Phytochrome-interacting factors orchestrate hypocotyl adventitious root initiation in *Arabidopsis*

Qianqian Li, Zhan Zhang, Chaoxing Zhang, Yaling Wang, Chubin Liu, Jiachen Wu, Meiling Han, Qiuxia Wang and Dai-Yin Chao
DOI: 10.1242/dev.200362

Editor: Yka Helariutta

Review timeline
Original submission: 13 January 2022
Editorial decision: 21 February 2022
First revision received: 25 March 2022
Accepted: 19 April 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200362

MS TITLE: Phytochrome-Interacting Factors orchestrate hypocotyl adventitious root initiation in *Arabidopsis*

AUTHORS: Dai-Yin Chao, Qianqian Li, Zhan Zhang, Chaoxing Zhang, Yaling Wang, Chubin Liu, Jiachen Wu, and Meiling Han

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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

The manuscript entitled "Phytochrome-Interacting Factors orchestrate hypocotyl adventitious root initiation in Arabidopsis" by Li et al., demonstrated that that the regulation of HAR formation by PIFs is dependent on the auxin biosynthesis-related genes YUCCA2 (YUC2) and YUC6, the auxin influx carrier-related genes AUX1 and LAX3, and the transcription factors (TFs) WOX5/7 and LBD16/29 for the hypocotyl adventitious root formation. PIFs directly bind to the promoters of these genes and activate their expression to initiate HARs.
These findings reveal a previously uncharacterized transcriptional regulatory network underlying HAR formation.

Comments for the author

There are some issues need to be addressed or improved:.
1) Does the dark treatment influence the transcription of PIF1/3/4/5 or the protein levels of PIFs?
2) Authors showed that PIFs regulated the transcripton of YUCCA2(YUC2) and YUC6, the auxin influx carrier-related genes AUX1and LAX3, and WOX5/7and LBD16/29 to generate root primordium HARs. Does the the dark treatment, which induces induced hypocotyl adventitious root formation, influence the expression of YUC2/6, AUX1, LAX3, WOX5/7 or LBD16/29?
3) Line 116: "PIF4-Flag and PIF5-Flag overexpression lines ..... did not produce HARs at all." This conclusion seems to be inconsistent. PIF4-Flag and PIF5-Flag overexpression lines produced adventitious roots in both dark and red light in Fig. 2B, please further determine whether the conclusion in Line 116 is correct.
4) In Figure 5, endogenous auxin but not exogenous auxin is able to rescue the defective HAR formation of pifq, how to explain this result? Why the exogenous auxin couldn't rescue the defective HAR formation in pifq?
5) PIFs-AUX1/LAX3 regulatory module regulates auxin distribution in hypocotyl or transport auxin from shoots to hypocotyl during dark-induced HARs?
6) PIF1-Flag or PIF4-Flag for plants should be italicized, e.g. "PIF1-Flag or PIF4-Flag seedlings" or "PIF1-Flag and PIF4-Flag transgenic plants".
7) Line 159:). Auhtors performed ChIP-qPCR with the PIF4-Flag overexpression line and found that the predicted PIF-binding fragments of the YUC2 and YUC6 promoters precipitated to a much greater degree in this line than in the PIF1-Flag transgenic line (Fig.4C,F). Authors suggest that it was most likely the result of the high level of PIF4, they should provide experimental evidence, not just prediction.
8) Line 255-256: "the abundance of IAA14 protein in this mutant was similar to that in phyB"might be not accurate (Fig. 9D). he abundance of Actin protein in phyB pifq quintuple mutant is much less than that in phyB in Fig. 9D which means that the total protein in phyB pifq is less. If the total protein up-sampled in phyB pifq is consistent with that in phyB, the abundance of IAA14 protein in phyB pifq would be larger than that in phyB. Thereby, these data suggest that PIFs might also regulate the stability of IAA14.
9) Fig. 10 and 11 should be integrated into the same Fig., and Fig. 12 and 13 should be integrated into the same Fig.
10) Please use "minus" (-) instead of dashes for EMSA data in the article.

