SHORTROOT-Mediated Intercellular Signals Coordinate Phloem Development in Arabidopsis Roots

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Plant Cell. Advance Publication February 28, 2020; doi: 10.1105/tpc.19.00455

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Review timeline:

TPC2019-00455-RA	Submission received:	June 14, 2019
	1st Decision:	July 25, 2019 *revision requested*
TPC2019-00455-RAR1	1st Revision received:	September 19, 2019
	2nd Decision:	January 24, 2020 *accept with minor revision*
TPC2019-00455-RAR2	2nd Revision received:	February 5, 2020
	3rd Decision:	February 7, 2020 *acceptance pending, sent to science editor*
	Final acceptance:	February 25, 2020
	Advance publication:	February 28, 2020

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.)

TPC2019-00455-RA 1st Editorial decision – *revision requested* **July 25, 2019**

We have received reviews of your manuscript entitled "SHORTROOT mediated intercellular signals that coordinate the phloem development in Arabidopsis roots." Thank you for submitting your best work to The Plant Cell. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

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

The overall opinion of the reviewers is that the manuscript reports interesting new findings appropriate for the journal, but that some revisions will be needed before it can be published. It is likely that a revised version will be sent to some of the same reviewers, so it will be important to address their concerns carefully.

The most important criticisms are as follows:

i) All three reviewers noted that the developmental phenotypes need to be quantified rather than just showing a single image.

ii) Some additional genetic experiments would be of interest, such as *shr nars1* double mutant, examination of additional nars1 alleles, and testing rescue of *shr* mutant phloem phenotypes by transgenes expressing NARS1 in specific domains.

iii) The model that NARS1 moves from more differentiated cells to the developing stele was deemed not to be sufficiently well-supported. Reviewers 1 and 3 suggested ways to try to document the NARS1 expression pattern better, or to detect NARS1 protein in cells to which it may have moved. (Perhaps fusing a bright fluorescent protein to the other end would give stronger signal.) Reviewers 2 and 3 also noted that you have not tested whether SHR binds to the NARS1 promoter.

iv) All three reviewers also suggested to provide a deeper discussion of alternative mechanisms. This will be especially important if further work along the lines suggested still leaves room for alternative interpretations other than the model that NARS1 moves from older to younger cells. In that case, you may need to discuss such alternatives more thoroughly, without making the strong claim that NARS1 is a "top-down" mobile regulator.

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-Please see the attached file for comments on the figures, which you can correct in the revision. You can download the attachment, open in acrobat and view comments. please apply the comments to all figures in the paper.

The reviewers also noted various other specific points that you should address in revisions.

Please note the following:

-The Plant Cell now requires authors to complete and submit an author revisions checklist upon submission of a revised manuscript. The aim of the checklist is to aid authors in preparing a high quality manuscript, facilitate the review and assessment of revised manuscripts, and help to ensure that journal standards are maintained across the board. If your manuscript is accepted, the completed checklist will be published as supplemental material attached to the article online. Please download a copy of the checklist (pdf fillable form) at this link, for submission with your revised manuscript: https://tpc.msubmit.net/html/Author_Revisions_Checklist.pdf.

-Supplemental materials should be restricted to large datasets and tables, presentation of replicates, and validation of reagents, methods, or genotypes. Any data that are used to support the major claims must be in the main manuscript. Supplemental figure legends must indicate what figure in the main manuscript is supported by the supplemental data presented. Please justify how each of the supplemental figures meet the criteria.

-Sampling methods and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc.), along with a clear description of and rationale for any statistical analyses conducted. The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

Given the nature of the comments, we are offering you 60 days to complete the revision. If a revision is not returned within this time frame, and if you have not been granted an extension, we will withdraw the manuscript, which will leave you free to submit the work elsewhere. If you need an extension, we encourage you to contact us at any point before submitting your revision.

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

TPC2019-00455-RAR1 1st Revision received **September 19, 2019**

Reviewer comments and **author responses**:

Reviewer #1:

The submitted manuscript by Zhou et al. describes the functions of the mobile transcription factor SHR in coordinating two asymmetric cell division (ACD) processes that are required for the generation of companion cells (CC) and sieve element (SE) in Arabidopsis phloem development. The study deploys a suite of genetic, cell and molecular biology tools to demonstrate a new functional scheme of SHR that acts as a positional signal, by turning on a distant NAC domain transcription factor, to drive the ACD of the phloem precursors. The experimental design and data presentation in the story were found solid and the context of the manuscript was well written and explained. Thus, the research topic of the manuscript should be appealing to the Plant Cell audience and the quality of the research shown here also appeared to be a good fit for potentially publishing at the Plant Cell.

