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A Two-Step Model for *de novo* Activation of *WUSCHEL* during Plant Shoot Regeneration

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Review timeline:

TPC2016-00863-RA	Submission received:	November 18, 2016
	1st Decision:	December 29, 2016 *revision requested*
TPC2016-00863-RAR1	1st Revision received:	February 8, 2017
	2nd Decision:	March 16, 2017 *accept with minor revision*
TPC2016-00863-RAR2	2nd Revision received:	March 17, 2017
	3rd Decision:	March 22, 2017 *acceptance pending, sent to science editor*
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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.)

TPC2016-00863-RA 1st Editorial decision – *revision requested* **Dec. 29, 2016**

We ask you to address all comments/concerns from the reviewers, and to pay particular attention to the following points:
- extend the ChIP analysis to evaluate binding of ARR-B across the length of the WUS promoter, or provide an acceptable explanation for why only select promoter regions were assessed.
- Elaborate on the convergence of ARR-B and HD-ZIPIII transcription factors on WUS regulation, paying attention to the specific comments from each of the Reviewers.
- Provide a dramatically revised text that includes more extensive and more detailed descriptions of all the data shown, whether in the main text or supplemental.
- The revised manuscript should also more accurately acknowledge prior publications in this field, particularly with respect to the role of WUS in regeneration.
- Reformat the supplemental data files, and ensure that all figures are high resolution and legibly labeled.
- Finally we ask you to connect your findings to what is known about WUS regulation during normal SAM initiation and maintenance. What are the parallels and what is different? Extensive experimentation along these lines may be outside the scope of this manuscript. However, we like you to seriously consider point 6 from Reviewer 1. Analysis of the suggested WUS reporter lines would clarify an important point namely whether the steady state and de novo activation of WUS occur via independent cis-elements as is currently implied. Otherwise, elaborate on this point in the revised text.

Please download the attached file and view in Acrobat in comment mode (not in browser window). In general, the figures need to be professionally illustrated to increase legibility. The fonts need also to be sized proportionally to the figures but still need to be read by the reader.

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

[Reviewer comments shown below along with author responses]

TPC2016-00863-RAR1 1st Revision received Feb. 8, 2017

Reviewer comments and **author responses**:

Reviewer #1:

The manuscript by Zhang et al. aims to elucidate the mechanism underlying the transcriptional activation of WUSCHEL during shoot regeneration. The authors present data from a diverse set of experiments including expression analyses by in situ hybridization and reporter genes, ChIP, transactivation assays, protein-protein interaction studies, as well as functional tests for shoot regeneration. The take home message is that cytokinin dependent Type-B ARRs transcription factors act together with HD-ZIP III transcription factors to induce WUS during shoot regeneration. The manuscript does contain a number of interesting and important observations, but currently suffers from severe limitations that need to be addressed before publication.

Point 1. A good part of the reported data is not novel, but has been published before, sometimes in a slightly different context. Examples include the role of WUS for cytokinin independent callus formation and in vitro development (Zuo, Plant Journal 2002), the expression of WUS and STM during shoot regeneration (Gordon, Development 2007), the inability of multiple ARR-B mutants to undergo shoot regeneration (Mason TPC 2005) and the even distribution of TCS output on SIM (the same authors). I appreciate that some of the results differ from those reported in the literature, but more care needs to be taken to explain what has been known before. For example, I cannot see that the role of WUS during regeneration is controversial as claimed: both references given in the manuscript report that wus mutants are unable to regenerate shoots. However, even in the abstract the authors claim that they have found evidence for the requirement of WUS for shoot regeneration.
Along these lines, I fail to see any new results with regards to WUS function in the entire first paragraph.

RESPONSE: In Gordon's paper, they wrote on Page 3545 "The average number of shoots formed in the wus-1 mutant (n=91) decreased to 5% of wild-type number of shoots (n=106; 0.25±0.08 versus 5.06±0.04)". However, Chatfield et al. showed that none of wus-/- (SAIL_150_G06) mutant can regenerate shoots. The difference between these two studies could be due to the fact that they used different wus mutants for shoot regeneration assays. Nevertheless, we did not claim that "the role of WUS during regeneration is controversial" in the revised manuscript (Page 4). We wrote "Consistent with a previous report (Chatfield et al., 2013), the wus mutant failed to regenerate shoots".

