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Peer Review Report for 10.1105/tpc.16.00027

Vascular Cell Induction Culture System Using Arabidopsis Leaves (VISUAL) Reveals the Sequential Differentiation of Sieve Element-like Cells

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	1st Decision:	Feb. 8, 2016 *revision requested*
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	2nd Decision:	Apr. 26 *accept with minor revision*
TPC2016-00027-RAR2	2nd Revision received:	May 2, 2016
	3rd Decision:	May 10, 2016 *acceptance pending, sent to science editor*
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REPORT:

(Note: The report shows the major requests for revision and author responses to major reviewer comments. Miscellaneous correspondence and confidential comments for the editors 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.)

TPC2016-00027-RA 1st Editorial decision – *revision requested* Feb. 8, 2016

Please put primary focus on additional genetic evidence of NAC020 negatively regulating APL in addition to more detail regarding marks of sieve element and companion cell differentiation.

Please note that the journal does require, as requested by Reviewer 2, 1) no additional nomenclatures (new gene names) should be created for genes that are already named in the literature, and 2) complete datasets that support the main figures (i.e. Figures 5A, 5C, 6A) should be added as supplemental files. In general, data and methods that are integral to the main conclusions of the article must be presented in the main manuscript; supplemental data (figures, tables, data sets) should provide direct support for a main figure or table in the manuscript. Please see the Instructions for Authors for detailed information on supplemental data and preparation of figures.

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

[Reviewer comments shown below along with author responses]

TPC2016-00027-RAR1 1st Revision received Apr. 7, 2016

Reviewer comments and author responses:

Reviewer #1 (Comments for author):

Kondo et al. describe an important advance in their manuscript: the establishment and characterization of a culture system for the induced transdifferentiation of mesophyll into phloem sieve elements. Building on their previous work of inducible xylem vessel formation in the same set up, the authors show that their culture system not only produces xylem vessels, but also sieve elements, and they go at length to demonstrate that these sieve elements are genuine (although not perfect; e.g. there appears to be no sieve plate formation). The also characterize the temporal progression of gene expression in the system, which allows them to come up with co-expression modules that work at particular time points during the transdifferentiation process. Finally, they prove that their method allows the identification of novel players in phloem differentiation by characterizing one particular gene, which clearly has an impact on sieve element differentiation in planta.

Overall, this is an excellent paper, it is well written, the experiments are performed meticulously, and the data support the conclusions. I have only a few suggestions that the authors should accommodate in a revised version:

Point 1. The authors make the point that sieve element formation requires cell division, which is nicely demonstrated. They also show that sieve element differentiation does not occur in the absence of bikini. I wonder how these two things are related? Their culture system also contains auxin and cytokinin, is the main role of one of those hormones to induce divisions so that sieve elements can reformed? in other words, if auxin or cytokinin levels are reduced, is there a shift in the relative abundance of xylem vessels versus sieve elements? I believe this could be tested quickly and would add some more insight.

> RESPONSE: Thank you very much for your valuable suggestion. We tested the effects of auxin and cytokinin on phloem SE differentiation after inducing procambial cells. Treatment with auxin or cytokinin was less effective to the ratio of xylem vessels and SEs than aphidicolin application (Figure S3). This data was added into the Results section.

Point 2. I believe it is not helpful to add to the nomenclature confusion by renaming factors that have already been published previously. So please refrain from renaming genes PNDX if they have already been described before.

> RESPONSE: Thank you very much for pointing [this] out. We corrected names of all PND family genes into NACXX (such as NAC020).

Reviewer #2 (Comments for author):

In the manuscript entitled "Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL) visualizes the sequential phloem differentiation process" by Kondo and co-workers, the authors clearly describe that the previously published in vitro xylem differentiation culture system can also be used to follow phloem cell differentiation. Moreover, they convincingly show that this in vitro system allows following the developmental process of SE differentiation in detail and enables them to extracting novel information from a combinatorial transcriptomics experiment. Overall, I find this a very clearly written manuscript containing a logical flow of the correct experiments with good figures. Nevertheless, the statements at the end of the manuscript need further experimental support (see comments below). Despite the shortcomings described below, this manuscript can be an important advance to the developmental biology field in general and vascular development in specific.

