

WIND1 Promotes Shoot Regeneration through Transcriptional Activation of *ENHANCER OF SHOOT REGENERATION1* in Arabidopsis

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Review timeline:

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	1 st Decision:	Sept. 16, 2016 <i>revision requested</i>
TPC2016-00623-RAR1	1 st Revision received:	Nov. 6, 2016
	2 nd Decision:	Dec. 12, 2016 <i>accepted with minor revisions</i>
TPC2016-00623-RAR2	2 nd Revision received:	Dec. 13, 2016
	3 rd Decision:	Dec. 15, 2016 <i>accepted</i>
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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.)

TPC2016-00623-RA 1st Editorial decision – *revision requested* Sept. 16, 2016

The reviewers think that your work is well done and contributes to the understanding of wound-induced shoot regeneration. Reviewer 3 is concerned with the novelty of the findings, and asks for revisions and additional evidence that will strengthen the novelty of the manuscript. Reviewer 1 asks for further evidence to address the connection between the effect of WIND1-regulated ESR1 on callus formation and on shoot regeneration. Please also address the additional minor points raised by the reviewers.

----- Reviewer comments:

Reviewer #1 (Comments for the Author):

The entire plant body plan can be regenerated from any plant tissue in response to external inductive cues. This extraordinary capacity has been immensely used in green culture industries for a long time. Besides external hormone application, wound plays a key role in initiating the formation of pluripotent regenerative mass and de novo organogenesis. While recent studies have begun to elucidate the underlying mechanisms of hormone-mediated signaling, how wound relays the regulatory signals to facilitate de novo organogenesis, remained elusive.

The high quality manuscript by Iwase et al. investigates the mechanism of wound-induced callus formation and regeneration. The authors convincingly show the key regulator WIND1 directly binds to the downstream target ESR1 to control the extent of callus formation and subsequent regeneration. I truly enjoyed reading several experiments towards probing the role of WIND1-mediated regulation that eventually impinges on CUC, a shoot-promoting factor, though ESR1.

In my opinion, the work provides a substantial and important contribution towards understanding how besides other external inductive cues, wound regulate de novo shoot regeneration.

1. Line #269-273: It is a nice result that XVE:ESR1 plants regenerate shoots only upon wounding and when they are grown without wound stress, they develop callus. However, I was wondering whether the callus formed in XVE:ESR1 without wounding retained the potential to make shoot on SIM.
2. Line #276-284: To maintain the flow of the story, the data discussing de novo root regeneration at wound sites can be moved to the end of results section.

3. Line #290-291: Authors should ascertain whether the reduction of shoot regeneration in the *esr1* mutant is because of the sheer reduction in the amount of callus formation. This can be done by careful correlation between amount of callus formed and number of shoot regenerated.

4. In the *esr1* mutant, callus formation upon wounding seems to be more severely impaired than those formed upon CIM treatment (Fig. 4 and Fig. 8). Authors should clarify this fact because it will highlight the major role of ESR1 in wound-induced callus formation than hormone-induced callus formation.

5. Line #406-409: The present data show that "the expression of PLT genes is not altered in *esr1-2* mutants, suggesting that they do not act downstream of ESR1". Although authors have already discussed the possibility of PLTs being upstream of ESR1, at present one cannot rule out the possibility that PLTs act in parallel or independent of ESR1 to regulate de novo shoot regeneration.

In addition, the *esr1* mutant makes callus on CIM and subsequently shoot on SIM albeit at a low frequency, suggesting that the mutant callus still retains pluripotency. Therefore, the expression of PLTs need not change significantly in *esr1* mutant callus as PLTs are essential to establish the callus pluripotency (Kareem et al., 2015). The authors may discuss these points.

