Auxin transport network underlies xylem bridge formation between the hemi-parasitic plant *Phtheirospermum japonicum* and host *Arabidopsis*

Takanori Wakatake, Satoshi Ogawa, Satoko Yoshida and Ken Shirasu DOI: 10.1242/dev.187781

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MS TITLE: Auxin transport network underlies xylem bridge formation between the hemi-parasitic plant Phtheirospermum japonicum and host Arabidopsis

AUTHORS: Takanori Wakatake, satoko yoshida, and Ken Shirasu

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

Wakatake et al., well described the importance of auxin flow mediated by auxin efflux and influx carries in the formation of xylem-bridge during haustorium development. Time-course observation with various reporter markers indicated that auxin response was restricted to the narrow path prior

to xylem-bridge formation. Also, the result of the inhibitor assay was confirmed by RNAi genetic analysis. Especially, the functional differences of PjPIN1 and PjPIN9 were elegantly shown from the both sides of genetic and expression analysis. I feel that the experiments are overall well-conducted and the manuscript is well-written along with the results. Moreover, this work is very interesting and scientifically important for developmental biologists, I think. Here I put few major and minor comments.

Comments for the author

Major remark
The authors showed DR5::Venus patterns when only treated with NPA and CHPAA in Fig.6. No control experiments were shown, making it difficult to understand the effect of the inhibitors. The authors should present a control image of DR5::Venus at 24, 48, 72 hpi. The authors discussed that CHPAA treatment does not disturb DR5 expression (line 217), but considering that the influx carriers potentially restrict high auxin response in the central domain, it is possible that CHPAA treatment expands DR5 signal domain. In addition, CHPAA treatment consequently triggered broad xylem bridge (Fig.2F), implying the alteration of auxin response. Therefore, I recommend the authors to compare DR5 response carefully with control samples, which may enable reinforcing the role of auxin influx carriers during xylem bridge formation.

Minor comments
1. Fig.2A–C
It is not easy to understand the stage classification shown in Fig.2. Please show a schematic for stage-I, II, III of xylem bridge formation.

2. Introduction Line94, Fig.2 and Fig.S1 legend
“～” should be “– ”, when showing the variation of the sample number.

3. Fig.6 legend
“(D) to (F) at 24, 48 or 72 hpi” should be “(D) to (F) at 48, 72 or 96 hpi”

Reviewer 2

Advance summary and potential significance to field

Parastic plants form vascular connections to host plants in which the haustorium of parasitic plants contributes to host invasion and vascular connections, by producing a xylem bridge (XB) between parasite and host xylem systems. In this manuscript, the authors examined how XB is formed between the haustorium of parasitic plants and host plant roots. The authors used an Orobanchaceae hemiparastic plant, P. japonicum and A. thaliana as a host plant. By focusing on the role of auxin and auxin transport system mediated by PIN family (auxin efflux) and AUX1/LAX family (auxin influx) during XB formation, the authors demonstrate that 1) auxin response sites with DR5 activity overlap with tracheary elements differentiation regions with PjCESA7 (cellulose synthesis reporter expression) during XB formation, 2) NPA, an auxin efflux inhibitor, blocks XB formation, indicating that auxin efflux within haustria is crucial for XB formation, 3) PjPIN1, PjPIN2, and PjPIN9 have specific expression/subcellular localisation pattern in parasite root and haustorium before/during XB formation suggesting their roles for XB formation, 4) PjLAX1, PjLAX2, and PjLAX5 also have specific expression/subcellular localisation pattern in parasite root and haustorium before/during XB formation, suggesting their presumptive role for XB connections, and 5) either PjPIN1-knockdown or PjPIN9-knockdown blocked XB formation, indicating that Both PjPIN1 and PjPIN9 positively regulate XB formation. Taken together, the authors propose a reasonable model of auxin and concentration gradient during haustorium development and conclude that cooperative action of auxin transporters is responsible for XB formation between parasite and host plants.
I think that this work contains significant findings on haustorium development in parasitic plants. Although the role of local auxin biosysnthesis in haustorium development of P. japonicum (Ishida et al., 2016, Plant Cell) is studied by the authors’ group, no works on the roles of auxin transport system have been studied in this plant. The authors succeeded in providing a reasonable model of auxin and concentration gradient during haustruim for study XB formation. I think that this manuscript has significant findings and will be very helpful for understanding the mechanisms

underlying the interaction between parasitic and host plants, particularly haustorium development and XB formation.