Reviewer 2

Advance summary and potential significance to field

In the paper ´Phytochrome-Interacting Factors orchestrate hypocotyl adventitious rootinitiation in Arabidopsis¨ by Li, etal, Authors demonstrate with clean and varied experiments the binding PHYTOCHROME-INTERACTING FACTORS to the promoters of YUC2, YUC6, AUX1, LAX3, WOX5, WOX7, LBD16 and LBD29. Authors also show that mutant in diverse PIF are affected the development of hypocotyl adventitious roots. Therefore, they demonstrate that PIFs act upstream of its direct transcriptional targets for the proper initiation and development of HARs. However a more detailed analysis of the phenotype is desirable.

Comments for the author

Major comments Although the major findings of this work are well sustained in the experiments and analyses shown, it is a pity that authors did not explode the phenotype in a more deep and complete manner. For example with larger photographs indicating the defects in HAR initiation, include the GUS analyses of DR5, and other markers in main figures. Confocal images to see if there are defects in cell divisions and orientation, the analyses of diverse reporters downstream auxin, crosses or transformation of pif mutants with DR5, WOX5, and other fluorescent markers are almost mandatory to analyze in more detail the defects in the mutant, especially if authors claim that a phenotype is the result of disrupting auxin biosynthesis, transport and downstream effectors. I am almost sure that there are several fluorescent markers for more than 50% of the genes are published and available.
I am positive that this extra information will be of great value to improve the manuscript and fulfill the type of data required for a paper dealing with such an interesting developmental trait and to justify its publication in Development.My opinion is that the message of the manuscript is suitable for publication after improving the phenotypic description.
Minor Comments Several figures could be mixed in bigger figures, or go to supplementary and have space for figures of more detailed analyses of HAR initiation.
Figure 5 too small, can be part of a bigger composed figure or supplementary.

Reviewer 3

Advance summary and potential significance to field

This is a generally clear and very well documented paper that shows a) that PIF expression profoundly affects adventitious root formation (HAR) and (b) that PIFs bind to the promoter of many genes including WOX5/7, some LBDs, and YUC 2 and 6 all of which have been associated with root formation. As such, it advances our understanding of this important area in useful and informative ways.

Comments for the author

I see two major issues that the authors should address.
First, the manner in which the results of PIF overexpression are described appears to be overly simplistic (Fig. 2). Overexpression of all PIFs examined lead to increased HAR in red light. In darkness, however, the issue is more complicated.
Overexpression of PIF1 and 3 increase HAR, while overexpression of PIF 4 and 5 decrease HAR formation. Currently the paper describes this, and then dismisses the effect of PIF4 and PIF5 in the dark, saying that there is more trypan blue staining, hence more cell death, with the results then being dismissed as a side effect of some toxicity.
It is possible, however, that the deep blue staining may arise from an increase in the number of dead xylem cells, especially as xylem typically forms in the region where the stain is located. Other data in this paper show that PIFs induce YUC synthesis, which is expected to increase levels of auxin, and prior work has shown that auxin promotes xylem formation. Thus the simplest explanation is that overexpression of PIF4 and 5 in the dark leads to high levels of auxin which promote xylem formation—but that those high levels of auxin do not suffice to cause these dark-grown hypocotyls to form HAR. This conclusion fits well with data shown later indicating auxin alone is not sufficient to induce HAR. Options other than toxicity in the PIF4 and 5 overexpressing lines should be discussed.
Second, the proposed model (Fig 14) is not entirely supported by the data. The model shows all PIFs promoting HAR in the dark, and not in the light. This agrees with prior work showing that HARs form more readily in the dark. But, as shown in Fig. 2, overexpression of all PIFs promoted HAR formation in the light. Thus, the actual effect of light appears reversed from the way it is displayed in the model.
This needs to be discussed and/or the final model should be revised.