Reviewer #3 (Comments for the Author):

The manuscript by Iwase et al follows on from their very interesting work published in 2011 where they identified WIND1 as an important factor in wounding and tissue reprogramming. Here, they identify ESR1 as a direct downstream target of WIND1 and show that ESR1 is upregulated by wounding. Furthermore, ESR1 promotes callus formation at the wound site and is required for shoot regeneration. Overall, the text is clear and well written. The experiments are thorough and presented well, and there is little that I can criticize about them. My main concern is the novelty underlying this study. Several important messages in this paper are already published mainly the well-established fact that ESR1 is important for shoot regeneration. In addition, it is already known that ESR1 is upregulated by 35S::WIND1 - Iwase et al., 2011; overexpressing ESR1 promotes shoot regeneration - Banno et al., 2001; and ESR1 upregulates shoot meristem genes such as CUC1 - Matsuo et al., 2009. As such, several important messages in this manuscript have already been published. The main novel finding, that WIND1 directly targets ESR1, is interesting, but might only appeal to a subset of the field and I would have expected additional novel findings in a long (11 figure) manuscript.

Some ideas to improve the manuscript or for future work:

- Eleven figures could be reduced to 5 or 6, concentrating on the most novel findings
- What proportion of the 35S::WIND1 phenotype is due to ESR1 overexpression? I.e., does the *esr1* mutant block the 35S::WIND1 overexpression phenotype?
- Are these findings more broadly relevant for ESR1 function? Does ESR1-SRDX have morphological defects?
- Can the effects of auxin vs wounding on ESR1 expression be dissected apart? For instance, by mutating the ARF5 binding sites vs mutating the WRAF sites? How does this affect morphology vs how does it affect wound response?
- WIND1 promotes cell dedifferentiation via the cytokinin pathway (Iwase et al 2011). Does ESR also? Can *esr* mutants block the TCS or ARR5 response? What is the relationship between WIND1, ESR1 and cytokinin?
- Is the WRAF motif common? Does WIND1 bind other WRAF motifs in other gene promoters? Does WIND1 transcriptionally regulate other WRAF-containing genes?

TPC2016-00623-RAR1 1st Revision received

Nov. 6, 2016

Reviewer comments and **author responses**:

Reviewer #1:

Point 1. Line #269-273: It is a nice result that XVE:ESR1 plants regenerate shoots only upon wounding and when they are grown without wound stress, they develop callus. However, I was wondering whether the callus formed in XVE:ESR1 without wounding retained the potential to make shoot on SIM.

RESPONSE: This is an interesting question and we now added new data showing that XVE:ESR1 callus regenerates shoots on SIM (Supplemental Figure 5A). We added the following sentence in the Results section to describe these data: "Nevertheless, these XVE:ESR1-derived calli are capable of regenerating shoots when transferred to SIM, indicating that they retain the potential to develop shoots (Supplemental Figure 5A)."

Point 2. Line #276-284: To maintain the flow of the story, the data discussing de novo root regeneration at wound sites can be moved to the end of results section.

RESPONSE: We agree with the reviewer on this and moved our discussion on these data to the end of the Results section. Following the suggestion from Reviewer 3, we now show this figure as Supplemental Figure 6.

Point 3. Line #290-291: Authors should ascertain whether the reduction of shoot regeneration in the *esr1* mutant is because of the sheer reduction in the amount of callus formation. This can be done by careful correlation between amount of callus formed and number of shoot regenerated.

RESPONSE: Our new data show that *esr1* mutants (and *ESR1-GFP* plants) develop callus comparable to WT on CIM (Supplemental Figure 5B) but they fail to regenerate shoots. We added the following sentence in the Results section to describe these data: "We should note, in contrast, that *esr1-2*, *ESR1-SRDX* and *ESR1-GFP* root explants develop callus comparable to WT on CIM (Supplemental Figure 5B), suggesting that ESR1 does not play major roles in hormone-induced callus formation *in vitro*."

Point 4. In the *esr1* mutant, callus formation upon wounding seems to be more severely impaired than those formed upon CIM treatment (Fig. 4 and Fig. 8). Authors should clarify this fact because it will highlight the major role of ESR1 in wound-induced callus formation than hormone-induced callus formation.

RESPONSE: As discussed above, our new data confirm this notion. Thus, in addition to the above sentence we added in the Results section, we also added the following sentence in the Discussion section: "Our data show that ESR1 is not essential for hormone-induced callus formation."