Comments for the author

I have several points to be considered by the authors.

1. The author succeeded in the production of PjPIN1 and PjPIN9 RNAi roots and showed their importance in XB formation and their different roles in haustrium development. I wonder whether DR5 auxin response maximum is altered in these RNAi roots. In addition, if it is possible, it will be more informative to produce the double RNAi line for PjPIN1 and PjPIN9, thereby demonstrating the importance of cooperative roles of PjPIN1 and PjPIN9 for haustorium development and XB formation.
2. The author found that several auxin transporters (PjPIN2 and PjLAX1) are specifically expressed in the tip region during haustorium development.
Particularly, PjPIN2 localization in the tip region is very unique and interesting, suggesting the critical role for accumulating auxin the tip region.
RNAi analysis of PjPIN2 (also PjLAX1) must be exciting if the knockdown of PjPIN2 causes a significant phenotype in haustorium development and XB formation. This will strengthen the authors' model in Fig. 9.
3. It is reported that inhibition of auxin transport by TIBA reduces haustorium development in Tryphysaria versicolor roots in the presence of HIF, DMBQ
(Tomilov et al., 2005, Plant Physiology, cited in References). The authors should mention the previous study using auxin transport inhibitors.

Minor points:
1. L.63; “indol-3-acetic acid” should be “indole-3-acetic acid”.
2. L.67; “PGP family” should be “PGP/ABCB family” or “ABCB family”. The other “PGP”s in the text should be edited.
3. L.68; Although “AUX/LAX family” has been used in many research papers (e.g., Swarup and Peret, 2012). However, based on recent review paper by Swarup and Bhosale (2019), “AUX/LAX family” should be “AUX1/LAX family”. The other “AUX/LAX”s in the text should be edited. Please see and cite the following paper. The group that originally found AUX1 in Arabidopsis (Malcolm Bennett’ group) uses “AUX/LAX family”.
Swarup R, Bhosale R. (2019) Developmental Roles of AUX1/LAX Auxin Influx Carriers in Plants. Front Plant Sci., 10:1306. doi: 10.3389/fpls.2019.01306.
4. L.94; “(48~72hpi)” should be “(48~72 hpi)” (Put a space).
5. L.208; “we concluded that …” is not suitable without the functional analysis of these PjLAXs. “we suppose that…” or “These experiments strongly suggest that …” is suitable here.
6. Fig. 1 legend title; “Auxin-responding cells respond to…” should be “Auxin-responding cells correspond to…”.
7. L.185; “Expression of the LAX gene family” should be “Expression of the AUX1/LAX gene family”.

First revision

Author response to reviewers' comments

First of all, we would like to thank the referees for their constructive criticism and feedback, which we believe have greatly improved our manuscript. Please kindly see our point-by-point replies to address specific concerns.

Response to reviewer comments

Reviewer 1

Major remarks
1. Comment: The authors showed DR5::Venus patterns when only treated with NPA and CHPAA in Fig.6. No control experiments were shown, making it difficult to understand the effect of the inhibitors. The authors should present a control image of DR5::Venus at 24, 48, 72 hpi.

Response: We apologize for the lack of important control data in the initial manuscript. We now include control images in Fig. 6.

2. Comment: The authors discussed that CHPAA treatment does not disturb DR5 expression (line 217), but considering that the influx carriers potentially restrict high auxin response in the central domain, it is possible that CHPAA treatment expands DR5 signal domain. In addition, CHPAA treatment consequently triggered broad xylem bridge (Fig.2F), implying the alteration of auxin response. Therefore, I recommend the authors to compare DR5 response carefully with control samples, which may enable reinforcing the role of auxin influx carriers during xylem bridge formation.

Response: We would like to thank the reviewer for this constructive suggestion. CHPAA treatment altered xylem bridge morphology when treated from 0 hpi and 24 hpi, but not from 48 hpi. We clarified this point in the manuscript line 125 because the previous manuscript did not include this information. Based on this observation, we examined the effect of CHPAA treatment from 0 hpi, instead of 48 hpi, on the DR5 expression. As a result, we observed expanded auxin responses in the plate xylem development site, which corresponds to the altered xylem bridge and the plate xylem morphology (Fig. 2G). This result supports our hypothesis that auxin influx carriers centralize high auxin responses. We added the new figure including this data (Fig. S8).

