HOMEOBOX PROTEIN52 Mediates the Crosstalk between Ethylene and Auxin Signaling During Primary Root Elongation by Modulating Auxin Transport-related Gene Expression

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Plant Cell. Advance Publication Oct. 17, 2018; doi:10.1105/tpc.18.00584

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Review timeline:

TPC2018-00090-RA	Submission received:	Feb. 2, 2018
	1st Decision:	April 18, 2018 *manuscript declined*
TPC2018-00584-RA	Submission received:	August 6, 2018
	1st Decision:	Sept. 9, 2018 *revision requested*
TPC2018-00584-RAR1	1st Revision received:	Sept. 26, 2018
	2nd Decision:	Oct. 3, 2018 *acceptance pending, sent to science editor*
	Final acceptance:	Oct. 14, 2018
	Advance publication:	Oct. 17, 2018

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.)

TPC2018-00090-RA 1st Editorial decision – *declined* April 18, 2018

We agreed this is an interesting paper that provides a possible link for the gap between ethylene signaling and auxin transport, which had been previously reported by multiple groups. Two reviewers raised criticisms regarding the role of HB52 in mediating ethylene regulation of several auxin transport genes (*PIN2*, *WAG1/2*); additional experimental evidence is required to support this conclusion. Genetic analysis evidence is also required to demonstrate the binding of EIN3 to the *HB52* promoter at specific sites, as well as HB52 binding to promoters of *PIN2* and *WAG1/2*. The quality and presentation of the data need to be improved (please see our instructions for authors) and we recommend also that the language style and manuscript structure be improved to maximize the impact of your work. You might consider using planteditors.com for example. We also agree with reviewer 3 that a model would be helpful in summarizing the major finding of the manuscript. Please address these and the other points raised by the reviewers in a revised manuscript.

Unfortunately, one of the invited reviewers failed to provide a timely review and we had to invite another reviewer. We sincerely apologize for the delay caused by this extra process. As far as we can tell, the delay was not deliberate and we hope you can understand this unfortunate situation. (In fact, one of our own papers was in the review process for more than 3 months before a first decision, so unfortunately these things happen.)

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

[Reviewer comments shown below along with author responses]

TPC2018-00584-RA Submission received August 6, 2018

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

The authors identified a transcription factor that regulates primary root elongation by mediating the crosstalk between ethylene and auxin. It provides some insight of the crosstalk between ethylene and auxin. However, the manuscript is more descriptive and the mechanism to illustrate how HB5 mediates crosstalk between ethylene and auxin is weak.

Point 1. Both Figure 1 and Figure 2 discuss the expression of *HB52*, which could be just one figure.

RESPONSE: Good suggestion. We combined these two figures.

Point 2.The binding of EIN3 to the promoter of *HB52* indicates that *HB5* is one the targets of EIN3, and the binding of HB5 to auxin transport genes was examined independently. However, how the auxin transport-related genes are influenced by HB52 in response to ethylene is unknown. Another point: The author did not provide evidence to show how ethylene regulates auxin transport mediated by HB5, such as the gene expression of PIN2, WAG1, WAG2 in the HB5 mutant and HB5ox mutant with or without ethylene treatments, the binding of HB5 to PIN2, WAG1, WAG2 in response to ethylene, the phenotype of the *pin2 hb5* double mutant et al.

RESPONSE: Excellent point. Other reviewers also pointed this out. We did the suggested experiment with vs. without ACC treatment and the results are shown in Figure 5C.

It's a good suggestion to demonstrate the binding of HB52 to the *PIN2*, *WAG1* and *WAG2* promoters in response to ethylene. This could be easily done if an HB52 antibody is available for ChIP-PCR assays. The lack of an HB52 antibody prevented us from doing the suggested experiment. However, the concern regarding the transcriptional regulation of PIN2, WAG1 and WAG2 by HB52 has been adequately addressed by in vitro biochemical (Fig. 6, 7, 8) and genetic analyses (Fig. 9).

We appreciated the suggestion of examining the *pin2 hb52* double mutant phenotype. However, we thought the *pin2 hb52* combination might not be the best to show their genetic relationship through phenotypic analysis because the primary root length of *pin1, wag1,* and *wag2* is similar to that of wild type regardless of the presence of ACC (Fig. 9). Under ACC treatment, *hb52* shows slightly longer primary roots than wild type (Fig. 2). Considering that the root phenotype of the *pin2 hb52* double mutants could be similar to wild type, we therefore constructed *OX35-2 wag1*, *OX35-2 wag2* and *OX35-2 pin2* double mutants whose root phenotypes could be distinguished from wild type under ACC treatment (Fig. 9).