Point 1. The genetic relationship between SHR and NARS1 was not thoroughly characterized. What is the double mutant phenotype in phloem development? How might SHR influence the expression of NARS1 at different regions of the roots, e.g. in distant differentiation zone vs. in local meristematic zone, if droplet digital RT-PCR might work on FACS sorted cells? Also, is it possible to perform in situ hybridization to detect the NARS1 transcripts in the roots?

RESPONSE: Thank you for your comments. Your comments were considered together with others and then we prioritized the experimental strategies that would address the relationship between NARS1 and SHR. To position NARS1 and SND2 amongst 24 phloem-enriched TFs that are under control of SHR, we performed time-course induction experiments of pSHR::SHR:GR using droplet digital RT-PCR. The data indicated that NARS1 expression is upregulated shortly after SHR induction (Fig. 4C). This result prompted us to re-establish and perform ChIP. ChIP data indicated that SHR binds to NARS1 promoter (Fig. 5A). Furthermore, when NARS1 was ectopically expressed in the stele of the *shr* mutant, phloem sieve element number increased (Fig. 8L,M). We think these data strongly support the direct relationship between SHR and NARS1. Even though we did not perform the specific experiments you suggested, we hope these alternative experimental data sufficiently addressed your questions.

Point 2. With regards to mutant phenotype characterization throughout the manuscript, the authors did not seem to pay sufficient effort to provide quantification data and statistical analysis to support their description.

RESPONSE: We modified the SE-ENOD immunolocalization to perform this experiment in a large scale. In Supplemental Figure 2, we present the quantitative analysis result scoring the phloem SE development in the major genotypes that were mentioned in the manuscript. In this analysis, we always found two phloem sieve elements in the wild type, while we found varied degrees of reduction in phloem sieve elements in the *shr*.

Point 3. The Discussion part appeared to be pretty light. Instead of the re-emphasized significance of the new findings, the audience will anticipate more insights into in-depth discussion of how SHR might distantly regulate the expression of NARS1.

RESPONSE: In the revised manuscript, we discussed the SHR-mediated molecular mechanisms of phloem development in depth, connecting with what we have found and what has been reported in the field.

Reviewer #2:

In general, this is a clear and well-written paper which addresses the question how the asymmetric cell divisions that lead to the formation of sieve elements (SEs) and companion cells (CCs) are controlled. After revision, it will be an important contribution to the field.

Point 1. While the quality of the figures presented is very good, my biggest concern is the lack of quantifications in this paper. Only in the last parts of the result sections the authors actually provide some numbers for some of their experiments. But even there they do not include wild type controls to compare these numbers with. It is absolutely necessary to state the number of samples analysed and the frequency of phenotypes observed compared to the appropriate controls. Otherwise, it is not possible to judge the quality of the data shown.

RESPONSE: This is the same response to the second comment of reviewer #1. We modified the SE-ENOD immunolocalization to perform this experiment in a large scale. In Supplemental Figure 2, we present the quantitative analysis result scoring the phloem SE development in the major genotypes that were mentioned in the manuscript. In this analysis, we always found two phloem sieve elements in the wild type while we found varied degrees of reduction in phloem sieve elements in the *shr*.

Point 2. The authors show that CC numbers are reduced in *shr* and can even be increased when PHB is absent or downregulated in the phloem in a *shr* background. First, the increase of CC numbers definitely needs to be compared to the wild type situation (see above). Moreover, I think the claim that SHR regulates CC numbers is a bit too strong. SHR regulates the number of cells between xylem and phloem and thus may well only indirectly regulate CC development. Compared to the regulation of the SE division this is a very small part of the manuscript and I think this part of the results should be discussed in a more conservative way.

RESPONSE: We agree with the reviewer thus limited our observation to the procambial cell division. The title of the result section was also changed to 'SHR-SCR-miR165/6 pathway regulates the procambial cell division'. In Supplemental Figure 2B, we included the scoring results of stele cell numbers in representative genotypes. There, you can find the statistically significant increase in the stele cell number of *shr phb* in comparison to the one of *shr*.