Minor points:
Lines 29-31 in the abstract should be rephrased, as the dependence of HAR formation on PIF-mediated AUX1 expression is at most tiny (Fig. 8C), and I don’t see any comparable data for the

PIF-mediated effect of WOX and LBD expression on AR number. It can be stated that PIFs bind these promoters and that expression of these genes has been previously shown to affect root formation, but stating that the connection between the two has been made requires more data. The sentence in line 277-279 should end after WOX5/7 is mentioned in line 278 because the final clause “which in turn triggers the initiation of HAR” is not supported by data shown in the results section. A full discussion of this possible connection could be put in the discussion, just not stated as a result.
Similarly, line 124 would be better stated as … these results suggest…. rather than… these results indicate…
Re line 139 -140: The current sentence implies that yucasin treatment somehow helps indicate that YUC 2 and YUC 4 are specifically involved. I believe that yucasin treatment inhibits all YUCs. Thus, the statement should be reworded to say: Supporting the conclusion that YUCs are required for… (This is just a minor issue of rewording.)
Re: line 147-148 Again, the conclusion appears somewhat overstated. The sentence says that HAR formation increases because PIFs increase expression of YUC2 and YUC6. However, the data show that YUC2 and YUC6 expression increases in the darkness for all PIFs --including PIF4 and 5 (Fig.3), while HAR formation does not (Fig. 2).
Line 708 (title for Fig, 5) The statement that exogenous auxin can not rescue HAR formation seems overstated given that only 0.05 uM IAA was applied. Higher concentrations could well have a stronger effect. The affirmative statement that endogenous auxin rescued HAR formation is fine.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript entitled "Phytochrome-Interacting Factors orchestrate hypocotyl adventitious root initiation in Arabidopsis" by Li et al., demonstrated that that the regulation of HAR formation by PIFs is dependent on the auxin biosynthesis-related genes YUCCA2 (YUC2) and YUC6, the auxin influx carrier-related genes AUX1 and LAX3, and the transcription factors (TFs) WOX5/7 and LBD16/29 for the hypocotyl adventitious root formation. PIFs directly bind to the promoters of these genes and activate their expression to initiate HARs. These findings reveal a previously uncharacterized transcriptional regulatory network underlying HAR formation.
Response: We really appreciate reviewer #1 for his/her effort to review our manuscript and his/her comments and suggestions.

Reviewer 1 Comments for the Author:
There are some issues need to be addressed or improved:
1) Does the dark treatment influence the transcription of PIF1/3/4/5 or the protein levels of PIFs?
Response: Thanks for this concern. Previous studies have reported that dark treatment results high accumulation of PIFs proteins in *Arabidopsis* seedlings (Lorrain et al., 2008; Shen et al., 2008; Soy et al., 2012; Ni et al., 2013), which is consistent with our observations. We had provided such information in our previous manuscript. Please see line 74 and line 91.

2) Authors showed that PIFs regulated the transcription of YUCCA2(YUC2) and YUC6, the auxin influx carrier-related genes AUX1and LAX3, and WOX5/7and LBD16/29 to generate root primordium HARs. Does the dark treatment, which induces induced hypocotyl adventitious root formation, influence the expression of YUC2/6, AUX1, LAX3, WOX5/7 or LBD16/29?
Response: Thanks for this concern. Indeed, these genes are upregulated in the darkness. We have provided these results in our revised manuscript. Please see Figure S7 and lines 153-156, 202-203, 279-280.

3) Line 116: "PIF4-Flag and PIF5-Flag overexpression lines ..... did not produce HARs at all." This conclusion seems to be inconsistent. PIF4-Flag and PIF5-Flag overexpression lines produced adventitious roots in both dark and red light in Fig. 2B, please further determine whether the conclusion in Line 116 is correct.

Response: Thanks for this concern. We are sorry for that we did not describe the results clearly. In fact, the sentence is "~80% of the hypocotyls of *PIF4-Flag* and *PIF5-Flag* overexpression lines …did not produce HARs at all". In the sentence, the subject is "~80% of the hypocotyls of *PIF4-Flag* and *PIF5-Flag* overexpression lines". However, we indeed forgot to restrict the condition as in darkness. To better describe this, we have modified this sentence of "~80% of the hypocotyls of *PIF4-Flag* and *PIF5-Flag* overexpression lines were abnormal (Fig. 2E) and hardly produce HARs under darkness." Please see lines 116-117.