Point 3. They claimed that HB52 is downstream of EIN3; the direct genetic evidence is to cross HB5ox into the *ein3* mutant, which is missing in the manuscript.

RESPONSE: We also appreciated this suggestion of creating the *ein3 HB52ox* double mutant, but we disagree with this for the following reason. *HB52* is driven by the 35S promoter and is not under the regulation of EIN3 in the *ein3 HB52ox* double mutant, making it hard if not impossible to determine their genetic relationship. Regardless of ethylene treatment, the double mutant *ein3 HB52ox* would be phenotypically similar to HB52ox. Therefore, we constructed the *35S:EIN3-GFP hb52* double mutant instead. We reasoned that if HB52 is a target of EIN3, in this double mutant the enhanced EIN3 should not relay the signaling onto HB52 due to the loss of HB52. Moreover, the root phenotype of *hb52* can be easily distinguished from that of wild type and *ein3* under ethylene treatment (Fig. 4).

Point 4. It is confusing that *WAG1,2* were upregulated in OX35-2, however, the GUS staining was largely reduced. WAG1,2 are kinases that phosphorylate PINs, and the phosphorylation is important for auxin transport. The question is whether the reduction in GUS staining in OX35-2 is due to the upregulation of WAG1/2 or is due to the regulation of other factors such as auxin biosynthesis, which were not examined in the manuscript.

RESPONSE: We appreciated the comment. In our opinion, the reduced GUS signal in the root tip of HB52ox is likely due to enhanced PIN2 activity that pumps auxin out of the root tip and elongation zone. As to auxin biosynthesis, we did observe a few genes involved in auxin biosynthesis being upregulated in the HB52ox line and downregulated in *hb52*, which suggests that the possibility that the downregulation of auxin biosynthesis is less likely in HB52ox lines. We would like to address HB52 and auxin biosynthesis in a follow-up study.

Point 5. Figure 7 and Figure 8 could be one figure.

RESPONSE: Good suggestion. We agree that it's better to combine Fig. 6, 7, 8 as one figure. Considering that each figure already contains five panels, it would make it too large a figure if we combined all three figures together, so

we left them as individual figures.

Point 6. Figure 10 doesn't seem to support the conclusion because *ox35-1pin2* displayed the *ox35-2* phenotype, not the *pin2* phenotype. Most likely, PIN2 is downstream of HB5?

RESPONSE: We appreciated the comment regarding the phenotype of the *OX35-2 pin2* double mutant. We agree that PIN2 is downstream of HB52. Genetic analysis of the primary root length of *OX35-2 pin2* showed that the inhibition of root elongation by HB52 overexpression is alleviated in the *pin2* genetic background. The root length of *OX35-2 pin2* is longer than that of OX35-2, although it's shorter than that of *pin2*. The difference is statistically significant (Fig. 9C). We repeated the experiment and obtained similar results (Fig. 9).

Point 7. Surprisingly, the EIN3ox phenotype shown in Figure 5 is not as strong as they presented before. This may be due to gene silencing? If it is the case, they should examine the protein expression of EIN3 in EIN3ox and any plants crossed with EIN3ox.

RESPONSE: We appreciated the comment. There is a possibility of gene silencing, but this is less likely. We think it's more likely due to the difference in growth and treatment conditions. Nevertheless, the root phenotype of EIN3ox was not significantly different from that of *35S:EIN3-GFPhb52* (Fig. 4B) and adequately addressed the genetic relationship between HB52 and EIN3.

Reviewer #3:

In this manuscript, the authors show a direct transcriptional link between the master transcriptional regulator of ethylene responses, EIN3, and several auxin transport-related genes (*PIN2*, *WAG1*, and *WAG2*).