Point 3. I am not convinced with the data suggesting that NARS expression in CCs controls the asymmetric division of the SE precursor cell. Obviously, the NARS protein is expressed at very low levels or highly instable (Expression of NARS fused to a fluorescent protein under control of its native promoter or the SUC2 promoter was not detectable). Thus, from these data it would be just as likely that NARS could be expressed in the sieve tube

precursors at very low levels. On the other hand, expression of NARS under control of the CC specific SUC2 promoter rescues the phenotype. However, it is difficult to interpret these data, since we do not see the fusion protein here, but instead some "punctate fluorescence in the stele". The authors also check regulation of phloem expressed transcription factors in the root tip below the SUC2 expression domain and see a different regulation of expression when compared to the situation in *nars*. However, they also see a partly different regulation when compared to plants expressing NARS under control of the S29 promoter. However, the S29 plants can also restore the *nars* phenotype and thus should look similar from a molecular point of view. Altogether, we are left with some questions marks here, and at least it would be good to discuss these issues and give alternative interpretations.

RESPONSE: We took this comment seriously and tried to visualize the GFP-NARS1 protein using the wholemount immunolocalization. This experiment was successful for visualizing erGFP expressed under *NARS1* promoter. In the case of *pNARS1::GFP:NARS1*, we found punctate signals in the stele. However, they were not distinctive enough from the background signal. Based on these data and the data presented in the manuscript, we think that GFP-NARS1might be unstable and modified, which needs more detailed investigations. Since we could not fully resolve the non-cell autonomous nature of NARS1, we modified our statement in the following way in the line 364, "These data collectively indicated that NARS1, generated in the CCs of differentiation zone, might function as a top-down signal, either mobile factor itself or a trigger of other mobile signal that locally regulates the ACD of SE precursors".

Point 4. L 337–346: I would take out this part altogether. Here we learn that SHR does not bind to the SND2 promoter, but it would be nice to see if it binds to the NARS promoter, especially if the claim is that NARS is a direct target of SHR. In addition, the fact that SND2 expression is downregulated after NARS expression does not strongly support the claim that NARS regulates SND2 somewhat directly.

RESPONSE: Thanks to your comment, we redid ChIP experiments with improved technical setups. In these experiments, we found that SHR consistently binds to NARS1 promoter. We also found the binding of SHR to SND2 promoter in two out of three experiments. Together with newly performed time-course induction experiments of SHR::SHR:GR, we are now confident that SHR directly turns on NARS1 expression. Even though SND2 promoter is bound by SHR, the delayed activation of SND2 by SHR suggests of the presence of other factor(s) required for SND2 induction. Combining these results with other genetic analyses, we propose that SHR, NARS1 and SND2 form a positive feedforward loop with positive feedback regulation between NARS1 and SND2.

Point 5. I am missing a discussion about the observed broadened expression of phloem markers in *nars*.

RESPONSE: S32 is the phloem SE marker that starts its expression broadly in the procambial cell right above the QC and then gets restricted to the phloem precursors and protophloem SE. In *nars1*, the division of phloem SE precursor does not occur but its inner cell divides. We think that the change in the procambial cell division can results in the spread of H2B-GFP in *nars1* to the inner part of phloem pole. Stable H2B-GFP would be diluted as the cells divide. Roots in *nars1* mutant are short, thus the dilution of H2B-GFP is likely slower than the wild type and *snd2* mutant. This might be the reason for higher intensity of H2B-GFP in *nars1* than in wild type and *snd2*.

Reviewer #3:

In their manuscript, Zhou et al. present new observations on the role of SHR in the regulation of primary root development. They investigate the effects of SHR on the periclinal cell divisions in the cell files that give rise to the protophloem and metaphloem, and the companion cells and the sieve elements. They further identify potential downstream effectors of SHR and propose a long-distance signaling mechanism that coordinates these formative divisions. The authors show in a variety of experiments that SHR movement into developing sieve elements and the activation of NARS1 is required for the formative divisions to occur. The part describing SHR requirement for phloem development is very solid. By contrast, regarding the part focusing on NARS1, additional observations and further experiments would be desirable to support the conclusions. Many questions could be answered with the use of the existing material and strengthen the model of mobile NARS1 as a long-distance signal as the only explanation for the observed effects and phenotypes. Moreover, in general, more quantifications of the phenotypes and detailed analyses of cell numbers and root length would make the importance of the described factors more evident. In detail:

The authors begin with a description of the defects in the *shr-2* mutant. They describe the phloem patterning defects and the observed differences in SE and procambium formation. However, the phloem patterning defects were partially already described in the here cited paper by Carlsbecker et al. 2010. The authors go into more detail, but it

should be pointed out that some aspects of these, including the changed expression of SE and CC marker genes, has already been described.