Minor comments
3. Comment: Fig.2A–C. It is not easy to understand the stage classification shown in Fig.2. Please show a schematic for stage-I, II, III of xylem bridge formation.

Response: We are sorry that the first manuscript did not include this information. We added this information to Fig. 2A.

4. Comment: Introduction Line94, Fig.2 and Fig.S1 legend. “～” should be “– ”, when showing the variation of the sample number.

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

5. Comment: Fig.6 legend. “(D) to (F) at 24, 48 or 72 hpi” should be “(D) to (F) at 48, 72 or 96 hpi”

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

Reviewer 2

Major remarks
1. Comment: The author succeeded in the production of PjPIN1 and PjPIN9 RNAi roots and showed their importance in XB formation and their different roles in haustrium development. I wonder whether DR5 auxin response maximum is altered in these RNAi roots. In addition, if it is possible, it will be more informative to produce the double RNAi line for PjPIN1 and PjPIN9, thereby demonstrating the importance of cooperative roles of PjPIN1 and PjPIN9 for haustorium development and XB formation.

Response: We agree that both suggested experiments will greatly push forward our understanding of the role of PIN genes during haustorium formation. However, these experiments are hampered

by technical problems. Currently, we lack a transgenerational transformation system in P. japonicum, therefore, we need to integrate the DR5 reporter system into the RNAi vector to see auxin response sites in gene knockdown hairy roots. During our vector construction, we noticed that the pHG8 vector is quite unstable and we found frequent mutation around the gateway recombination sites. Thus, we think the pHG8 vector is not suitable for further modification to combine the DR5 reporter system. Both PjPIN1 and PjPIN9 are expressed in the root meristematic region and therefore supposed to be important for root apical meristem maintenance. Knockdown of both genes could result in defects in hairy root formation and/or growth. We think effective knockdown of both PjPIN1 and PjPIN9 to examine their roles during haustorium development is difficult using our current system.

2. Comment: The author found that several auxin transporters (PjPIN2 and PjLAX1) are specifically expressed in the tip region during haustorium development. Particularly, PjPIN2 localization in the tip region is very unique and interesting, suggesting the critical role for accumulating auxin the tip region. RNAi analysis of PjPIN2 (also PjLAX1) must be exciting if the knockdown of PjPIN2 causes a significant phenotype in haustorium development and XB formation. This will strengthen the authors' model in Fig. 9.

Response: In our observation, PjPIN2 expression decreases after 24 hpi and no expression is observed at 48 hpi in haustoria. Therefore, we expect that PjPIN2 is rather related with haustorium initiation but not xylem bridge formation. We agree that it is indeed exciting, as suggested, to see how PjPIN2 and PjLAX1 change the auxin flow in the root meristematic region and establish the new local auxin maximum at the tip region of initiating haustoria in combination with auxin biosynthesis enzyme PjYUC3 (Ishida et al., 2016). This manuscript, however, focuses on roles of auxin transporters in xylem bridge formation and we believe that RNAi experiments of PjPIN2 (and PjLAX1) are beyond the scope of this paper.

3. Comment: It is reported that inhibition of auxin transport by TIBA reduces haustorium development in Triphysaria versicolor roots in the presence of HIF, DMBQ (Tomilov et al., 2005, Plant Physiology, cited in References). The authors should mention the previous study using auxin transport inhibitors.

Response: We would like to thank the reviewer for pointing out this. Tomilov et al. reported the effect of TIBA on haustorium initiation in response to HIF treatment in T. versicolor, whereas we report the effect of NPA on the xylem bridge formation with host plants in this study. We added the texts to clarify this difference in the discussion part (Line: 286-288).

Minor points
4. Comment: 1.L.63; “indol-3-acetic acid” should be “indole-3-acetic acid”.

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

5. Comment: 2.L.67; “PGP family” should be “PGP/ABCB family” or “ABCB family”. The other “PGP”s in the text should be edited.

Response: We would like to thank the reviewer for this correction. We carefully checked our manuscript and changed “PGP family” to “ABCB/PGP family”. (Line: 67, 76, 77, 309, 310, 313)

6. Comment: L.68; Although “AUX/LAX family” has been used in many research papers (e.g., Swarup and Peret, 2012). However, based on recent review paper by Swarup and Bhosale (2019), “AUX/LAX family” should be “AUX1/LAX family”. The other “AUX/LAX”s in the text should be edited. Please see and cite the following paper. The group that originally found AUX1 in Arabidopsis (Malcolm Bennett’ group) uses “AUX/LAX family”. Swarup R, Bhosale R. (2019) Developmental Roles of AUX1/LAX Auxin Influx Carriers in Plants. Front Plant Sci., 10:1306. doi: 10.3389/fpls.2019.01306.

