on SPA1-PIF4 interaction would lend further strength to the notion that it is SPA1 kinase activity per se that is required for PIF4 stabilisation.

Author response: We have now added in vitro kinase assay using both WT SPA1 and mSPA1 with PIF4 (Fig. 5A). The results show that mSPA1 has reduced or no phosphorylation activity toward PIF4 compared to wild type SPA1. However, both wild type and mSPA1 displayed similar interaction with PIF4 in in vivo co-IP assays (Fig. 4C).

(3) Related to point (2): Destabilisation of PIF proteins, including PIF4, usually involves proteasomal degradation. Can the reduction in PIF4 accumulation in spaQ/mSPA1 be prevented by treatment with proteasome inhibitors (MG132/bortezomib)?

Author response: We have now added the data (Fig. S5), showing that the degradation of PIF4 can be prevented in both spaQ/SPA1 and spaQ/mSPA1 with bortezomib treatment. In fact, the lower band potentially the unphosphorylated form of PIF4 is stabilized in the spaQ/mSPA1 background, while both forms are stabilized in the spaQ/SPA1 background, suggesting that the phosphorylation of PIF4 by SPA1 is critical for PIF4 stabilization at high ambient temperature.

(4) SPA proteins usually act as co-factors of the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) E3 ubiquitin ligase, and cop1 mutants show a strong reduction in PIF4 protein accumulation similar to spaQ (Gangappa and Kumar, 2017). It is thus interesting to consider how these proteins may cooperate in the control of thermomorphogenesis, but this is not touched upon in the manuscript. Addressing this question experimentally might be beyond the scope of this manuscript, but it would be an important point to consider in the discussion.

Author response: Previously, we have shown that HFR1 is stabilized in cop1 background and the increased level of HFR1 contributed to the reduction in PIF levels in cop1 background (Xu et al., 2017, Development). We suspect similar mechanisms is operating in spaQ background. We have added a sentence in this regard.

Minor points

(1) p. 3, l. 11: The authors refer to cryptochromes as thermosensors. However, while cryptochromes can clearly affect temperature-responsive processes (Ma et al., 2016), in contrast to phytochromes they have not been shown to be bona fide temperature sensors.
This sentence should be reworded.

Author response: This has been corrected.

(2) p. 6, l. 14: The authors test several thermo-responsive marker genes for expression in spaQ. Have these genes also been identified in their RNA-seq as temperature- and/or SPA-dependent?

Author response: The RNA-seq experiment displayed temperature-dependent difference for only two genes: IAA29 and CYP79B2 in wild type, which was performed at 22 vs 28 for 24 h. Thus, we performed RT-qPCR assays for these two along with two additional growth genes (PRE5 and SAUR15) at 22 vs 28 for 4 h. All of these four genes showed temperature-dependent difference in wild type but not in spaQ background (Fig. S3). These differences might be due to a difference in the amount of time at 28C (4h for RT-qPCR vs 24h for RNAseq) and/or due to the intrinsic differences in response time for different genes (e.g., some genes respond early vs some respond later).

(3) Figures 1B, 3B and 4C: One-way ANOVA is usually followed by a post-hoc test for multiple comparisons across the data set. The type of post-hoc test should be stated.

Author response: This has been corrected. We performed post-hoc analyses and stated ‘Tukey’s HSD test’ in figure legends.

(4) Fig. 2A: There appears to be a mismatch in numbers in the Venn diagram. While total numbers of up- and down-regulated genes for WT and spaQ add up to total numbers of all (co-)regulated genes in the respective genotype, this is not the case when gene numbers are considered that are specific to only WT, only spaQ or both; e.g. 425 genes up-regulated in both WT and spaQ + 231 genes down-regulated in WT and spaQ does not equal 735 genes co-regulated in WT and spaQ.

Author response: The difference in differentially expressed gene numbers comes from opposite pattern of expression. There are 2 types of genes which are up-regulated in WT but down-regulated in spaQ, and down-regulated in WT but up-regulated in spaQ. These are categorized into group II and III in Fig 2D, respectively. Therefore, group I (425), group II+III (97) and group IV (231) add up to 735.

(5) Fig. 4D/Methods: The origin of the native PIF4 antibody should be stated.

Author response: This has been corrected. We used anti-PIF4 (AS 16 3955, Agrisera).