-Point 1. Figure 1 A: The scheme showing the different tissues in the root could be clearer; why are there white "blocks" in the Figure on top of the periclinally dividing cells?

RESPONSE: The root scheme was revised and the white blocks, indicating T division, were explained in the figure caption.

Point 2. The authors suggest a delay in phloem differentiation, based on the observed later onset of pAPL::erGFP expression in the *shr-2* background. To support this claim, the later differentiation could be quantified by counting the numbers of cells in the cell file between the QC and the first cell with erGFP signal. Showing one microscopy image to support a hypothesis without further information on the variability and the numbers of observations is maybe a bit too suggestive.

RESPONSE: Unfortunately, we failed in germinating *pAPL::erGFP; shr-2*, which we generated years ago. Generating a line is on the way. Instead, we corrected the statement in line 135 as follows to avoid misleading: "Expression of *pAPL::erGFP* in the *shr-2* started not in the meristem but in the elongation zone, suggesting a delay in phloem differentiation in comparison to neighboring cell types (Figure 1G)."

Point 3. Often it remains unclear whether the cells that are adopting SE fate in the *shr-2* or other mutant backgrounds are becoming fully differentiated? The expression of the ENODL9 marker suggests so, but the observed maintained marker gene expression does not. One common criterion to determine the differentiation of the protophloem is the observable increased staining of the cell wall in the differentiated PP due to the formation of the secondary cell wall (Truernit et al 2008, Plant Cell). This can be easily done in live cell imaging with the Propidium Iodide staining used by the authors and would help to emphasize the, besides the lacking formative division, normal SE differentiation.

RESPONSE: In Figure 6A–C, we included the images of SE stained with propidium iodide. In the *nars1* and *snd2*, we did not find a potential defect in SE differentiation, indicated by the absence of stained sieve plates.

Point 4. The transversal section of the root expressing pAPL::erGFP suggests, due to the lack of GFP signal in one side of the root, the absence of one of the SE poles. Phenotypes like this have been reported for severe phloem developmental mutations, but the authors do not further discuss if this developmental phenotype of absent phloem strands was observed and in what frequency?

RESPONSE: In the revised manuscript, we scored the status of sieve elements in representative genotypes by performing immunolocalization in a large scale (Supplemental Figure 2A). In our SE phenotyping, we found individuals in various genotypes with only one phloem pole, which we named class 2. We found that about 20% of *shr-2* mutant shows class 2 phenotype.

Point 5. In Figure 2, the authors observe a recovery of reduced stele cell number in the pUAS::MIR165A *shr-2* J0571 lines. A quantification of the numbers of cells in the different cell layers and the stele for this and other discussed mutants (*shr-2*, *scr-4*, the pS32 rescues etc.) would add to our understanding of the effects. Additionally, as a partial rescue of the *shr-2* cell number phenotype is observed in the pUAS::MIR165a and *phb-6* background, how does the root length respond to this? Root length measurements as a general indicator for proper root growth and differentiation could be provided here.

RESPONSE: In the revised manuscript, we scored the stele cell number in the available representative genotypes (Supplemental Figure 2B). The root lengths for *shr-2 phb-6* were previously presented in the paper by Sebastian et al. (2015), which we published in PLoS Genetics.

Point 6. The authors observe a reduced number of procambial cell files in the *scr-4* and pCRE1::PHBem-GFP line, additional to the *shr-2 phb-6* mutant. The introduction of the SUC2::erGFP marker would be of interest to see, if CC formation is affected in these lines. Furthermore, the expression of later described SND2 and NARS1 genes could be investigated in this background, as the sporadic lack of one SE in some of the roots suggests at least a partial involvement of the SHR-SCR-miR165/166 signaling module.

