A Robust Auxin Response Network Controls Embryo and Suspensor Development through a bHLH Transcriptional Module

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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.)

TPC2018-00518-RA 1st Editorial decision – *revision requested* Aug. 9, 2018

Whereas the reviewers were generally positive and felt that your data supports conclusions, it seems that they would like to see a better and/or more focused characterization of the bHLH functions. As currently written, a strong story about these does not emerge. I think that a focus on bHLH49 or better characterization of bHLH49 would help here.

In addition to the reviewer comments, I have a few comments that I would like you to address:
1- There is extensive use of red and green in figures throughout the manuscript. Could this be altered to accommodate those who are colorblind? There are websites that suggest other combinations.
2- There needs to be a better description of microscopy images in the legends. For example, it is not clear what color signal correlates with what reporter or fluorescent dye.
3- There is a typo in the figure legend for Fig. 8 - I believe you meant to type "post-embryonic".

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

[Reviewer comments shown below along with author responses]

TPC2018-00518-RAR1 1st Revision received Nov. 8, 2018

Reviewer comments and **author responses**:

Reviewer #1:

In this manuscript, the authors use a transcriptomic approach to characterize changes in gene expression associated with ectopic embryo development in the suspensor that is caused by expression of a stabilized form of the IAA12 (bdl) protein. Their work demonstrates that repression of auxin response in the suspensor results in a dramatic reprogramming of gene expression. In particular, they show that the expression of many genes involved in auxin

metabolism, transport and signaling is altered, indicating that the auxin system has robust homeostatic mechanisms. This part of the manuscript is very well done. In the second part of the study, the focus is on genes that regulate ectopic embryo formation. The authors assemble a collection of transcription factor and signaling proteins that are misregulated in the *bdl* material relative to the wild type. They then focus on four bHLH genes and perform a series of genetic studies to explore the function of these genes in embryogenesis and auxin signaling. At this point, the story becomes rather non intuitive. All four genes are upregulated in the *bdl* line and downregulated by auxin treatment. Interestingly, one of these, *bHLH49*, appears to be a direct target based on a CHX experiment. However, they are upregulated in the embryo-proper of *bdl* proembryos rather than suspensors. Looking at *bHLH49* in particular, this implies that, despite the fact that it is a direct auxin target, its expression is not affected in the suspensor, where the auxin response is inhibited. Further, over-expression in the pro-embryo results in non-cell-autonomous formation of embryos in the suspensor. The authors note that ectopic embryo formation involves intensive communication between the suspensor and the proembryo, and this study certainly provides support for this concept.

The more important question is what is the role of the bHLHs in embryogenesis and suspensor identity? The analysis of recessive mutants provides some evidence that each of these genes is required for aspects of suspensor formation. In addition, ectopic expression of these genes, particularly *bHLH49*, appears to cause the formation of extra proembryos. In this case, it is not clear if over-expression in the proembryo or suspensor is important.

This manuscript describes a lot of work and some useful data. I would be supportive of publication if the authors can provide more clarity with respect to the bHLH story, particularly *bHLH49*.

Point 1. Is this gene acting non cell autonomously to promote embryo identity in the suspensor? This could be addressed by over-expressing the gene with the suspensor-specific promoter.

RESPONSE: In our manuscript, we describe strong misexpression defects in embryos misexpressing the *bHLH49* gene from the ubiquitous *RPS5A* promoter. It can indeed not be concluded if the phenotype is caused by misexpression in the pro-embryo, suspensor or both. To address this question, we have expressed *bHLH49* in only the suspensor from the M0171 driver, which did not induce these phenotypes (now mentioned in the manuscript). Unfortunately, despite generating over 400 promoter-reporter fusions in the past decade (e.g. Schlereth *et al., Nature* 2010; Rademacher *et al., Plant J.* 2011; Rademacher *et al., Dev. Cell* 2012; Wendrich *et al., Plant Reprod.* 2015; Möller, *et al., PNAS* 2017; Wendrich *et al., PNAS* 2017; Palovaara *et al., Nature Plants* 2017; this manuscript), we have never encountered a single promoter that drives expression in the entire pro-embryo and not in the suspensor. The only promoter we know of that might confer such expression in *DRN* (e.g. Slane *et al., Development* 2014). However, in our hands, lines driving expression from the *DRN* promoter are highly variable, with suspensor expression being very common. Therefore, we simply cannot do the inverse experiment, expressing *bHLH49* from only the pro-embryo. We did express it from the Q0990 driver, which confers expression in the inner lower tier cells (Radoeva *et al., Plant Physiol* 2016), but this did not induce the defect induced by *RPS5A*-driven expression. We have added new experimental data, in which we describe that expression of *bHLH49* from the suspensor-specific M0171 or ARF13 promoters did not induce embryo defects, which is consistent with *bHLH49* misexpression acting in the pro-embryo.