Point 5. Line #406-409: The present data show that "the expression of PLT genes is not altered in *esr1-2* mutants, suggesting that they do not act downstream of ESR1". Although authors have already discussed the possibility of PLTs being upstream of ESR1, at present one cannot rule out the possibility that PLTs act in parallel or independent of ESR1 to regulate de novo shoot regeneration.

RESPONSE: We agree with the reviewer on this and modified the respective sentence in the Discussion section as below: "Instead, PLTs may function upstream of, or in parallel to, ESR1 and it will be interesting to investigate these functional relationships in future studies."

Point 6. In addition, the *esr1* mutant makes callus on CIM and subsequently shoot on SIM albeit at a low frequency, suggesting that the mutant callus still retains pluripotency. Therefore, the expression of PLTs need not change significantly in *esr1* mutant callus as PLTs are essential to establish the callus pluripotency (Kareem et al., 2015). The authors may discuss these points.

RESPONSE: Unlike the *esr1-1* allele, which shows relatively mild phenotypes, the *esr1-2* allele, which we used for the expression analysis, produces hardly any shoots on SIM (Figure 6B). Thus, we believe *esr1-2* callus, with normal expression of PLTs, may retain reasonable levels of pluripotency but they cannot progress through the shoot programme without functional ESR1. We added the following sentence in the Discussion section to discuss these points: "Given that the levels of *PLT3*, *PTL5*, *PLT7* expression are comparable between WT and *esr1-2* mutants, *esr1-2* calli likely retain reasonable levels of pluripotency but they cannot progress through the shoot programme without functional ESR1."

Reviewer #3:

Point 1. My main concern is the novelty underlying this study. Several important messages in this paper are already published mainly the well-established fact that ESR1 is important for shoot regeneration. In addition, it is already known that ESR1 is upregulated by 35S::WIND1 - Iwase et al., 2011; overexpressing ESR1 promotes shoot regeneration - Banno et al., 2001; and ESR1 upregulates shoot meristem genes such as CUC1 - Matsuo et al., 2009. As such, several important messages in this manuscript have already been published. The main novel finding, that WIND1 directly targets ESR1, is interesting, but might only appeal to a subset of the field and I would have expected additional novel findings in a long (11 figure) manuscript.

RESPONSE: We thank the reviewer for acknowledging that our work is thoroughly performed and well presented. We want to point out, however, that most studies on ESR1 are based on overexpression (Banno et al., 2000; Matsuo et al., 2009) and whether "ESR1 is important for shoot regeneration" has never been shown before this study. Also, ESR1 is one of 1,163 genes up-regulated more than 5-fold in 35S:WIND1 plants (Iwase et al., 2011); thus, we believe demonstrating WIND1's direct regulation on ESR1 is an important advance, providing the first molecular link between wound stress and shoot regeneration.

Point 2. Eleven figures could be reduced to 5 or 6, concentrating on the most novel findings.

RESPONSE: Following the reviewer's suggestion, we moved 3 figures to Supplemental Data (Supplemental Figure 1, 6, 7). We think the remaining 8 figures are central to the manuscript, demonstrating wound-induced ESR1 expression (Figure 1), WIND1's direct control on ESR1 (Figure 2), the role of WIND1-ESR1 in callus formation (Figure 3, 4), and the role of WIND1-ESR1 in shoot regeneration (Figure 5-8).

Point 3. What proportion of the 35S::WIND1 phenotype is due to ESR1 overexpression? I.e., does the *esr1* mutant block the 35S::WIND1 overexpression phenotype?

RESPONSE: Our new data show that the *esr1-2* mutation partly blocks callus phenotypes in 35S:WIND1 (Figure 4C). Although testing the contribution of downstream genes in an overexpression system may not be most informative to assess their physiological functions *in vivo*, these data do support our view that ESR1 is one of the important targets acting downstream of WIND1. We added the following sentences in the Results section to describe these data. "In addition, we examined whether the *esr1-2* mutation blocks callus formation induced by WIND1 overexpression. As shown in Figure 4C, 35S:WIND1 T1 plants display weak, intermediate and strong callus formation which we previously classified as type I, type II and type-III, respectively (Iwase et al., 2011a). As expected, WIND1 overexpression in *esr1-2* mutants causes milder callus phenotypes, producing ~10% of WT-like T1 plants without any visible callus formation (Figures 4C)."