For the same reason, we did not claim that we found the evidence for the requirement of WUS for shoot regeneration. 1) In the abstract, we wrote "The homeodomain transcription factor WUSCHEL (WUS) is essential for de novo establishing shoot stem cell niche. We found that the WUS-positive cell (WUS^+) marks shoot progenitor during regeneration.". 2) On Page 4, we wrote "we show that WUS-positive (WUS^+) cell marks shoot progenitor during shoot regeneration.".

We agree with the reviewer that there are not new findings in the first paragraph. However, this paragraph serves as a logical starting point for us to investigate the expression pattern of WUS rather than those of other genes during regeneration. We could remove this paragraph if the reviewer strongly recommends doing so.

Point 2. The authors claim that cells in with REV and TCS activity overlap will give rise to WUS expression. From the data presented this is a non-result, since both markers seem to be expressed almost everywhere! In addition, there is no evidence for the claim that WUS is expressed in cells that express REV and ARR1.

RESPONSE: Our in situ hybridization analyses have shown that REV and PHB are not expressed everywhere in the callus (Figure 6C and 6D; Figure S14A).

Because ARR1 is expressed everywhere in the callus, the WUS^+ cell must reside in $ARR1^+$ region. To clarify that WUS is only activated in REV^+ cells, we show two more pictures for REV-DsRED WUS-GFP reporters (Figure S16A). As you can see, the WUS^+ signal is overlapped with REV^+ signal. We never observed that WUS is activated outside of REV^+ cells.

In the first submission, we used two "high-magnification" pictures (Figure 7K and 7L) to show that the WUS-GFP^+ cell is overlapped with the REV-$DsRED^+$ cell. Indeed they give an impression to the reviewer that DsRED signals

(red) are presented everywhere within the callus because we only showed a very small region within the callus. To clarify whether REV-DsRED reporter is expressed everywhere in the callus, we showed two more pictures for REV-DsRED reporter (Figure S16A). We include the bright-field pictures for comparison. The REV-DsRED signals only appear at some cells within the calli (please see merged pictures). To further address this concern, we added PHB-sGFP reporter data in the revision. As shown in Figure S16B, the PHB reporter is only expressed in a small region within the callus.

Point 3. The attempts to map ARR-B binding onto the WUS promoter in vivo by ChIP are fairly poor since only low resolution ChIP-PCR data is provided, which is compatible with widespread binding.

RESPONSE: We selected the conserved regions for ChIP-PCR analyses according to our phylogenetic shadowing analyses (Figure 2E). The reasons for doing this is that the activation of WUS by B-ARR seems to be evolutionarily conserved and B-ARRs may therefore bind to the conserved cis-elements in the WUS promoters in Arabidopsis close relatives. Nevertheless, to address this concern, we performed ChIP-PCR by analyzing all the WUS promoter regions (Figure 2D). As you can see, ARR1/2 only binds to the regions i and j (previously d and e) in the WUS promoter.

Point 4. The experiments described in Fig 2 are not explained sufficiently: what are the promoter deletions used in panel B?

RESPONSE: To address this concern, we revised Figure 2. We added Figure 2B "Diagrams of the B-type ARR effector and WUS reporter constructs for transient expression analyses in (C)".

Point 5. The finding that ARR-Bs and HD-ZIPs interact is really interesting. However the standard interpretation of the genetic data would suggest that this interaction is not relevant. That providing more of partner A in the absence of partner B causes the rescue of loss of partner B certainly is not compatible with the idea of a relevant complex as claimed by the authors. Rather it suggests that both inputs act in parallel.

RESPONSE: Both B-type ARRs and HD-ZIP III are functionally redundant. We refer to HD-ZIP IIIs as A and to B-type ARRs as B. The rev mutant is not a case of "in the absence of A" because PHB and PHV are still functional. Therefore, it is reasonable to find that overexpression of ARR2 increases shoot regenerative capacity in the rev mutant (Figure 4F) because more ARR2 can bind to PHB or PHV to elevate regenerative capacity.

We ruled out the possibility that B-type ARR and HD-ZIP III regulate each other's expression because the expression of B-type ARRs is not disturbed in the hd-zip iii mutant and vise versa (Figure S14). These results indicate that B-type ARR and HD-ZIP III act in parallel or act in the same complex. To distinguish between these two possibilities, we performed a shoot regeneration assay using the phb phv rev triple mutant on SIM with different levels of cytokinin. If B-type ARR and HD-ZIP III act in parallel, we would expect that the increased level of cytokinin (increasing the B-ARR function/activity) would compensate for the low shoot regenerative capacity in the phb phv rev triple mutant. However, what we found is that the increased level of cytokinin did not suffice to induce shoot regeneration in phb phv rev triple mutants (Figure 4D). This observation indicates that 1) both B-type ARRs and HD-ZIP III are essential for shoot regeneration and 2) B-type ARR and HD-ZIP III function as partners in shoot regeneration and the role of these TFs in the activation of WUS is interdependent. This hypothesis is supported by our finding that B-type ARRs bind to HD-ZIP III.