4) In Figure 5, endogenous auxin but not exogenous auxin is able to rescue the defective HAR formation of pifq, how to explain this result? Why the exogenous auxin couldn't rescue the defective HAR formation in pifq?
Response: Thanks for this concern. Auxin accumulation in a certain cell is determined not only by external auxin levels but also by transport and metabolism of auxin. The observation that exogenous auxin is not able to rescue the HAR phenotype of *pifq* indicated that the auxin influx is impaired in *pifq* mutant. Consistent with this, we found that expression of *AUX1* and *LAX3*, encoding auxin influx carriers, requires PIFs. Given that exogenous auxin is not able to enter the cell through AUX1/LAX3 in *pifq* mutant, it is reasonable that exogenous auxin is not able to rescue the HAR phenotype of *pifq*. In contrast, endogenous auxin does not require influx of auxin and high level of auxin in the cells is able to trigger HAR.

5) PIFs-AUX1/LAX3 regulatory module regulates auxin distribution in hypocotyl or transport auxin from shoots to hypocotyl during dark-induced HARs?
Response: Thanks for this concern. Previous study has reported that AUX1 promotes auxin transport from shoots to roots via phloem (Marchant et al., 2002). In addition, AUX1 and LAX3 are also involved in regulating auxin influx from the meristem and cotyledons into the hypocotyls (Vandenbussche et al., 2010). Our results showed that PIFs significantly regulate the expression of *AUX1* and *LAX3* in the shoot and hypocotyl (Please see Figure 5G and H and Figure S9 of our revised manuscript) and auxin distribution is indeed altered in *pifq* (Please see Figure S5 in our revised manuscript). Based on these evidences, PIFs-AUX1/LAX3 module regulates auxin transport from shoots to hypocotyl and auxin accumulation in HAR primordium.

6) PIF1-Flag or PIF4-Flag for plants should be italicized, e.g. "PIF1-Flag or PIF4-Flag seedlings" or "PIF1-Flag and PIF4-Flag transgenic plants".
Response: We really appreciate these suggestions. We totally agree with the reviewer #1 and we have corrected these as suggested. Please see lines 164, 167, 169, 231, 511, 736, 761, 763, 798, 799, 822 in our revised manuscript.

7) Line 159:). Auhtors performed ChIP-qPCR with the PIF4-Flag overexpression line and found that the predicted PIF-binding fragments of the YUC2 and YUC6 promoters precipitated to a much greater degree in this line than in the PIF1-Flag transgenic line (Fig.4C,F). Authors suggest that it was most likely the result of the high level of PIF4, they should provide experimental evidence, not just prediction.
Response: Thanks for this remind. We are sorry that we did not make the results more obvious, but Fig 2C and D had shown that the protein level of PIF4-Flag is significantly higher than PIF1-Flag. Here we have cited the data of Fig 2C,D in the end of this sentence, as "We also performed… most likely the result of the high level of PIF4 (Fig 2C,D)." Please see line 169-170.

8) Line 255-256: "the abundance of IAA14 protein in this mutant was similar to that in phyB"might be not accurate (Fig. 9D). The abundance of Actin protein in phyB pifq quintuple mutant is much less than that in phyB in Fig. 9D, which means that the total protein in phyB pifq is less. If the total protein up-sampled in phyB pifq is consistent with that in phyB, the abundance of IAA14 protein in phyB pifq would be larger than that in phyB. Thereby, these data suggest that PIFs might also regulate the stability of IAA14.
Response: We appreciate this concern. We totally agree with this opinion and we have modified this sentence as suggested. Please see line 266-267.

9) Fig. 10 and 11 should be integrated into the same Fig., and Fig. 12 and 13 should be integrated into the same Fig.
Response: We really appreciate this suggestion. In the new version, Fig. 10 and 11 were integrated into Fig. 7, and Fig. 12 and 13 have been integrated into Fig. 8.