Point 2. What about the loss-of-function phenotype in the suspensor? Is this phenotype also due to non-cell autonomous action?

RESPONSE: This is a good question, and unfortunately not one we can answer. As reported in the manuscript, the penetrance of the embryo phenotype in the *bhlh49* mutant is 15-25%, and complementation with the bHLH-tdTomato transgene restores this to "background" levels of 5%. Ideally, domain-specific complementation would rescue to the same degree if action can be fully recapitulated. As discussed in the previous point, we cannot perform pro-embryo-specific complementation, and would be limited to suspensor-specific expression. Given the very weak expression of the (complementing) endogenous *bHLH49* promoter, complementation with suspensor-specific expression is very unlikely, and at best a partial complementation could be expected. Because partial complementation would be almost impossible to score, we have decided not to pursue this further.

Point 3. I think the authors can do a better job of highlighting the surprising nature of their results. For example, they don't note that *bHLH49* appears to be a direct target of auxin, but responds in the proembryo rather than the suspensor where the auxin response is being altered. I think we need a bit more than just noting that there is lots of communication between the proembryo and suspensor.

RESPONSE: We have modified the text to highlight this aspect more. On the other hand, we spend a significant part of the manuscript describing how perturbation of auxin response systemically alters auxin accumulation and response patterns. We agree that any statement in this area lacks precision. However, we feel that without quantitative spatial and temporal analysis of all members to the more than 10 gene families whose expression is altered, as well as modeling of their effects, we cannot go beyond stating that there is convergent and systemic rewiring of auxin biology. As discussed below, we do now provide direct evidence that (as predicted by the transcriptional responses) auxin levels are upregulated in M0171>>bdl embryos.

Point 4. The authors made transgenic lines for 68 genes, which is formidable, but they don't provide any details. How many independent lines did they look at. Where they looking at T1s? More details are required.

RESPONSE: We apologize for this oversight. We have now included this information in the manuscript. All embryos were by definition analyzed in T2 in up to 8 independent T1 plants, and confirmed in the T3 generation in at least 2 lines.

Reviewer #2:

The manuscript by Radoeva, Lokerse, et al. showed that auxin homeostasis and response are rearranged during embryogenesis when the auxin response is specifically blocked in the suspensor. And they identified auxin-regulated bHLH transcription factors, forming a network involved in embryo and suspensor development. Their experiments were well done and findings are novel. But, I think that the functional analysis of bHLH transcription factors in embryo and suspensor development is insufficient as follows:

Point 1. Auxin-dependent expression analysis (Fig. 5B) was performed in roots, not during embryogenesis.

RESPONSE: Indeed, we did not show that *bHLH49/60/63/100* are regulated by auxin treatment in embryos. Doing so through qPCR is unrealistic, given the amount of material needed. We did attempt to culture ovules carrying pbHLH-n3GFP transgenes on auxin-containing medium, but the very low survival rate (see also Sauer and Friml, *Plant J.,* 2004) and difficulties with imaging expression patterns and intensity in embryos among culturing-induced defects precluded the collection of clear data.

Point 2. The authors identified only one allele of *bhlh60* and *bhlh63* and did not complement their phenotypes with the introduction of *bHLH60* and *63*.

RESPONSE: Indeed, we restricted our analysis to only those available insertion lines that could be expected to have strong downregulation. We did not attempt mutant complementation for the simple reason that phenotypic penetrance in the *bhlh60* and *bhlh63* mutants was very low (13% and 12%, respectively) and qualitatively very mild. Given that even in wild-type, these very mild phenotypes are observed at a low rate (of up to 5%), the range to evaluate complementation is too small to make firm statements.

Point 3. Expression analysis of embryo-identity genes, such as *WUS* and *STM*, was not performed in the *bhlh* mutants. I think that these problems need to be improved for publication.

RESPONSE: Again, detailed analysis of these mutant phenotypes was hampered by the low phenotypic penetrance. In our experience, analyzing changes in reporter gene expression by mRNA in situ hybridization or fluorescent reporters in a background where only 1 in every 8 embryos shows a mutant phenotype is simply impossible.

Point 4. According to the results of whole-genome transcript profiling, it is predicted that the auxin response and level are upregulated during embryogenesis, but there are no data that support this prediction. The authors used only Dr5/DR5v2 as an auxin response marker. To investigate auxin levels, expression analysis of DII-Venus and quantitative analysis of endogenous IAA would be helpful.