Point 4. Are these findings more broadly relevant for ESR1 function? Does ESR1-SRDX have morphological defects?

RESPONSE: ESR1 is implicated in the control of embryonic development based on cotyledon formation defects in *esr1* mutants (Chandler et al., 2007). We confirmed these defects in ESR1-SRDX plants but unlike many other SRDX plants that display much stronger phenotypes than knockout mutants, ESR1-SRDX plants do not show other major developmental phenotypes. We added the following sentence in the Results section to describe these results. "We confirmed the cotyledon formation defects, previously described for *esr1-2* mutants (Chandler et al., 2007), in the ESR1-SRDX plants. As expected, we also found clear defects in callus formation from leaf explants, demonstrating the requirement of ESR1 for wound-induced callus formation (Figures 3A and 3B)."

Point 5. Can the effects of auxin vs wounding on ESR1 expression be dissected apart? For instance, by mutating the ARF5 binding sites vs mutating the WRAF sites? How does this affect morphology vs how does it affect wound response?

RESPONSE: To dissect auxin from wounding, we tested the effect of an auxin transport inhibitor NPA on ESR1 activation at wound sites. As we show in Supplemental Figure 2E, NPA does not block ESR1 expression at wound sites, suggesting that local auxin transport does not contribute to the ESR1 activation at wound sites. We agree that testing the impact of ESR1 promoter mutations *in planta* is interesting, but we believe this is beyond the scope of this manuscript, given that we still do not know exactly how exogenous auxin enhances ESR1 expression and generation of the respective transgenic plants would require at least twelve months from now. We added the following sentences in the Results section to describe these results. "Liu et al. (2014) has recently shown that wounding induces auxin accumulation at wound sites of Arabidopsis leaves. To uncouple the effect of wounding from auxin accumulation, we tested whether inhibition of auxin transport by N-1-naphthylphthalamic acid (NPA) interferes with ESR1 activation at wound sites. As shown in Supplemental Figure 2E, 1 μ M NPA does not block ESR1 expression at wound sites, suggesting that local auxin transport does not contribute to ESR1 activation at wound sites."

Point 6. WIND1 promotes cell dedifferentiation via the cytokinin pathway (Iwase et al., 2011). Does ESR also? Can *esr* mutants block the TCS or ARR5 response? What is the relationship between WIND1, ESR1 and cytokinin?

RESPONSE: Our new data show that the *esr1* mutation does not block ARR5 expression after wounding (Figure 4E). Instead, we show that the *arr1 arr12* mutation does block ESR1 induction (Figure 4D), suggesting that ESR1 functions downstream of cytokinin signalling. We added the following sentences in the Results section to describe these data: "We have previously reported that WIND1 promotes callus formation via the B-type ARABIDOPSIS RESPONSE REGULATOR (ARR)-mediated cytokinin signalling pathway (Iwase et al., 2011a). To test the functional relationship between ESR1 and cytokinin signalling, we tested the ESR1 expression in *arr1 arr12* double mutants defective in B-type ARR signalling (Mason et al., 2005). Interestingly, our RT-qPCR analysis revealed that wound-induced activation of ESR1 gene is compromised in *arr1 arr12* while the *esr1-2* mutation does not interfere with the expression of a cytokinin-responsive ARR5 gene (Argyros et al., 2008) (Figures 4D and 4E). Together, these results suggest that ESR1 functions downstream of B-type ARR-mediated cytokinin signalling."

Point 7. Is the WRAF motif common? Does WIND1 bind other WRAF motifs in other gene promoters? Does WIND1 transcriptionally regulate other WRAF-containing genes?