RESPONSE: Thank you for this comment. However, considering the time given for the revision and people involved in this manuscript, I had to prioritize the experiments. Comment #2 given by Reviewer 2 also suggested to weaken

the statement for the CC regulation by SHR-miR165/6-HDZIPIII pathway. Thus, we revised the Result section titled 'SHR-SCR-miR165/6 pathway regulates the procambial cell division', which is more focused on the procambial cell division partly including the division for CC formation (see the paragraph from line 159 to 171).

The influence of SHR-SCR-miR165/166 signaling module on the SE, however, was further investigated as you suggested. We measured the expression of *NARS1* and *SND2* in *scr-4* and *pCRE1::PHBem-GFP* using realtime RT-PCR. As you predicted, expression of NARS1 and SND2 was decreased in those lines. Thank you for this comment. This result was described in lines 409-415.

Point 7. The naming convention for the microRNA resistant PHB should be unified, as it is referred to as PHBem in the pCRE1 construct (line 176) and as PHB-M with the pS32 promoter (line 189). To support their claim that PHB mRNA has to be excluded from the SE pole to allow normal procambial proliferation, the authors could additionally to the pS32::PHB-M:GFP construct describe the effect of this expression in the WT background.

RESPONSE: PHBem and PHB-M are different silent mutations of PHB as shown in the figure below. This figure was taken from the Figure S4 by Sebastian et al. (2015), which we published in PLoS Genetics. PHB-M was originally developed and reported in the paper by Carlsbecker et al. (2010) and PHBem was developed and reported in the paper by Sebastian et al. (2015). PHBem is more resistant to miR165/6 than PHB-M. We used these to investigate the dosage-dependent regulation of root meristem activities by PHABULOSA.

It would have been nicer if we investigated pS32::PHB-M:GFP in the wild type. But, we considered its potential overlap with the study reported by Miyashima et al. (2019) in Nature, which deals with the interaction between PEARs and HD-ZIP III in the regulation of procambial cell division. And, in the same context as our response to Comment #6, we did not pursue this aspect in the revision.

Point 8. In the experiments with the immobilized SHR expressed in the stele and the SE poles, the authors describe the occurrence of roots with more than two SEs. It would be interesting to know with what frequency this phenotype occurs and if the additionally formed SEs are continuous along the root or just appearing as "islands"?

RESPONSE: Distribution of phloem SEs in *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* is available in Supplemental Figure 2A. In the *shr* mutant, SE number decreases overall, however, its distribution varies. Class 5 is the closest to the wild type pattern since it has two SEs in each phloem pole, but SEs are not aligned perpendicular to the xylem axis like in the wild type. Based on our scoring, ~75% of shr-2 individuals have reduced SE number (mainly belonging to Class 2 and 3). *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* line recovers the SE number significantly, and about 43% shows reduced SE number, mainly belonging to Class 4. Detailed explanation about classifying SE phenotype is available in lines 116–125.

Because we handled with quantitative distribution of SE phenotype, we performed SE-ENOD immunolocalization for many individuals, not along the longitudinal axis of a single root. Thus, it is not clear to us whether Class 4 phenotype, for example, appears as an island. However, in our small-scale analyses of SEs along the root of *shr*, *shr phb* and wild type, not presented in our manuscript, additional SEs did not seem to appear as islands. There was a tendency of the increase in SE near the hypocotyl, though. Because of this tendency, we tried to analyze SEs in the root maturation zone that just shows differentiation of metaxylem.

Point 9. The observation that induced callose formation in the phloem blocks SHR movement and subsequently the rescue of the SE phenotype in *shr-2* is interesting. However, in this line not only SHR but other molecules are also prevented from entering the developing SEs which might explain this observation. Ultimately, the expression of immobilized SHR from the S32 promoter in this background should allow a rescue of this phenotype even if callose deposition is induced and would validate the assumption.

RESPONSE: We agree with your concern. We considered this experiment as the additional support for the SHR movement to the phloem pole being needed for division for SE, which we showed with *pS32::SHRΔNLELDV:nlsGFP; shr-2*. That's why we only described what we focused on in this experiment. However, as you pointed out, blocking the symplastic transport using EPM promoter interferes with not only SHR movement into the phloem but also others that are moving into the phloem and moving along the phloem. Since our study is proposing the mechanism that requires for both, the expression of immobilized SHR from the S32 promoter in the condition callose over-accumulate in the phloem probably won't rescue the phloem phenotype.