Point 1. The authors present convincing genetic and gene expression evidence showing that *HB52* is a direct target of EIN3 and that HB52 regulates the expression of *PIN2*, *WAG1*, and *WAG2*. What the presented experiments do not prove is that the ethylene-mediated regulation of *PIN2*, *WAG1*, and *WAG2* is mediated by HB52. Equally consistent with the authors' results would be a model in which HB52 regulates auxin transport and therefore distribution in an ethylene-independent manner. Alterations of HB52 would result in abnormal auxin distribution in these mutants even in the absence of ethylene. In this scenario, the altered ethylene response in the *hb52* mutants would be a consequence of abnormal 'basal' auxin gradients rather than the inability of this hormone to modify these gradients. To differentiate between these two possibilities, the authors should examine the expression levels of *PIN2*, *WAG1*, and *WAG2* in the wild type and *hb52* mutants (hypo and hypermorph mutants) in the presence and absence of ethylene. The experiment that the authors did do was to examine the levels of these auxin transport genes (*PIN2*, *WAG1*, and *WAG2*) in the wild type and different *hb52* mutants but did not compare the effects of ethylene (presence versus absence of ethylene). These experiments show that if the levels of HB52 are low, the expression of these auxin transport genes is also low, and if high, they are also high. This indicates that HB52 regulate the levels of these genes *(PIN2*, *WAG1*, and *WAG2*), but not that their regulation by ethylene is mediated by HB52. I feel this is a critical (and simple) experiment that is needed to show that the ethylene-triggered induction of *PIN2*, *WAG1*, and *WAG2* expression is HB52 dependent.

Another important point that needs to be addressed is the use of non-standard assays to monitor ethylene sensitivity. Most ethylene mutants have been characterized using the triple response assay where plants are germinated in the dark and grown for three days in the presence or absence of 10 ppm of ethylene. Unless there is a good reason for doing the ethylene sensitivity assays at later developmental stages and in light-grown plants, the standard triple response assays should be used to monitor ethylene sensitivity.

RESPONSE: Excellent point. We appreciate the comments and suggestion. We did the suggested experiment with vs. without ACC treatment and the results are shown in Figure 5C, which supports the notion that HB52 is indeed involved in ethylene-triggered induction of *PIN2*, *WAG1* and *WAG2*.

Point 2. It is very odd that there is not a reason given to why this particular transcription factor was chosen to be investigated.

RESPONSE: In the process analyzing the role of Arabidopsis ERF1 in ethylene-auxin crosstalk, we found the ethylene-responsive homeodomain factor HB52. Meanwhile, our lab was also working on other homeodomain factors including EDT1/HDG11 and HB17. Therefore we decided to investigate the function of HB52.

Point 3. "In addition, we initially generated HB52 overexpression lines under control of CaMV 35S promoter, which displayed aberrant flower development and thereby an infertile phenotype." These results should be shown.

RESPONSE: We did not include these results because these lines were not used for our study. We mentioned this observation in the text as a reminder for colleagues, which is good enough in our opinion. We can include the results as a supplemental figure if you insist.

Point 4. "In addition to phenotypes in root elongation and response to ACC treatment, we also observed collapsed root meristem in the overexpression lines (Figure S2A)". The authors should better define what collapsed root meristem means. Based on the images in Figure S2A, I can see a reduction in the number of Lugol staining columella cell layers but not necessarily a disruption of the root proximal meristem. Better images will be needed to show a collapse of the proximal meristem.

RESPONSE: Good point. We agree that "collapsed meristem" was not an accurate description of the phenotype. To avoid any misleading, we change the phrase to abnormal meristem.

Point 5. "These partial loss function and gain-of-function mutants displayed an ACC-resistant phenotype in root elongation relative to the wild type (Figure 3).". What is the phenotype of these mutants in the standard triple response assay (3-day-old seedlings grown in the presence of 10 ppm of ethylene)? As mentioned above, I would like to see the phenotype of these mutants in the standard triple response assay.

RESPONSE: We appreciate the comment and suggestion. We did the triple response assay and presented the results in Figure S5. The triple response is altered in the overexpression lines.

Reviewer #4:

In this manuscript, the authors examined the role of homeodomain gene *HB52* in mediating ethylene inhibition of root growth by modulating the expression of genes involved in auxin transport. Ethylene was shown to modulate auxin transport through the auxin transporter PIN2, but the molecular link between ethylene signaling and PIN2 is missing. The study established that HB52 mediates the ethylene-induced regulation of *PIN2* expression. They first showed that HB52 is expressed in roots (Fig 1) and is induced by the plant hormone ethylene (Fig 2). They showed that the ethylene-regulated transcription factor EIN3 binds to the *HB52* gene promoter by ChIP and yeast one-hybrid assays (Fig 4). Genetic studies indicated that while the loss-of-function HB52 mutants displayed reduced ethylene-mediated root inhibition, the HB52 overexpression lines had reduced root growth (Fig 3). Double mutant analysis indicated that the short root phenotype of gain-of-function ethylene mutants (*ctr1* and EIN3OX) are partially suppressed by *hb52* mutation, indicating that HB52 function downstream of EIN3 to mediate ethylene-mediated root growth inhibition (Fig 5). To explore the mechanism by which HB52 inhibits root growth, the author examined the auxin distribution in the HB52 mutants using auxin-reporter DR5:GUS and found that while in WT plants, ethylene treatment led to auxin transport from the root tip to elongation zone, auxin failed to transport out of the root tip in the *hb52* mutant and the auxin level appears to be reduced in HB52 OX lines (Fig 6), leading to the hypothesis that HB52 regulates genes involved in auxin transport in roots. Indeed, they found that *PIN2*, *WAG1* and *WAG2* are downregulated in *hb52* but increased in HB52OX lines (Fig 6). They further provided evidence showing that HB52 binds to the *PIN2*, *WAG1* and *WAG2* gene promoters by in vitro binding, ChIP and yeast one-hybrid assays (Fig 7-9). Finally, the authors provided genetic evidence that *pin2*, *wag1* and *wag2* mutants partially suppressed the shoot-root phenotypes of HB52 OX lines, supporting the notion that PIN2, WAG1 and WAG2 act downstream of HB52 in ethylene-mediated inhibition of root growth. The study filled an important gap by identifying HB52 as the link between ethylene signaling and genes involved in auxin transport. Most of the genetic and molecular studies are well carried out and well presented. I have some comments for the authors to consider to further strengthen the manuscript:

Point 1. The HB52 mutant phenotypes should be further characterized. The authors did provide root elongation phenotypes in the absence or presence of ethylene in Fig. 3. The cellular bases of these phenotypes should be characterized, i.e. which cells in which zones are affected? Are the cells shorter or are the numbers of cells reduced? These should be done in the context of auxin levels revealed by DR5-GUS (Fig 6B) to determine if the cellular phenotypes can be explained by the auxin concentration changes in different regions.

RESPONSE: We appreciate the comments and suggestions. We conducted new experiments and presented cell

number and cell length results in Figure S3.

Point 2. Related to Point 1, the authors should discuss why auxin levels are reduced in the root tip and elongation zones in the HD52 overexpression lines shown in Fig 6B. Is it possible that HD52 overexpression led to increased expression of WAG1/WAG2/PIN2, which pumped auxin out of the root tip and even elongation zone?

RESPONSE: Good point. We also believe that enhanced PIN2 levels due to HB52 overexpression pumps auxin out of the root tip and elongation zone. We added a discussion as suggested.

Point 3. The binding of EIN3 to the *HB52* promoter at the cis2 site is demonstrated by ChIP and yeast one-hybrid assays (Fig. 4). Additional evidence such as mutation of cis2 in both yeast one-hybrid and transient promoter-reporter assays in plants should be provided.

RESPONSE: We did the suggested experiments and presented the results in Fig 3D, E.

Point 4. Similar mutational analysis of the promoter elements in yeast one-hybrid and transient promoter assays should be performed to further prove that HB52 binds to the *PIN2*, *WAG1* and *WAG2* gene promoters (Fig. 7-9). The authors should also discuss why in each of the three promoters, only one out of two/three putative HB52 sites is functional.

RESPONSE: We did the suggested experiments and presented the results in Fig 6D and E, Fig 7D and E, Fig 8D and E. Why in each of the three promoters is only one out of two/three putative HB52 sites functional? Most likely, the other predicted binding sites are not real HD binding sites, since they were not bound by HB52 in planta or in yeast.

Point 5. The authors should explain why HB52 overexpression only partially suppressed the *ctr1-1* or 35S:EIN3 phenotype (Fig. 5).

RESPONSE: The ethylene signaling pathway is quite complex and regulates many downstream genes besides *HB52*. Therefore, the phenotypes of *ctr1-1* or 35S:EIN3 plants were been partially suppressed by partial loss of HB52 (*hb52*, not HB52 overexpression).

Point 6. The authors should explain why *pin2*, *wag1* and *wag2* only partially reverse the HB26 overexpression phenotype (Fig. 10).

RESPONSE: In our study, we just analyzed whether HB52 was able to bind to the promoters of *WAG1*, *WAG2* and *PIN2* and activate their expression. We speculate that HB52 may regulate other genes related to the auxin-signaling pathway, and thus *pin2*, *wag1* and *wag2* could only partially reverse the phenotype of the HB52 overexpression lines.

Point 7. It would be helpful to draw a model to summarize the major findings of the study.

RESPONSE: Good suggestion. An action model was added.

TPC2018-00584-RA 1st Editorial decision – *revision requested* Sept. 9, 2018

We ask you to pay attention to the following points in preparing your revision.