10) Please use "minus" (-) instead of dashes for EMSA data in the article.
Response: Thanks for this remind. We have changed this as suggested. Please see Fig. 4, 6 and 8 in the revised version.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C. and Fankhauser, C. (2008). Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. *Plant J.* **53**, 312-323. doi:10.1111/j.1365-313X.2007.03341.x
Marchant, A., Bhalerao, R., Casimiro, I., Eklof, J., Casero, P.J., Bennett, M. and Sandberg, G. (2002). AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. *Plant Cell* **14**, 589-597. doi:10.1105/tpc.010354
Ni, W., Xu, S.L., Chalkley, R.J., Pham, T.N., Guan, S., Maltby, D.A., Burlingame, A.L., Wang, Z.Y. and Quail, P.H. (2013). Multisite light-induced phosphorylation of the transcription factor PIF3 is necessary for both its rapid degradation and concomitant negative feedback modulation of photoreceptor phyB levels in Arabidopsis. *Plant Cell* **25**, 2679-2698. doi:10.1105/tpc.113.112342
Shen, H., Zhu, L., Castillon, A., Majee, M., Downie, B. and Huq, E. (2008). Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME-INTERACTING FACTOR1 from Arabidopsis depend upon its direct physical interactions with photoactivated phytochromes. *Plant Cell* **20**, 1586-1602. doi:10.1105/tpc.108.060020
Soy, J., Leivar, P., Gonzalez-Schain, N., Sentandreu, M., Prat, S., Quail, P.H. and Monte, E. (2012). Phytochrome-imposed oscillations in PIF3 protein abundance regulate hypocotyl growth under diurnal light/dark conditions in Arabidopsis. *Plant J.* **71**, 390-401. doi:10.1111/j.1365-313X.2012.04992.x
Vandenbussche, F., Petrasek, J., Zadnikova, P., Hoyerova, K., Pesek, B., Raz, V., Swarup, R., Bennett, M., Zazimalova, E., Benkova, E., et al. (2010). The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in Arabidopsis thaliana seedlings. *Development* **137**, 597-606. doi:10.1242/dev.040790

Reviewer 2 Advance Summary and Potential Significance to Field:
In the paper "Phytochrome-Interacting Factors orchestrate hypocotyl adventitious root initiation in Arabidopsis" by Li, et al, Authors demonstrate with clean and varied experiments the binding PHYTOCHROME-INTERACTING FACTORS to the promoters of YUC2, YUC6, AUX1, LAX3, WOX5, WOX7, LBD16 and LBD29. Authors also show that mutant in diverse PIF are affected the development of hypocotyl adventitious roots. Therefore, they demonstrate that PIFs act upstream of its direct transcriptional targets for the proper initiation and development of HARs. However a more detailed analysis of the phenotype is desirable.
Response: We really appreciate reviewer #2 for his/her effort to review our manuscript and his/her comments and suggestions.

Reviewer 2 Comments for the Author:
Major comments
Although the major findings of this work are well sustained in the experiments and analyses shown, it is a pity that authors did not explode the phenotype in a more deep and complete manner. For example with larger photographs indicating the defects in HAR initiation, include the GUS analyses of DR5, and other markers in main figures. Confocal images to see if there are defects in cell divisions and orientation, the analyses of diverse reporters downstream auxin, crosses or transformation of pif mutants with DR5, WOX5, and other fluorescent markers are almost mandatory to analyze in more detail the defects in the mutant, especially if authors claim that a phenotype is the result of disrupting auxin biosynthesis, transport and downstream effectors. I am almost sure that there are several fluorescent markers for more than 50% of the genes are published and available.
Response: We really appreciate these constructive suggestions. We totally agree with the reviewer #2. Actually, we had generated various transgenic plants in wild type and *pifq* backgrounds, including *YUC2pro:GUS*, *YUC6 pro:GUS*, *WOX5 pro:GUS*, *LBD16 pro:LBD16-GUS*, *LBD16 pro:LBD16-GFP*, *AUX1 pro:AUX1-GFP* and *LAX3 pro:LAX3-GFP*. We performed GUS staining assays and also confocal microscopy observation for more details of root primordia. GUS staining assays showed that PIFs regulate the expression of *YUC2* and *YUC6* in the hypocotyls, which is consistent with the

RT-qPCR data (Figure S6 of the revised version). In addition, *WOX5* and *LBD16* are expressed in HAR primordia in wild type but not in *pifq* mutant background (Figure 7E,F of the revised version). We did not find any HAR primordia in *pifq* mutant under confocal microscope. Similarly, the confocal images showed that LBD16-GFP was expressed in HAR primordia and localized in the nuclei, while no GFP fluorescence was observed in *pifq* (Figure 7G of the revised version). Moreover, AUX1-GFP was expressed in pericycle and HAR primordia cells and localized in the plasma membrane, while LAX3-GFP was expressed in stele but not in HAR primordia (Figure 5G,H of the revised version). We have provided these results. Please see lines 150-153, 198-201, 280-284.