RESPONSE: This is a very good point. We have now analyzed the ratiometric R2D2 (DII/mDII) auxin activity sensor in M0171>>bdl embryos and added the data to the revised manuscript. As predicted by the transcriptome changes, there is a strong increase of auxin levels in the embryos, yet a full suppression of the auxin response (as reported by DR5 activity) until the heart stage of embryo development.

Point 5. The expression analysis of *DR5* in pRPS5A-bHLH49 was performed under normal conditions (without auxin). Was there no change in *DR5* expression even when pRPS5A-bHLH49 was treated with auxin?

RESPONSE: We have performed the experiment as suggested, and did not find a change in auxin responsiveness of DR5 in the pRPS5A-bHLH49 background. This has now been added to Supplemental Figure S4F, G, M, N.

Reviewer #3:

The manuscript by Radoeva et al., entitled: "A robust auxin response network controls embryo and suspensor development through a bHLH module" reports on the identification and characterisation of regulators of suspensor identity maintenance and embryonic cell fate establishment. Their analysis focused on auxin signaling-dependent regulators of the bHLH family, as a result of a microarray analysis of isolated embryos expressing the auxin resistant mutant *bdl* driven by the suspensor-specific driver line M0171. Identification of the key regulators of cell fate is of importance for organ regeneration studies, as used in protocols in crops upon transformation. Any data helping to improve these protocols, by identification of TFs involved in cell proliferation or cell specification, are definitely welcome. The analysis is well conducted and data are properly interpreted, despite some discrepancies detailed below.

Point 1. Blocking auxin signalling in the suspensor affected auxin homeostasis: activation of auxin production, inactivation of auxin degradation and transport, as well as some signalling components. Some ARFs are up-regulated: are these repressors? For example, *ARF13* is suspensor-specific (Rademacher 2011) but is upregulated by inhibition of the signalling response in the suspensor. Any hypothesis? Of course, the DR5 reporter is off in this M0171>>bdl line, confirming the inhibition of the auxin response. But did you also have a look at the R2D2 reporter to con/infirm the scheme in Fig. 2J for an increase in auxin levels in this line?

RESPONSE: There is indeed massive rewiring of auxin biology upon local response inhibition. Most of these make intuitive sense when considering that regulation converges upon homeostasis of auxin output. The only exceptions are a few ARFs and a single Aux/IAA (IAA17). Given that the biological function of these ARFs and their role in gene regulation in the embryo is not clear (for example Rademacher *et al., Dev. Cell* 2012), and because the effect is rather small (2-fold upregulation), we did not pursue this aspect further.

As suggested by the reviewer, we did analyze the ratiometric R2D2 (DII/mDII) auxin activity sensor in M0171>>bdl embryos and added the data to the revised manuscript. As predicted by the transcriptome changes, there is a strong increase of auxin levels in the embryos, yet a full suppression of response (as reported by DR5 activity) until the heart stage of embryo development.

Point 2. Following the analysis of the transcriptional dataset for regulators of the S>E developmental program, the authors focused on transcription factors, with the hypothesis that if they are positive regulators of suspensor identity maintenance or repressor of embryo identity induction, they should be up-regulated if they are normally expressed in embryo cells, and down-regulated if they are normally expressed in suspensor cells. They identified 4 bHLH proteins (49, 60, 63, 100) fitting this hypothesis. For some reason that doesn't appear well justified (difficult to judge from the picture in 4H that there is no expression in the suspensor, which could also be an argument for 63, etc.), they excluded bHLH153 from the analysis. Some better arguments would be required for this exclusion.

RESPONSE: There is a very simple reason for not including the *bHLH153* gene in further functional analysis: the gene is expressed in the pro-embryo and downregulated in *M0171>>bdl* embryos (2.2-fold down; Table S2). We did in fact analyze insertion lines for *bHLH153* in a different context and reported (Wendrich *et al., Plant Reproduction* 2015) that there is no obvious embryo phenotype.

Point 3. They characterised in more details the function of *bHLH49*, as well as *60*, *63*, and *100*, in the S>E transition, with analysis of auxin-dependent expression, mutant and RPS5A-induced expression. The analysis showed that *bHLH49* seems to be a direct target and be repressed by auxin, while the 3 others are indirectly induced, probably through bHLH49, at least for *bHLH60* and *100*. However, this expression analysis (Fig 5B) does not fit with the expression patterns (Fig 5C) of *bHLH60* and *63*, which are upregulated by auxin and by ectopic expression of *bHLH49*, and when blocking auxin signalling. Also, the effect appears to be non-cell autonomous, as their *bdl*-dependent induction occurs in protoderm cells for suspensor cells proliferation. Nevertheless, consistent with the suspensor phenotypes in M0171>>bdl, *bHLH49* mutant embryos display a shift in E-S junction with what appears to be embryonic cells proliferation.