1- Reanalyze the MS versus ACC data set by making the direct comparison between these two data sets with correct statistical analysis as suggested by Reviewer 3, and properly explain how these results support your model.
2- Provide a transient assay using mutated promoters as suggested by Reviewer 4.
3- Revise the current mechanistic model, re-evaluate the relative significance of this branch in root ethylene response and its coordination with the previously established ethylene-auxin biosynthesis branch.
4- Manuscript writing should also be carefully revised.
5 - Please use a professional illustrator to make the figures more legible for the reader and to ensure consistent formatting in all components of the figure.

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

[Provided below along with author responses]

TPC2018-00584-RAR1 1st Revision received Sept. 26, 2018

Reviewer comments on previous submission and **author responses**:

Reviewer #3

Point 1. I am concerned that the authors do not follow or are not familiar with the ethylene research literature. I have requested "In the introduction, the description of the ethylene signaling pathways is not up-to-date. The new functions of EIN2 in the regulation of translation and epigenetics should be mentioned." The authors did include the seminal publication by Dr. Guo's lab on translational regulation but failed to mention the work from Dr. Alonso and Qiao's labs.

RESPONSE: Sorry for not including these important publications. We have added the requested citations.

Point 2. I am also concerned the authors have not properly analyzed or interpreted the new results regarding the effects of ethylene on the expression of *PIN2*, *WAG1* and *2* in the HB52 loss and gain-of-function lines. The authors stated that: "As shown in Figure 6C, the transcriptional levels of *PIN2*, *WAG1*, and *WAG2* but not the other genes examined were reduced in the hb52 mutants and elevated in OX35-2 relative to the wild-type with or without ACC treatment. This result indicates that HB52 may directly or indirectly regulate *PIN2*, *WAG1*, and *WAG2* at the transcriptional level."

First, the authors are probably referring to Figure 5C, not 6C. I had requested that the authors examine the effects of altering the levels of HB52 on the effects of ethylene on the expression levels of *PIN2*, *WAG1* and *2*. The way it is displayed in Figure 5c, it is difficult to tell if there are any statistically significant effects, as the requested direct comparisons were not done. Nevertheless, it seems that the reduction or increase in the levels of HB52 does not have an obvious effect on the induction of these auxin-related genes by ethylene, and therefore, the claim that HB52 mediates the regulation of these genes by ethylene is not supported by the data. Surprisingly, the authors acknowledge including these new data, but they did not properly analyze or discuss it.

RESPONSE: Very good point! We greatly appreciate your comments! Indeed, we did not present the data well in Figure 5C. We have remade Figure 5C so that direct comparison and statistical significance between the control and ACC treatment are clearly shown. The transcript levels of *PIN2*, *WAG1* and *WAG2* were significantly reduced in *hb52* and increased in the overexpression line.

Point 3. In summary, I think the presented data do not convincingly show that HB52 mediates the ethylene-induced regulation of *PIN2*, *WAG1*, and *WAG2*. Some of the claimed ethylene defects of the loss and gain-of-function HB52 mutants are pretty mild and could be due to basal alteration of the auxin levels in the root rather than a direct alteration of the ethylene-induced regulation of the transcriptional levels of the auxin-related genes. In other words, HB52 may regulate auxin homeostasis, and alteration of the levels of this gene may have some effects on the ethylene response, however, I do not think model proposed by the authors in which HB52 mediates the ethylene-induced regulation of *PIN2*, *WAG1* and *WAG2* is supported by the data.

RESPONSE: We think that our data of genetic and biochemical analyses do support that HB52 mediates the crosstalk between ethylene and auxin by transcriptionally modulating *PIN2*, *WAG1*, and *WAG2* during primary root elongation.

Reviewer #4:

The authors did most of the experiments I suggested and adequately addressed all of my questions.

Point 1. I would like to have one clarification from the authors. I suggested that the authors mutate promoter elements and test them in yeast one-hybrid and in tobacco transient assays in Figs 3, 6-8. I only see mutant data with yeast one-hybrid assays but not in the transient assays. Were the transient assays done with mutant promoters as indicated in the response letter?

RESPONSE: Thank you so much for your comments and suggestions! How could we have missed the mutated binding sites in the transient assays? What a blunder! We have redone the transient assays with the mutated binding sites.

TPC2018-00584-RAR1 2nd Editorial decision – *acceptance pending* **Oct. 3, 2018**

We are pleased to inform you that your paper entitled "Arabidopsis HB52 mediates the crosstalk between ethylene and auxin by transcriptionally modulating *PIN2*, *WAG1*, and *WAG2* during primary root elongation" 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 **Oct. 14, 2018**