Point 1. As mentioned above, the main comments relate to the statements made towards the end of the manuscript. I fully agree that the subclade of 5 NAC genes represent important factors controlling phloem differentiation based on published work and work in this manuscript. I also agree that NAC020 appears to be induced before the other NACs

in VISUAL and in the root based on expression of NAC045 and NAC020. Although the fact that overexpression of NAC020 leads to apl related phenotypes and is able to repress some of the pAPL-GUS expression, there is no clear result from the Q-PCR in Figure 7A (see minor comment 3). Moreover, there is no loss-of-function data, or any other more direct molecular indication of NAC020 repressing APL. I would have liked to see some additional experiment to strengthen their claim. For example, the authors could use the inducible NAC020 overexpression to see loss of APL transcript levels in Arabidopsis. Related to this, a Q-PCR experiment using the inducible line in combination with CHX could indicate direct repression. Alternatively, the authors could try to show direct interaction by e.g. ChIP-PCR. Most importantly, loss-of-function phenotypes would be very helpful to make a strong point here. At the moment, there is no mention of nac020 mutants (or combinatorial mutants with the closest homolog) in the manuscript. Another way to strengthen their point would be to e.g. try and (partially) complement the apl mutant with NAC020 or NAC045/NAC086 (inducible) overexpression lines. Alternatively, the authors could try to revert the NAC020 overexpression phenotype by overexpressing APL as well. I am not suggesting the authors should perform all these experiments, but at the moment I find their claim very strong based on the experimental evidence present in the manuscript (NAC020 overexpression).

RESPONSE: Thank you very much for your valuable suggestion. Further to examine the relationship between NAC020 and APL, time-course qRT-PCR analysis was performed after estradiol-induction. Although NAC020 expression was promptly up-regulated within 3 or 6 hours, decreases in APL expression was detected at 24 hours after induction (Figure 7B), suggesting that NAC020 may repress APL expression indirectly. Based on this result, we weakened our conclusion.

We also intended to examine the phenotype of its loss-of-function mutant. However, no mutant for NAC020 was available from the stock center. Then we analyzed NAC020-SRDX (NAC020 fused with the chimeric repressor domain) transgenic lines in VISUAL, in collaboration with Drs. Mitsuda and Takagi. Interestingly, NAC020-SRDX suppressed the expression of phloem-related genes including APL but did not affect the xylem-related gene IRX3 in VISUAL (Figures 7B and 7C). This result supports our idea that NAC020 is involved in controlling APL expression. However, both inducible-NAC020ox and NAC020-SRDX down-regulated APL expression (Figures 8A and 8B), suggesting the possibility that NAC020 acts as a transcriptional repressor. These results and notions were also added to the Results and Discussion sections.

Point 2. A second major remark is that, in its current form, it is very unclear which genes were found in what dataset. I strongly urge to include additional tables in the supplemental data that include ALL the gene lists for each experiment (VP, VX, apl, SEOR, etc.); for the overlaps described in Figures 5A, 5C and for the co-expression networks in Figure 6A. At the moment, it is impossible to evaluate how the selections of gene list were done or to judge if the selection criteria were actually followed. Moreover, even if the raw data becomes available upon publication, it would make it very difficult for the reader to see how a selection was done, without doing the entire analysis again. In this case, much more information would have to be given in order to reach the exact same datasets. It seems wise to avoid this confusion and simply present the reader with all information. Even more so, one of the key strengths of this work is the creation of a repository for the research community of the transcriptional changes that occur during phloem differentiation. Having clear and complete tables is absolutely crucial in this respect.

RESPONSE: Thank you for pointing [this] out. Genes identified in this study were listed in Supplementary Tables for readers (Supplementary Table1 and Table 2).

Point 3. Given the fact that NAC020 (PND1) is suggested to be an early phloem differentiation gene, while NAC028 (PND2) in the same subclade in the phylogenetic tree in Figure 6C is a late differentiation gene, it would be important to show the expression patterns for all 5 NAC genes. One would expect that NAC020 and NAC028 share the expression in the young root meristem based on phylogeny; although the expression in VISUAL would suggest NAC028 to be a later gene. Making a simple promoter-GUS/GFP line could clarify this matter.