To further support our conclusion, we found that HD-ZIP III alone could not activate WUS expression in the transient protoplast assays (Figure 4I).

Point 6. The regions identified by ChIP and transactivation assays as bound by B-ARRs map closely to, but still outside the element identified by Baurle et al. to be sufficient for WUS expression in the SAM. The maximum of REV binding even lies outside the region defined by the Laux lab, despite the fact that this does contain a canonical HD-ZIP III binding motif. The authors should discuss these results more carefully. In addition, they need to test their hypothesis that steady state and de novo activation work via independent cis-elements by analyzing the discussed deletion construct and the minimal element defined by the Laux lab in their setting. These reporter lines exist and the experiments should be trivial.

RESPONSE: Thanks for these helpful and constructive comments. Whether the steady state and de novo activation of WUS work via independent cis-elements is an open question and may be outside the scope of this manuscript. It

will take at least half a year for us to perform experiments to address this question. To discuss our results more carefully, we revised our Discussion section: 1) Based on our and Laux' lab data, we conclude that D5 region is important for WUS expression. All the cis-elements responsible for WUS activation or maintenance reside in this region. 2) We do NOT rule out a role of 57-bp region in de novo activation of WUS. It is possible that this region and ARR1/2 binding regions cooperatively regulate WUS de novo activation in response to cytokinin during regeneration. 3) Whether the same cis-elements or regulatory regions are responsible for de novo activation or maintenance of WUS expression in SAM is currently unknown. Although the analyses of the suggested WUS reporter lines will be helpful, ectopic heterologous enhancer assays cannot address the necessity of an element in its native chromatin environment. Therefore, we propose that the saturating mutagenesis of the regulatory sequences within D5 region by CRISPR/cas9 approach in the native genomic context will shed light on how the diverse cis-elements in D5 region cooperatively regulate WUS expression during normal plant development and shoot regeneration.

On Page 13:

"It is currently unknown whether the same cis-elements or regulatory regions are responsible for de novo activation or maintenance of WUS expression in SAM. By promoter deletion analyses, Bäurle and Laux reveal that the D5 region (-541 to -726 bp) is absolutely necessary for WUS expression in the stem cell niche (Bäurle and Laux, 2005). The genomic fragment carrying the D5 deletion does not rescue the wus phenotype and abolishes WUS expression in the inflorescence meristem. This result is in a good agreement with our data that ARR1 and ARR2 activate WUS through binding to two fragments (-550 to -620 bp and -700 to -760 bp) within D5 region (Supplemental Figure 6). Using a series of synthetic reporters that carries tetrameric tandem repeats of WUS promoter fragments, Bäurle and Laux further identify a 57-bp regulatory region (-712 to -655 bp) that confers WUS transcription in the SAM stem cell niche (Bäurle and Laux, 2005). Surprisingly, this 57-bp regulatory region does not overlap with the ARR1/2 binding regions. Does this 57-bp regulatory region also play a role in de novo activation of WUS during shoot regeneration? Similarly, are the two ARR1/2 binding regions identified here involved in the maintenance of WUS expression in SAM during normal plant development? To answer these questions, we should dissect whether the 57-bp region is sufficient to drive WUS expression during shoot regeneration. In addition, the saturating mutagenesis of the regulatory sequences by clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/cas9) approach in the native genomic context will reveal how the diverse cis-elements in D5 region cooperatively regulate WUS expression (Canver et al., 2015)."

Reviewer #2:

The manuscript entitled "A Two-Step Model for de novo Activation of WUS during Plant Shoot Regeneration" shows that WUSCHEL (WUS) is essential for de novo establishing shoot stem cell niche. During shoot regeneration, the WUS signal marks the shoot progenitor. Cytokinin activates WUS expression in two steps. Firstly, a cytokinin rich environment promotes the removal of repressive histone mark H3K27me3 at the WUS locus in a cell-cycle dependent manner. Secondly, the B-type ARRs directly activate WUS expression through interacting with HD-ZIP III transcription factors. On the whole, the data are clearly presented and convincing. The manuscript is well written. The mechanisms underlying hormonal regulation on WUS expression and subsequent regeneration of stem cell niche is a very important question in plant regeneration. The positive correlation between cytokinin and WUS expression has long been suggested. However, the underpinning mechanisms remain unknown. Thus, this manuscript is very interesting and will attract the general interests of plant scientists. I have several suggestions for the authors to improve the manuscript as follows.