Point 10. The main part of the paper focuses on the identified downstream effectors of SHR that play a role in its effect on formative cell division regulation in the SE precursors. NARS1 and SND2 were identified as transcription factors of interest. SND2 shows expression and also protein localization in the developing and early SE. NARS1 promoter activity is localized towards the CC in the differentiation zone while no GFP was observable for the protein fusion. It is not clear why the authors chose an N-terminal GFP tag here while for SND2 a C-terminal tag was used. Furthermore, considering the hypothesis that NARS1 is transported towards the developing SE and has presumably a major role in coordinating the formative cell divisions, more efforts should have been undertaken in order to localize the protein outside its zone of expression. The authors have used immunolabeling to detect the ENODL9 protein in other experiments. Using the antibodies against the GFP tag of NARS1 could provide a better signal for localizing the protein.

RESPONSE: A part of this comment is quite similar to the comment #3 by Reviewer 2, thus we repeat a part of this response. The reason for choosing N-terminal tag of GFP for NARS1 was because we could not visualize GFP tagged protein even under CRE1 promoter when we expressed NAR1 tagged with GFP in the C-terminus. This was explained in lines 352–354.

During the revision period, we made more efforts to visualize the GFP-NARS1 protein using the whole-mount immunolocalization. This experiment was successful for visualizing erGFP expressed under NARS1 promoter.

In the case of *pNARS1::GFP:NARS1*, we found punctate signals in the stele. However, they were not distinctive enough from the background signal. Based on these data and the data presented in the manuscript, we think that GFP-NARS1might be unstable and modified, which needs more detailed investigations. Since we could not fully resolve the non-cell autonomous nature of NARS1, we modified our statement in the following way in the line 364, "These data collectively indicated that NARS1, generated in the CCs of differentiation zone, might function as a top-down signal, either mobile factor itself or a trigger of other mobile signal that locally regulates the ACD of SE precursors" .

Point 11. The authors identified T-DNA insertion lines for SND2 and NARS1. While snd2 has no detectable root phenotype, the *nars1-2* mutant shows reduced root length and a missing periclinal division in the SE precursor cell file. The neighboring procambial cell file however undergoes the formative division. Considering the weak nature of the *nars1-2* allele, it would be interesting to see how a complete null allele would behave (or a stronger allele, Kunieda et al. 2008, Plant Cell).

RESPONSE: Periclinal cell division of SE precursor and SE differentiation status for *nars1-1* (SM-3-28017), a line reported by Kunieda et al. (2008), were analyzed and shown in Supplemental Figure 6F. Its phloem phenotype was consistent with *nars1-2*, which was described in lines 334–336.

Point 12. The results presented in Figure 6 are among the most interesting of the paper, as here the authors describe the aberrant cell division phenotype of the *nars1-2* mutant. Here the authors provide some quantification of the occurrence of the phenotype (line 319). The results and the different mutants analyzed however seem not to represent the whole picture. For example, although the authors claim NARS1 and SND2 to be the main downstream targets of SHR in the context of SE proliferation control, the *nars1* and *snd2* mutations were not analyzed in the *shr-2* background. The *nars1 snd2* double mutant seems not to be different from the *nars1* mutant and the missing SND2 among the genes induced by S29::GFP-NARS1 raises the question why only SND2 and not any of the other putative downstream targets of NARS1 was selected for further analysis?

RESPONSE: We acknowledge that the manuscript is still far from understanding SHR mediated regulatory networks comprehensively. To get closer to these, however, we investigated the dynamics of SHR-dependent phloem-enriched TFs upon the activation of SHR in time course. Together with ChIP experiments, we found NARS1 is a direct target of SHR that shortly responds to SHR activation. Our additional ChIP experiments indicated that SHR also binds to *SND2* promoter (Supplemental Figure 12C), however, its activation of *SND2* expression needs other components (Supplemental Figure 5). Furthermore, in the line ectopically expressing SND2 throughout the stele, we found the increase in *NARS1* expression. We already know that *SND2* expression significantly decreases in the *nars1* mutant. These data together indicate that *SHR*, *NARS1*, and *SND2* form a positive feedforward loop, and within this *SND2* promotes *NARS1* as positive feedback. We need to investigate this regulatory motif further to understand its biological meaning. But, based on other simulation studies, we think that SND2 might function as a modulator of NARS1 *in vivo* to make NARS1 robustly work for SE formation. That means SND2 is not absolutely

required for SE precursor division in the artificial experimental system, *S29::GFP-NARS1; nars1-2*.