I am positive that this extra information will be of great value to improve the manuscript and fulfill the type of data required for a paper dealing with such an interesting developmental trait and to justify its publication in Development. My opinion is that the message of the manuscript is suitable for publication after improving the phenotypic description.
Response: We appreciate these constructive suggestions. We have addressed these concerns by completing cell biology experiments and revising our manuscript thoroughly. Please see Figure 5G and H, Figure 7E-G and Figure S6 in the revised version. Please see lines 150-153, 198-201, 279-284.

Minor Comments
Several figures could be mixed in bigger figures, or go to supplementary and have space for figures of more detailed analyses of HAR initiation.
Response: We appreciate these comments. We have rearranged the figures as suggested. In more detail, Fig 5 and 9 were changed as Fig S8 and S11, respectively; Fig. 8 and S7 were integrated into Fig. S10; Fig. 10 and 11 were integrated into Fig. 7; Fig. 12 and 13 were integrated into Fig. 8.

Figure 5 too small, can be part of a bigger composed figure or supplementary.
Response: We appreciate these suggestions. We have rearranged the figures as suggested. The original Figure 5 was changed as Figure S8.

Reviewer 3 Advance Summary and Potential Significance to Field:
This is a generally clear and very well documented paper that shows a) that PIF expression profoundly affects adventitious root formation (HAR) and (b) that PIFs bind to the promoter of many genes including WOX5/7, some LBDs, and YUC 2 and 6, all of which have been associated with root formation. As such, it advances our understanding of this important area in useful and informative ways.
Response: We really appreciate reviewer #3 for his/her effort to review our manuscript and his/her comments and suggestions.

Reviewer 3 Comments for the Author:
I see two major issues that the authors should address.

First, the manner in which the results of PIF overexpression are described appears to be overly simplistic (Fig. 2). Overexpression of all PIFs examined lead to increased HAR in red light. In darkness, however, the issue is more complicated. Overexpression of PIF1 and 3 increase HAR, while overexpression of PIF 4 and 5 decrease HAR formation. Currently the paper describes this, and then dismisses the effect of PIF4 and PIF5 in the dark, saying that there is more trypan blue staining, hence more cell death, with the results then being dismissed as a side effect of some toxicity.

It is possible, however, that the deep blue staining may arise from an increase in the number of dead xylem cells, especially as xylem typically forms in the region where the stain is located. Other data in this paper show that PIFs induce YUC synthesis, which is expected to increase levels of auxin, and prior work has shown that auxin promotes xylem formation. Thus the simplest explanation is that overexpression of PIF4 and 5 in the dark leads to high levels of auxin which promote xylem formation—but that those high levels of auxin do not suffice to cause these dark-grown hypocotyls to form HAR. This conclusion fits well with data shown later indicating auxin alone is not sufficient to induce HAR. Options other than toxicity in the PIF4 and 5 overexpressing lines should be discussed.
Response: We really appreciate these suggestions. This is a valuable comment. We totally agree with this opinion. We have changed our description and discussed this explanation as suggested.

Please see line 119-122, 319-323.

Second, the proposed model (Fig 14) is not entirely supported by the data. The model shows all PIFs promoting HAR in the dark, and not in the light. This agrees with prior work showing that HARs form more readily in the dark. But, as shown in Fig. 2, overexpression of all PIFs promoted HAR formation in the light. Thus, the actual effect of light appears reversed from the way it is displayed in the model. This needs to be discussed and/or the final model should be revised.
Response: We appreciate these suggestions. Actually, this proposed model is for the wild type but not gene modified plants. In red light, the wild type seedlings are not able to form HAR. Indeed, PIFs overexpression lines are able to promote HAR formation under red light, but this phenomenon is because of over accumulation of PIFs. To make this more accurate, we changed description of the legend of this figure. Please see the new figure legend in the revised manuscript.