Point 1. The ARR1 signal was evenly distributed throughout the callus at early stages of shoot regeneration. However, it seems that the signal was restricted to discrete regions and enriched in the regenerated meristem at the late stages. Do HD-ZIP III transcription factors play roles in restricting the regionalization of ARR? I expect this could be discussed more.

RESPONSE: We addressed this point in the Discussion.

"Interestingly, ARR1 reporter activities are restricted to discrete regions and enriched in the regenerated meristem at the late stages. This observation suggests that miR165/6-targeted HD-ZIP III transcription factors play roles in restricting the regionalization of ARR1. In this scenario, the co-localization of B-type ARRs and HD-ZIP III may contribute to the maintenance of WUS expression in developing SAM."

Point 2. What is the mechanism of the interaction between ARRs and HD-ZIP III transcription factors on the regulation of WUS expression? Did the three HD-ZIP III transcription factors bind to the WUS promoter and regulate its transcription? The authors may analyzed their potential association by ChIP or add discussion here.

RESPONSE: In Figure 5B, we have shown that REV bound to the WUS promoter.

Reviewer #3:

In the manuscript by Zhang and colleagues, de novo shoot development from Arabidopsis hypocotyls is examined using a variety of markers, live imaging, and in situ hybridizations at various time points. The authors focus on how the homeodomain protein WUSCHEL (WUS) is activated during de novo organogenesis, as it has been known for many years that WUS is important in this process.

They make several observations-
1-WUS appears to come on in small clusters (possibly single cells) instead of being broadly expressed as previously reported (Gordon et al., 2007).
2-ARR1 and ARR2 appear to bind the WUS promoter.
3-WUS activation seems dependent on the status of H3K27me3 in the promoter, and this mark is slowly decreased in a division dependent manner.
4-ARR1 and ARR2 physically interact with the HD-ZIP III class of transcription factor, PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV). These HD-ZIP are also shown to be necessary for shoot development in this system.
5-Although REV and ARR2 have a broad overlap in developing callus, WUS is only induced in a small subset of these overlapping cells.

Overall, the data presented are of good quality. The results are a step forward in understanding regeneration from explants, at least in Arabidopsis, although it is probably not indicative of what happens in a plant in vivo. This may reduce its general interest and make it a bit more specialized.

Point 1. Many of the experiments, especially for ChIP PCR, are based on overexpression constructs, which likely leads to artifacts.

RESPONSE: We agree with the reviewer's comment. Due to the low abundance of plant transcription factors, most labs used HA-/FLAG-/Myc-tagged overexpression lines for ChIP analyses. This approach will definitely bring some artifacts. For B-type ARRs, they are highly expressed in the callus during shoot regeneration. Therefore, it is unlikely that overexpression construct will bring artifacts in ChIP-PCR. For HD-ZIP III, the amount of these transcription factors is low in vivo (they are only expressed in some cells within the callus). To avoid the artifacts brought about by constitutive overexpression construct, we used an INDUCIBLE overexpression construct (35S::FLAG-GR-rREV) (Figure 5).

Point 2. What is meant that the WUS construct could rescue 83% of the time (page 7)? Is that 83% of primary transgenics or 83% of the time in a homozygous transgene, homozygous wus mutant?

RESPONSE: We are sorry for this confusion. It means that 83% of the homozygous wus mutant can be rescued. We clarify this in the text on Page 5: "ProWUS:WUS was able to rescue the wus phenotype (87%, n=38, wus -/-).". The primers used for genotyping were added to Supplemental Table 2.

Point 3. In Figure 2, what is the WT bar representing? Is that wild-type explant ChIP with anti-HA or anti-FLAG? There should be controls for both.

RESPONSE: We are sorry for this confusion. The levels in wild type were normalized to 1.0 for both anti-HA and anti-FLAG samples. To clarify this, we revised Figure 2D.

Point 4. ARR1 and ARR2 are shown to interact with PHB/PHV and REV, but an ARR1/2 mutant was not tested in the system. Further discussion seems to imply that all type B ARRs are interacting with these HD-ZIP III proteins. Without showing this, it should not be discussed as such. This is also a problem using the TCS reporter, as it may or

may not be read out for all of the type B-ARRs and therefore cannot act as a proxy for the presence of an HD-ZIP III interacting B-type ARRs. The failure of ARR2 in situ hybridizations is disappointing. Were reporters for ARR1 and ARR2 tried?