Reviewer 2 Advance summary and potential significance to field
PhyB and PIF4 are very important factors for regulating plants thermomorphogenesis. In this manuscirpt, the authors showed that SPA proteins act as positive regulators of thermomorphogenesis by destabilizing phyB and directly phosphorylating PIF4 protein. PIF4, PHY, CRY, COP1 were reported to be involved in thermomorphogenesis before, SPA was reported to directly interact with COP1 and enhance COP1 activity, while photoreceptors CRY and PHY repress COP1 activity via SPA1, so it is not surprising that SPAs are involved in temperature response. The authors group reported that SPA1 acted as a serine/threonine kinase and directly phosphorylated PIF1 in vitro and in vivo. They showed that SPAs were necessary for the light-induced phosphorylation, ubiquitination and subsequent degradation of PIF1 and phyB interacted with SPA1 to enhances the recruitment of PIF1 for phosphorylation (Nature Communications). It is not surprising either that SPA could phosphorylate PIF4.
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the expression level of SPA1 gene was repressed at high temperature. I am confused about this result. Given SPA1 is essential for thermomorphogenesis, why the expression level was down-regulated in response to elevated temperatures? Whether SPA gene drives by its native promoter can complement the thermomorphogenic response in spaQ mutant?

Author response: We do not have a transgenic line expressing SPA1 from its native promoter. However, this is not uncommon scenario as many times a gene is necessary to respond to a signal, but the expression of the same gene is down-regulated in response to that signal as part of a negative feedback regulation. For example, phyA is necessary for controlling a large number of gene expression in response to red light, but the expression of phyA is drastically down-regulated under red light. In our case, we do not have antibody against native SPA1. The gene expression data show a modest down-regulation of SPA1, but the TAP-SPA1 driven by 35S promoter did not show a reduction in protein level most likely due to over expression and/or lack of post-translational regulation of SPA1 by high ambient temperature.

2. The fig4D and 4E showed that PIF4 protein level was rescued in the 35S:LUC-SPA1/spaQ at high temperature. However, the fig3C and 3D showed that SPAs were essential for stabilization of PIF4 at both normal and elevated temperature. So why the SPA1 protein did not rescue the PIF4 protein level at the normal temperature in fig4D?

Author response: There might be two reasons for this difference. First, the expression level of the native PIF4 is different than that in overexpressed PIF4-myc lines. Second, perhaps SPA1 is not sufficient to increase PIF4 level at normal temperature due to genetic redundancy, but it can do so at elevated temperature. Please note that the native PIF4 level is barely detectable even in Col-0 at 22C (Fig. 4D).

3. Does SPA1 phosphorylate PIF4 in vivo? Fig4D is not of good enough quality, which does not distinguish the phosphorylated and unphosphorylated forms of native PIF4. The authors may check the phosphorylation and stabilization of tagged PIF4 in spaQ expressing either the wild type or the mutant form of LUC-SPA1.

Author response: We have now added proteasome inhibitor treatment data (Fig. S5), which shows clear separation of PIF4 double band in 35S:LUC-SPA1/spaQ. In 35S:LUC-mSPA1/spaQ, we could only detect the unphosphorylated band using bortezomib treatment, indicating that the phosphorylation of PIF4 by SPA1 is critical for PIF4 stabilization.

4. The figS3A and S3B showed SPA1 interacted with PIF4 by yeast two hybrid assay and pull-down assay. Does SPA1 directly interact with PIF4 in vivo? I think more solid evidences and assays in vivo are needed.

Author response: We have now added results from in vivo co-IP assay (Fig. S4), showing that PIF4 interacts with SPA1. Also, we added mSPA1 and PIF4 double transgenic line in this assay that shows mSPA1 still interacts with PIF4 similar to wild type SPA1.

5. The previous works showed that phyB interacted with SPA1 to promote the formation of PHYB-SPA1-PIF1 trimolecular complex and enhance the phosphorylation of PIF1(Paik et al., 2019). Whether phyB, SPA1 and PIF4 form a complex in regulating thermomorphogenesis?

Author response: We have now added this assays in Fig. 4D. The result shows that SPA1 can form SPA1-PIF4-phyB ternary complex in vitro in response to light.

6. The authors showed that SPAs regulated global gene expression at high ambient temperature. Does these genes are also regulated by PIF4?

Author response: We have now added the comparison between SPA-and PIF4-regulated differentially expressed genes (Fig. S4). The results show that they do overlap significantly, but both display distinct gene expression pattern suggesting other factors involved in the process.

Second decision letter

MS ID#: DEVELOP/2020/189233

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AUTHORS: Sanghwa Lee, Inyup Paik, and Enamul Huq
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

See original review for information on significance.

*Comments for the author*

The authors have added key experiments to the manuscript and satisfactorily addressed all my concerns. I recommend the manuscript for publication.

Reviewer 2

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