Minor points:

Lines 29-31 in the abstract should be rephrased, as the dependence of HAR formation on PIF-mediated AUX1 expression is at most tiny (Fig. 8C), and I don't see any comparable data for the PIF-mediated effect of WOX and LBD expression on AR number. It can be stated that PIFs bind these promoters and that expression of these genes has been previously shown to affect root formation, but stating that the connection between the two has been made requires more data.
Response: We appreciate this suggestion. We have rephrased this description as suggested. Please see lines 32-34.

The sentence in line 277-279 should end after WOX5/7 is mentioned in line 278, because the final clause "which in turn triggers the initiation of HAR" is not supported by data shown in the results section. A full discussion of this possible connection could be put in the discussion, just not stated as a result.
Response: We appreciate this suggestion. We have changed this description as suggested. Please see line 291.

Similarly, line 124 would be better stated as … these results suggest…. rather than… these results indicate…
Response: We really appreciate these suggestions raised by the Reviewer #3. We have replaced "indicate" with "suggest". Please see line 127.

Re line 139 -140: The current sentence implies that yucasin treatment somehow helps indicate that YUC 2 and YUC 4 are specifically involved. I believe that yucasin treatment inhibits all YUCs. Thus, the statement should be reworded to say: Supporting the conclusion that YUCs are required for… (This is just a minor issue of rewording.)
Response: We really appreciate these suggestions raised by the Reviewer #3. We have modified this sentence, as "Supporting the conclusion that *YUC* genes are required for..." Please see line 142.

Re: line 147-148 Again, the conclusion appears somewhat overstated. The sentence says that HAR formation increases because PIFs increase expression of YUC2 and YUC6. However, the data show that YUC2 and YUC6 expression increases in the darkness for all PIFs --including PIF4 and 5 (Fig.3), while HAR formation does not (Fig. 2).
Response: We really appreciate these suggestions raised by the Reviewer #3. We have deleted this sentence.
Line 708 (title for Fig, 5) The statement that exogenous auxin can not rescue HAR formation seems overstated given that only 0.05 uM IAA was applied. Higher concentrations could well have a stronger effect. The affirmative statement that endogenous auxin rescued HAR formation is fine.
Response: We really appreciate these suggestions raised by the Reviewer #3. Actually we applied IAA with 0-0.2 μM to wild type and *pifq* mutant. We found exogenous application of IAA is able to promote HAR formation. However, the HAR phenotype of *pifq* mutant couldn't be rescued even with high concentration IAA application. Please see Figure S8B,C and line 178-179.

Second decision letter

MS ID#: DEVELOP/2021/200362

MS TITLE: Phytochrome-Interacting Factors orchestrate hypocotyl adventitious root initiation in *Arabidopsis*

AUTHORS: Qianqian Li, Zhan Zhang, Chaoxing Zhang, Yaling Wang, Chubin Liu, Jiachen Wu, Meiling Han, Qiuxia Wang, and Dai-Yin Chao
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

In this study, authors found that PIFs are required for darkness-induced HAR formation through directly binding to the promoters of genes involved in root formation, including auxin biosynthesis genes YUCCA2 (YUC2) and YUC6, the auxin influx carrier genes AUX1 and LAX3, and the transcription factors WOX5/7 and LBD16/29, to activate their expression. This study reveals a previously uncharacterized transcriptional regulatory network underlying HAR formation.

Comments for the author

I am happy with the revisions and no further comments.

Reviewer 2

Advance summary and potential significance to field

Authors took all my suggestions in considerations to improve their manuscript. I am happy to see all the new panels, figure organization and extra sumplementary figures related to my previous requests.

Comments for the author

Authors took all my suggestions in considerations to improve their manuscript. I am happy to see all the new panels, figure organization and extra sumplementary figures related to my previous requests.
I do believe that the new version of this manuscript deserves to be published in Deevelopment