RESPONSE: Previous studies from my lab and other labs have shown that four B-type ARR transcription factors, ARR1, ARR2, ARR10, and ARR12, play essential roles in shoot regeneration in Arabidopsis (Mason et al., 2005; Ishida et al., 2008; Zhang et al., 2015). Although the single mutant does not exhibits defects in shoot regeneration, the double and triple mutants do show reduced shoot regenerative capacity. For example, we show that ARR2 and ARR12 redundantly regulate shoot regeneration and that the arr1 arr10 arr12 triple mutant lost regenerative capacity (Figure S5C).

We have shown that ARR10 and ARR12 can also interact with PHB/PHV/REV in planta by BiLC assays (Figure S12C). We agree with reviewer that it is not appropriate to all the B-type ARRs can interact with HD-ZIP III proteins. Therefore, we revised the abstract and text to clarify that only four B-type ARRs (ARR1, ARR2, ARR10 and ARR12) are involved in WUS activation: For example, In the abstract: "Subsequently, the B-type ARRs (ARR1, ARR2, ARR10 and ARR12), which encode the transcriptional activators in cytokinin signaling pathway,".

Actually, we have already shown the ARR1 reporter in the supplemental data (Figure S11Q-X). As you can see, the expression pattern of ARR1 mimics that of the TCS reporter.

Point 5. The manuscript needs to be heavily edited, as there are grammatical errors throughout.

RESPONSE: Thanks. We have corrected the grammatical errors throughout the text.

TPC2016-00863-RAR1 2nd Editorial decision – *accept with minor revision* **March 16, 2017**

This acceptance is contingent on revision based on the few remaining comments of our reviewers. Particularly:

1) Include data on the genetic interaction of ARR2-SRDX with rev6, as this will substantially strengthen the claim on the ARR-HD-ZIP synergy

2) Address the two minor editorial changes mentioned by Reviewer 2

TPC2016-00863-RAR2 2nd Revision received **March 17, 2017**

Reviewer comments and **author responses**:

Reviewer #1:

The authors have addressed most of my concerns, although the connection to the in vivo situation remains unclear. Also, the overlap to published work remains, but the more careful wording and the long discussion put the new findings into appropriate context.

Point 1. I strongly advise that the data on the genetic interaction of ARR2-SRDX with rev-6 should be included, since it substantially strengthens the claim on the ARR-HD-ZIP III synergy.

RESPONSE: We added these data in the revision (Page 11-12). Accordingly, we added one Figure in the Supplemental Data.

Reviewer #2:

The authors have greatly improved the manuscript in this version and responded to the editor's and reviewers' concerns. I think the manuscript meets the standard of the journal. But I have two points here.

Point 1. In Figure 5, ChIP analysis indicates that the occupancy of both ARR2-3xFLAG and FLAG-GR-rREV peaked at day 3 and decreased at day 10 after transfer to SIM. Does this suggest that the binding of ARR2 and REV on the

WUS promoter is required for the initiation of WUS transcription but not for its maintenance? The authors should explain this point.

RESPONSE: The reduced occupancy of ARR2 and REV on the *WUS* promoter could be due to a dilution effect. As shown in this figure, the calli undergo massive cell divisions on SIM. As a result, there are huge differences in the number of cells in the calli on day 0 and 10. A large proportion of the cells used in ChIP-PCR on day 10 are non-meristematic callus cells (cells that do not express *WUS*). This dilution effect may lead to the false impression that there is a reduction in the enrichment of ARR2 and REV on the *WUS* promoter.

Point 2. In Supplemental Figure 16A, the WUS+ cell (green) is marked by GFP. However, ProWUS:3xVENUS-N7 was described in the legends. The readers might get confused with the conflicting descriptions. I would recommend making this point clear.

RESPONSE: Fixed.

Reviewer #3:

I have read the revised manuscript and find it much improved. In general, my comments and concerns were partially addressed, which I appreciate, although I still think the authors run the risk of getting artifacts from their overexpression constructs. The manuscript still needs a thorough editing in many spots, as there are still some rather odd sentence structures and the lack of necessary articles. The manuscript will be interesting to those working on shoot regeneration, although whether these same mechanisms and interactions happen during normal tissue patterning in the shoot is still unclear.

TPC2016-00863-RAR2 3rd Editorial decision – *acceptance pending* **March 22, 2017**

We are pleased to inform you that your paper entitled "A Two-Step Model for de novo Activation of *WUS* during Plant Shoot Regeneration" 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 **April 7, 2017**