RESPONSE: We agree. Because the response to auxin (in roots) is indirect (Figure 5B), one cannot directly infer causality of regulation. An additional caveat is that regulation by additional auxin in roots is not the same as inhibition of the response in the embryo. We took care to limit our conclusions related to the auxin-induced regulation of *bHLH60/63/100* and regret that we have apparently given the impression that there is an easy explanation for the auxin/bHLH49 regulation of genes. In our interpretation, there is no simple explanation. We have refrained from further elaborating this aspect to avoid over-interpreting the data.

Point 4. The authors continued the analysis by ectopic expression of the bHLH proteins with the RPS5A promoter for embryonic overexpression. Why did they not generate either embryo-specific (one of the promoters developed in your lab, Wendrich 2015) or suspensor-specific (M0171) expression of bHLH49? This would have provided further information on the function of bHLH49 in the S>E shift. With the use of the RPS5A promoter, one cannot judge if the observed phenotypes (fig 7) are due to the TF function in the embryo or in the suspensor, as it was suggested to have non-cell autonomous activity.

RESPONSE: It can indeed not be concluded if the phenotype is caused by misexpression in the pro-embryo, suspensor or both. To address this question, we have expressed bHLH49 in only the suspensor from the M0171 driver, which did not induce these phenotypes (now mentioned in the manuscript). Unfortunately, despite generating over 400 promoter-reporter fusions in the past decade (e.g. Schlereth *et al., Nature* 2010; Rademacher *et al., Plant J.* 2011; Rademacher *et al., Dev. Cell* 2012; Wendrich *et al., Plant Reprod*. 2015; Möller, *et al., PNAS* 2017; Wendrich *et al., PNAS* 2017; Palovaara *et al., Nature Plants* 2017; this manuscript), we have never encountered a single promoter that drives expression in the entire pro-embryo, and not in the suspensor. The only promoter we know of that might confer such expression in *DRN* (e.g. Slane *et al., Development* 2014). However, in our hands, lines driving expression from the *DRN* promoter are highly variable, with suspensor expression being very common. Therefore, we simply cannot do the inverse experiment, expressing bHLH49 from only the pro-embryo. We did express from the Q0990 driver, which confers expression in the inner lower tier cells (Radoeva *et al., Plant Physiol* 2016), but this did not induce the defect induced by *RPS5A*-driven expression. We have added new experimental data, in which we describe that expression of bHLH49 from the suspensor-specific M0171 or ARF13 promoters did not induce embryo defects, which is consistent with bHLH49 misexpression acting in the pro-embryo.

Point 5. Also, it would be indicative to know the status of auxin homeostasis in the RPS5A-bHLH49 root/embryo. The DR5 pattern is correct in the line - bHLH49 would be downstream of auxin signalling as hypothesized, what about R2D2 as an indicator of auxin levels? It appears that *bHLH49* is repressed by auxin, but *RPS5A-bHLH49* is oversensitive to auxin, as measured by root length: is *bHLH49* repressed upon auxin treatment? Is the root morphology changed in RPS5-bHLH49 by auxin application? Is it due to inhibition of cell division (auxin would inhibit/rescue the cell proliferation phenotype of bHLH49)? In that case, does auxin also affect other *bHLH40* overexpression phenotypes, such as fruit development, fertility? In addition, *bHLH60* and *100* are up-regulated and down-regulated by bHLH49 overexpression but both are auxin-inducible. Any hypothesis?

RESPONSE: These are all good questions, all asking if the regulation of *bHLH49* by auxin is an important part of auxin action in roots. The loss of function data urges us to be modest in this regard. The *bhlh49* loss of function mutant has minute effects on auxin-dependent root growth (Figure 8A). Conversely, the RPS5A-bHLH49 has dramatic root phenotypes, but it is not clear whether these relate only to an altered auxin response. At least at the level of DR5 expression, the auxin response appears normal. We have now performed two additional experiments: first, we treated RPS5A-bHLH49 / DR5-GFP roots with auxin and found that the auxin response is normal (Supplemental Figure S4F, G, M, N). Second, we grew RPS5A-bHLH49 seedlings on auxin to see if this could restore

normal development, but this did not rescue root growth (Supplemental Figure S4 A-E, H-L). Thus, while auxin-regulation in embryos and roots, and gain- and loss-of-function phenotypes place *bHLH49* downstream of auxin, this gene is not the major output in auxin-dependent root growth.