RESPONSE: Thank you very much for your valuable comments. According to your suggestion, we made promoter-GFP T1 lines for NAC028 and NAC057 and compared their expression patterns. Indeed, NAC028 and NAC057 displayed slightly later expression patterns than NAC020 (Figure S11), which is consistent with our co-expression network analysis. This result was added to the Result section.

Reviewer #3 (Comments for author):

In this manuscript, the authors present the VISUAL system as a tool to ectopically induce vascular tissues in cotyledons. This tissue culture system has been already presented in 2015, where the authors induced differentiation of tracheary elements (TEs) in leaf disks. In this manuscript, on the other hand, it has been newly shown that this method can also induce phloem sieve elements (SEs)-a mixture of TEs and SEs. Multiple molecular evidences are shown to support that this method reliably recapitulates what happens in the Arabidopsis root in-vivo. On top of that, the authors propose a new molecular component NAC020 which down-regulates the expression of APL, a master regulator of the phloem development, when it is overexpressed. VISUAL is potentially very useful tool capable of overcoming limitations when working with Arabidopsis seedlings, and provide a very nice temporal resolution of early and late events during the phloem development. However, at this stage, I have concerns and questions on matters described below.

Point 1. Although APL is considered as a master regulator of the phloem development, it has been speculated (as the authors wrote in the discussion) that there are many aspects that APL is not involved. In the PND1 inducible overexpression study, the authors grew plants 7 days in the induction media. This experiment may not strongly support the idea of APL down regulation by PND1, because there is a possibility that PND1 regulates other phloem-related genes besides APL. This might cause abnormal SEs differentiation, which leads to the reduction of APL expression. I strongly encourage the authors to analyze further this interesting interaction. For example:

1) Short time induction (30 min or 1 hour), or time-course induction of inducible overexpressors.

2) Check APL expression in pnd1 loss-of-function mutant background.

3) It would be nice to describe pnd1 loss-of-function mutant phenotype.

4) Downstream regulators of PND1 (transcriptome analysis of PND1 inducible line or mutants, or ChIP-seq experiment is recommended)

> RESPONSE: Thank you very much for your valuable suggestion. Further to examine the relationship between NAC020 and APL, we performed time-course induction using XVE::NAC020 seedlings. Because turnover of GUS proteins is not so quick, we measured APL expression levels by qRT-PCR analysis after estradiol-induction. Although NAC020 expression was promptly up-regulated within 3 or 6 hours, decreases in APL expression was detected at 24 hours after induction (Figure 8B). suggesting that NAC020 may repress APL expression indirectly. Based on this result, we weakened our conclusion.
>
> We also intended to examine the phenotype of its loss-of-function mutant. However, no mutant for NAC020 was available from stock center. Then we analyzed NAC020-SRDX (NAC020 fused with the chimeric repressor domain) transgenic lines in VISUAL, in collaboration with Drs Mitsuda and Takagi. Interestingly, NAC020-SRDX suppressed the expression of phloem-related genes including APL but did not affect the xylem-related gene IRX3 in VISUAL (Figures 7B and 7C). This result supports our idea that NAC020 is involved in controlling APL expression. However, both inducible-NAC020ox and NAC020-SRDX down-regulated APL expression (Figures 8A and 8B), suggesting the possibility that NAC020 acts as a transcriptional repressor. These results and notions were also added to the Results and Discussion sections.

Point 2. It would be highly informative if the authors could present more validation data. For instance, how BAM3, OPS, BRX, and/or CVP2 are expressed in VISUAL system? Are they induced at the time point speculated in their transcriptome data?

> RESPONSE: Thank you very much for pointing [this] out. We summarized characteristics of each Module genes (Module-I to -IV) (Figure S10) and then compared expression profiles of BAM3, OPS, and CVP2 (Figure S10). CVP2 showed very similar expression profiles to Module-I genes (Figure S10). In addition, OPS displayed slightly earlier expression pattern than Module-I genes (Figure S10). These comparison suggest that Module-I genes show similar characteristics to well-known early phloem regulators such as CVP2 and/or OPS, validating our categorization of early phloem genes. This notion was added into the Results and Discussion sections.