RESPONSE: The VWRE-like motif is found in ~50% of genes in Arabidopsis but there are only 229 genes, including ESR1, that have two closely located VWRE motifs within the 1-kb promoter. Among them, we found that ~10% of genes are up-regulated more than 2-fold by WIND1 overexpression, suggesting that WIND1 may target them using VWRE motifs. We will investigate their functional relationships in future studies and for this manuscript we added the following sentences in the Discussion section: "In addition to ESR1, we found 228 other genes in Arabidopsis that carry two closely located VWRE-like motifs within the 1-kb promoter, ~10% of which are induced more than 2-fold by WIND1 overexpression (Iwase and Ryman, unpublished results). These

genes are putative targets of WIND1 and future studies should investigate their functional relationships to WIND1."

TPC2016-00623-RAR1 2nd Editorial decision – *accepted with minor revisions*

Dec. 12, 2016

We have received reviews of your manuscript entitled "WIND1 promotes shoot regeneration through transcriptional activation of ESR1 in Arabidopsis." 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. Please address the additional minor points raised by the reviewers.

TPC2016-00623-RAR2 2nd Revision received

Dec. 13, 2016

Reviewer comments and **author responses**:

Reviewer #1:

Point 1. The authors have addressed my comments adequately. I will be happy to read this manuscript in the Plant Cell. However, I would suggest the authors be careful in their interpretation of *esr1-2* or ESR1-SRDX mutant phenotypes with respect to shoot regeneration. One can clearly see greening of regenerative mass and green foci in the mutant callus when treated with high cytokinin (Fig. 6), therefore, it is very likely that the mutant has already regenerated shoot promeristems or shoot primordia. Of course, it is severely impaired in shoot outgrowth and one can not see regenerated shoot outgrowth. The authors should be cautious when they describe the phenotype; it will be helpful for readers. They can consider describing it in the results or discussion section.

RESPONSE: We have added the following sentence in the Discussion to address this point: "Since *esr1* mutant calli turn green and develop some green foci (Figure 6), they might be able to develop shoot promeristems and/or shoot primordia although they are severely impaired in shoot outgrowth."

Reviewer #3:

The revised manuscript by Iwase et al. adds additional data and clarifies a couple points. The manuscript is certainly improved, and the reduction of figures also helps. The majority of my points have been addressed with data; thanks for this.

Point 1. ESR1 is well-known as being important for shoot regeneration (hence the name of the gene) so I'm a bit puzzled as to why the authors are arguing this point (Review 3 response 1). Key evidence includes *esr1* mutants reducing shoot regeneration rates (Matsuo et al 2011), over expressing ESR1 enhancing shoot regeneration (Banno et al., 2001), and ESR1-SRDX inhibiting shoot regeneration (Matsuo et al 2008). This manuscript uses ESR1-SRDX "confirming the requirement of ESR1 in in vitro shoot regeneration" (line 310-311) and it's unclear to me why similar results in previously published papers don't count. Perhaps the authors are unaware of this paper? It would be appropriate to cite and acknowledge the previously published ESR1-SRDX results in this manuscript: Matsuo N, Banno H. 2008. The Arabidopsis transcription factor ESR1 induces in vitro shoot regeneration through transcriptional activation. Plant Physiol Biochem. 2008 Dec;46(12):1045-50.

RESPONSE: We have cited this reference and discussed the relevance of our findings over previously described results. We have added the following sentences in the Discussion: "Matsuo and Banno (2008) reported that overexpression of ESR1-SRDX chimeric proteins blocks shoot regeneration, suggesting that ESR1, potentially together with other redundant transcriptional regulators, promotes shoot regeneration. Our observation further highlights the functional importance of ESR1 since loss of ESR1 in *esr1* mutants or ESR1-SRDX expression by its own promoter is sufficient to cause severe regeneration defects (Figure 6)".

TPC2016-00623-RAR2 3rd Editorial decision – *accept*

Dec. 15, 2016

We are pleased to inform you that your paper entitled "WIND1 promotes shoot regeneration through transcriptional activation of ESR1 in Arabidopsis" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor

Dec. 22, 2016