Point 6. Finally, following your discussion around line 544, it would be interesting to analyse the effects of bHLH49 (loss and gain-of-function) in the case of induced somatic embryogenesis and during organ regeneration from hypocotyl explants (f.e. Inoue 2001). This system also involves cell dedifferentiation and cell fate reestablishment. This could be a nice follow-up for this project.

RESPONSE: This is an excellent point, and we have now elaborated this point in the Discussion. We assume the referee refers to the 2011 Iwase et al. paper that describes the role of the WIND1 transcription factor, and have cited this paper in our Discussion.

TPC2018-00518-RAR1 2nd Editorial decision – *accept with minor revision* Nov. 26, 2018

On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the minor improvements suggested by Reviewer 3, which involve small changes to the text and clarification of a few points.

TPC2018-00518-RAR2 2nd Revision received Nov. 27, 2018

Reviewer comments and **author responses**:

Reviewer #1:

I am satisfied with the revisions that the authors have made and recommend publication.

Reviewer #2:

The manuscript by Radoeva, Lokerse, et al. was sufficiently revised according to the reviewers' comments and succeeded in uncovering the function of bHLH49 in early embryogenesis.

Reviewer #3:

I reviewed the revised manuscript by Radoeva et al., entitled: "A robust auxin response network controls embryo and suspensor development through a bHLH module". The text was greatly improved, especially the transition between the two parts of the manuscript. The authors responded to my comments on the initial version. I'm quite satisfied with the answers. I still have minor comments.

Point 1. In Figure 1, there is some GFP signal visible and noted in the legend. Please indicate what is it linked to. A similar remark goes for Fig 3I and J.

RESPONSE: The GFP signal reflects the activity of the M0171 enhancer trap that drives mGAL4:VP16 expression, which in turn activates the *UAS-erGFP* gene. This information has now been added to the figure legend on lines 725/726.

Point 2. With respect to the Venn diagram in Fig. 1, are they any genes that would be up-regulated at one of the time points and down-regulated at the other?

RESPONSE: We thank the reviewer for this question, because it motivated us to rethink how to best display this data. Instead of Venn diagrams, we have chosen to display the data as an UpSet plot. This plot shows all possible intersections between up- or down-regulated genes between the two datasets (72 hour and 96 hour time points). We feel that this brings more clarity and shows that a very small number of genes are oppositely regulated between time points. Only 21 genes are up-regulated at 72 hours and down-regulated at 96 hours, and only 35 are down-regulated at 72 hours and up-regulated at 96 hours. This is in contrast to the 277 genes that are upregulated at both time points and 127 that are downregulated at both time points. We have updated Figure 1, changed the figure

legend (lines 728/729), mentioned how the plot was generated in the Experimental Procedures (lines 666-668), and added a reference to the paper describing the software tool (lines 926/927).

Point 3. Line 212: "enhanced auxin responsiveness". I am puzzled with this expression for the following reasons:
- Auxin signalling is blocked by stabilized BDL
- Auxin receptor genes are mostly down-regulated
- Some of the ARF are upregulated and some are downregulated.

Can you clarify or reword?

RESPONSE: We agree. The predicted effects on auxin conjugation, biosynthesis and transport are highly convergent, but on the auxin response they are more mixed. Our notion was mostly based on the coordinate downregulation of several Aux/IAA genes, which are evident negative regulators. The AFB4 and AFB5 receptors may or may not be canonical receptors, and their role is less clear. In the case of the ARFs, there is a mixed transcriptional response. For this reason, we have modified the statement on line 209, now stating that there is likely "altered" auxin responsiveness.

Point 4. *bHLH100* is downregulated in M0171>>bdl. In fig. 5, you controlled by expression pattern the microarray results. For that, you used a bHLH100p:nVenus reporter that shows already very low signal in WT. So it is very difficult to see that it is down-regulated in M0171>>bdl. Why didn't you use the bHLH100p:3nGFP line, which displays a stronger signal in WT?

RESPONSE: This is indeed unfortunate. The reason for using a Venus reporter here (and for the other *bHLH* genes) is that the antibiotic resistance gene in the n3GFP reporter transgenes is the same as is already present on the M0171 enhancer trap transgene. Thus, we needed to construct new reporters, and chose to not only change antibiotic resistance, but also the fluorophore, such that expression could more easily be separated from the erGFP expression in the M0171 line. Unfortunately, we could not recover lines with stronger expression.

TPC2018-00518-RAR2 3rd Editorial decision – *acceptance pending* **Dec. 4, 2018**

We are pleased to inform you that your paper entitled "A robust auxin response network controls embryo and suspensor development through a bHLH module" 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 **Dec. 19, 2018**