Point 3. Sieve plate formation is one of the most critical steps of the SEs differentiation. Besides, this process is the most unique feature that phloem possesses over other cell types. It appears that sieve plates would not form in

VISUAL, but I wonder how much in detail this has been analyzed. Can the authors observe sieve pore like structure with the accumulation of callose at the junction of cells in SE clusters? I strongly recommend that the authors provide high magnification images of the callose staining experiment.

RESPONSE: Thank you for your comments. We observed callose deposition after aniline blue staining with confocal microscope in detail. We could observe dot-patterned signals but not sieve plate-localized signals in SE clusters (Figure S5). However, it is difficult to prove that there is no phloem sieve pores in the induced SEs. In plants, SEs are connected to each other and produce sieve plates in between. By contrast, in VISUAL, most SEs are formed without polar orientation. Therefore, the formation of complete sieve plates with sieve pores may not occur, and instead, abnormal callose deposition may occur in VISUAL SE-like cells. Based on these, we changed the phrase into “sieve plate-like structures were not detected as far as we observed.”.

Point 4. Along these lines, cell wall thickening, in addition to callose deposition and enucleation, is one of the remarks of SEs differentiation. However, their chemical properties and mechanisms in which they are formed are less studied. Can the authors in their TEM images tell whether SEs undergo cell wall thickening in their culture system? If so, is there a group of genes related to the cell wall thickening up- or down-regulated in their time course transcriptome data?

RESPONSE: Thank you very much for your valuable comment. TEM images indicated that some SEs actually develop thick primary cell wall. However, because of the variety of wall thickness, we could not strongly conclude that SEs induced in VISUAL undergo obvious cell wall thickening. On the other hand, we found that our phloem-related genes lists (especially late phloem genes) include several cell wall remodeling factors such as XTH10 (encoding xyloglucan endotransglucosylase/hydrolase). Further studies are required to understand cell wall thickening during SE differentiation in VISUAL.

Point 5. The authors draw a conclusion that companion cell (CC) differentiation is not induced in VISUAL system, mainly based on the fact that the expression of three genes-SUC2, SULTR1;3, and AHA3 is not dramatically induced in the system. However, in figure 4A and H, several of genes belonging to the category of CC specific genes (Brady's data set SUC2 sorting) are appearing >4 fold increased in the heat map (marked red in SUC2 column). Please explain why they chose only three among how many? Are those three genes among top candidates that are highly up-regulated by bikinin treatment? In addition, regarding the conclusion about cell division, not being required for CC differentiation, APL is not only expressed in the SEs but also in the CCs. The fact that aphidicolin inhibits the expression of APL makes me to think that there is an inhibitory mechanism in which CCs as well as SEs differentiation is compromised. Can the authors address this question?

RESPONSE: Thank you for your comments. To comprehensively analyze the expression patterns of CC-related genes, we newly extracted CC-related genes based on cell-type specific microarray data with SUC2 marker (in the same way as S4 or S32 marker). These CC-related genes were far less overlapped with up-regulated genes in VISUAL than xylem or SE-related genes (Figure 5A). This result clearly indicates that CC differentiation is not completed in VISUAL. As you point out, our discussion of companion cells is not correct. Therefore, we rewrote the usefulness of further investigation on cell division in the Discussion section.

Point 6. Can the authors explain how often they can see TE and SE in one cell cluster? Is it rare or very common? Providing a quantitative data is highly recommended. Additionally, it is intriguing that the division process is required only for the phloem SE differentiation. When aphidicolin is treated in the procambum-induced cotyledons, what is interesting is that there is no ectopic or additional xylem TEs formed in the position of phloem SE clusters. Is there any positional preference in the formation of SEs and TEs? For instance, it appears that TEs tend to be induced close to the vein and in more aerial part of the leaves in case of the phloem SEs (Fig 1K). Can the authors explain this?

RESPONSE: Thank you very much for your comments. We newly observed total 1,000 cell clusters visualized with SEOR1-YFP. About 16% of cell clusters consisted of TEs and SEs, whereas others did not include TEs (Figures S6B-S6F). This result indicates that TE-SE mixed clusters are not majority in VISUAL.