Response: We would like to thank for this correction. We checked our manuscript carefully and changed the text accordingly (Line: 25, 68, 73, 188, 190, 590, 593).

7. Comment: L.94; “(48~72hpi)” should be “(48~72 hpi)” (Put a space).

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

8. Comment: L.208; “we concluded that …” is not suitable without the functional analysis of these PjLAXs. “we suppose that…” or “These experiments strongly suggest that …” is suitable here.

Response: We agree with the reviewer and have corrected the sentence as suggested.

9. Comment: Fig. 1 legend title; “Auxin-responding cells respond to…” should be “Auxin-responding cells correspond to…”.

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

10. Comment: L.185; “Expression of the LAX gene family” should be “Expression of the AUX1/LAX gene family”.

Response: We would like to thank the reviewer for this correction. We changed the text accordingly.

Second decision letter

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AUTHORS: Takanori Wakatake, Satoshi Ogawa, Satoko Yoshida, and Ken Shirasu
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

Wakatake et al., well described the importance of auxin flow mediated by auxin efflux and influx carries in the formation of xylem-bridge during haustorium development. Time-course observation with various reporter markers indicated that auxin response was restricted to the narrow path prior to xylem-bridge formation. Also, the result of the inhibitor assay was confirmed by RNAi genetic analysis. Especially, the functional differences of PjPIN1 and PjPIN9 were elegantly shown from the both sides of genetic and expression analysis. I feel that the experiments are overall well-conducted and the manuscript is well-written along with the results. Moreover, this work is very interesting and scientifically important for developmental biologists. Here I support the publication in Development.

Comments for the author

The authors have addressed all of my concerns. Great job.

Reviewer 2

Advance summary and potential significance to field

Parastic plants form vascular connections to host plants in which the haustorium of parasitic plants contributes to host invasion and vascular connections, by producing a xylem bridge (XB) between parasite and host xylem systems. In this manuscript, the authors examined how XB is formed between the haustorium of parasitic plants and host plant roots. The authors used an Orobanchaceae hemiparastic plant, P. japonicum and A. thaliana as a host plant. By focusing on the role of auxin and auxin transport system mediated by PIN family (auxin efflux) and AUX1/LAX family (auxin influx) during XB formation, the authors demonstrate that 1) auxin response sites with DR5 activity overlap with tracheary elements differentiation regions with PjCESA7 (cellulose synthesis reporter expression) during XB formation, 2) NPA, an auxin efflux inhibitor, blocks XB formation, indicating that auxin efflux within haustria is crucial for XB formation, 3) PjPIN1, PjPIN2, and PjPIN9 have specific expression/subcellular localisation pattern in parasite root and haustorium before/during XB formation suggesting their roles for XB formation, 4) PjLAX1, PjLAX2, and PjLAX5 also have specific expression/subcellular localisation pattern in parasite root and haustorium before/during XB formation suggesting their presumptive role for XB connections, and 5) either PjPIN1-knockdown or PjPIN9-knockdown blocked XB formation, indicating that Both PjPIN1 and PjPIN9 positively regulate XB formation. Taken together, the authors propose a reasonable model of auxin and concentration gradient during haustorium development and conclude that cooperative action of auxin transporters is responsible for XB formation between parasite and host plants.
I think that this work contains significant findings on haustorium development in parasitic plants. Although the role of local auxin biosysnthesis in haustorium development of P. japonicum (Ishida et al., 2016, Plant Cell) is studied by the authors' group, no works on the roles of auxin transport system have been studied in this plant. The authors succeeded in providing a reasonable model of auxin and concentration gradient during haustruim for study XB formation. I think that this manuscript has significant findings and will be very helpful for understanding the mechanisms underlying the interaction between parasitic and host plants, particularly haustorium development and XB formation.

Comments for the author

In this revised manuscript by Wakatake et al., the authors appropriately revised the first manuscript with additional data, according to the comments by previous two reviewers, thereby improving the quality of the manuscript.
I understand the difficulty in the establishment of a transgenerational transformation system in P. japonicum. I hope that it will work in P. japonicum in the future.