Aphidicolin treatment down-regulated SE-related genes but not TE-related genes (Figure. 2Q), suggesting that aphidicolin does not promote ectopic xylem differentiation. However, we could not exclude the possibility that future SE clusters can change the cell fate into TEs. But aphidicolin itself suppresses the formation of SE clusters

by inhibiting cell division. Therefore, further investigation with early SE markers using time-lapse imaging techniques is necessary for answering the question. We would like to clarify this point in the future.

As for positional preference of TEs and SEs in cotyledons, our observation revealed that TEs and SEs differentiation occurs in a random-manner. Therefore, we think that detailed analysis on spatial relationship between SE and TE differentiation is a next issue in order to understand multipotency of vascular stem cells. This notion was added into the Discussion section.

Point 7. Bonke et al (2003) originally reported that APL also represses xylem formation and found TEs in SE positions in the apl mutant. Is there ectopic xylem formed in the position of the phloem when PND1 is overexpressed in the Arabidopsis root?

RESPONSE: Thank you very much for your valuable comment. We made cross-sections for NAC020ox and NAC020-SRDX transgenic plants. However, we did not see any ectopic xylem cells at phloem position (Figure S12), probably due to the partial suppression of APL expression by NAC020. This result was added to the Results section.

Point 8. The authors nicely categorized genes that are involved in various stages of the phloem development. In particular, genes in Cluster III might be a key to explain why cell division is so important for SEs differentiation. It would be worth describing this aspect in the discussion.

RESPONSE: Thank you very much for your suggestion. Despite the importance of cell division in SE differentiation, we do not know why cell division is required. We found that early phloem members include a cell-cycle-related gene CDC2C. Genetic approaches focusing on CDC2C might reveal the importance of cell division in SE differentiation. This notion was added into the Discussion section.

TPC2016-00027-RAR1 2nd Editorial decision – *request revision* Apr. 26, 2016

We ask you to pay attention to the following points in preparing your revision - particularly the comments of Reviewers 2 and 3).

TPC2016-00027-RAR2 2nd Revision received May 2, 2016

Reviewer comments and author responses:

Reviewer #1 (Comments for author):

From my point of view the authors have adequately addressed my concerns and have improved the paper with new data that further confirm their major conclusions.

RESPONSE: Thank you very much for reviewing our manuscript.

Reviewer #2 (Comments for author):

Although the authors have addressed most of my concerns, the manuscript still lacks a good explanation in the materials and methods or supplemental files of how all the different gene sets were selected. Please indicate how this was done and what exact parameters were used to do so. Only this way it will be possible for others to repeat the selection. This is important as the manuscript provides an excellent resource to the community.

RESPONSE: According to your suggestion, we added the methods how gene sets were selected (Table S1).

Reviewer #3 (Comments for author):

At this stage there are two points:

Point 1. I would encourage the authors to completely remove the newly emerged NAC020-SRDX aspect. This element introduces several problems: (1) We don't know how the SRDX changes the activity of the transcription factor, (2) we don't know the number of target genes. It is possible (perhaps even likely) that the effect of the SRDX is not specific for NAC020. (3) The authors observe downregulated APL expression in this line, similarly as they see

downregulation in the overexpression line. This is counterintuitive. I think the story would be better without this element.

RESPONSE: Thank you for your advice. We discussed this comment with the editor. According to the suggestion from the editor, we decided to keep the results of NAC020-SRDX, but thoroughly weakened the description based on the results obtained with NAC020-SRDX in the text.

Point 2. As a minor point, the authors cannot conclude there is cell wall thickening or true sieve plates in the SE-like cells and should mention these aspects clearly in the text. I encourage them to use the term "sieve element like cells" throughout the text.

RESPONSE: According to your suggestion, we added the information about cell wall thickening into the Results section. Moreover, considering these aspects, we changed SEs into “SE-like cells”.

TPC2016-00000-RAR1 3rd Editorial decision – *acceptance pending*	May 17, 2016

We are pleased to inform you that your paper entitled "Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL) visualizes the sequential differentiation of sieve element-like cells" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor	May 18, 2016