Point 13. In Figure 7 the authors show the results of ectopic NARS1 and SND2 expression. While the rescue of the *nars1* mutant is shown for early SE expression of NARS1, these constructs were not introduced in the *shr-2* background. A compensation of the SE proliferation phenotype by these ectopically expressed genes would provide more conclusive evidence for a major role of these genes in this process. As the rescued SUC2::NARS1 nars1-2 line as well shows a lack of a detectable fusion protein fluorescence, I remain skeptical of the proposed transport of this protein. Further efforts should be undertaken in order to attempt detection of the protein outside its zone of expression. An immobilization of the NARS1 protein could be attempted, e.g. by adding several GUS tags or by screening for putative transport signals within the protein and modifying these. Also, is it possible that the NARS1 coding region contains elements that confer its wider expression? I believe this cannot be excluded at this point.

RESPONSE: In the revised manuscript, we generated *pCRE1::GFP:NARS1; shr-2* and found the increase in SE number (Fig. 8L,M and Supplemental Figure 2A). The number of individuals used for analyses is limited though. Nevertheless, our data indicate that the NARS1 plays a key role in the division for SEs as downstream of SHR.

Our efforts to visualize NARS1during the revisionary period have been described in the response to your comment #10. Even with more sensitive imaging as shown below, we still do not have confident results indicating that NARS1 is moving. Because of these, we do not rule out further modifications of NARS1 protein, which will need another layer of investigation beyond this manuscript.

Point 14. Furthermore, the pCRE1::SND2 construct or another ectopic expression of SND2 in the SE should be introduced in the *nars1* mutant to see to which degree it compensates these. The plant lines expressing SND2 from the CRE1 promoter are not sufficiently discussed as they seem to have a broader phenotype than the suggested occasionally occurring additional SEs. The general number of cells in the stele in Figure 7K seems to be increased.

RESPONSE: In response to your comment, we revisited the immunolocalization image to score the stele cell number. However, we did not find a significant difference between the line expressing *pCRE1::SND2:GFP* and the wild type Col-0. Instead, we frequently found ectopic xylem vessel formation. We did not comment on the xylem phenotype because this aspect is beyond the scope of this manuscript.

Point 15. In line 328, the authors refer to the expression of NARS1 shown in Figures 5 and 6. However, the native promoter activity is shown in Figure 4.

RESPONSE: Thank you for pointing out the error. In the revision, the referred figures were corrected to Figures 5 and 7 (see line 342).

Point 16. For the performed ChIP assay summarized in Supp. Fig. 8, it is not clear why the authors did not analyze the binding of SHR to the NARS1 promoter and only show the negative results for binding to the SND2 promoter. A previous study looking for direct targets of SHR (Cui et al 2011, Plant Physiology) does not identify NARS1 as a direct target of SHR.

The induction of SHR target genes over 24 h and 48 h respectively seems to be fairly long to suggest a direct activation of NARS1 by SHR. From the provided expression data, it is not discernable if SHR is actually expressed in the differentiation zone of the root and could therefore directly activate NARS1.

RESPONSE: Thank you very much for these comments! Your and other reviewers' comments prompted us to analyze the expression of *NARS1* and *SND2* as well as other phloem-enriched TFs dependent on *SHR* as explained in the response to your comment #12. Expression dynamics for 5 early responders including *NARS1* are shown in Figure 4C and others are available in Supplemental Figure 5. Based on the time-course result and your comment on ChIP, we redid the ChIP experiments with the improved skillset and found the consistent binding (3 out of 3 independent experiments) of SHR to the *NARS1* promoter, which is shown in Figure 5A. Supplemental Figure 1F and G show the SHR expression at transcription and protein levels. There, SHR is clearly expressed in the differentiation zone. Taking these data together, we conclude that SHR directly regulates *NARS1* expression in the phloem CC in the differentiation zone.

Point 17. In summary, the manuscript provides new and interesting insights into the role of SHR in orchestrating formative divisions in the developing phloem and procambial cell files. However, many of the conclusions drawn seem to be a bit far fetched at this point, considering the data. Often alternative explanations are not considered. E.g.

could NARS1 not be the direct target of SHR, but a secondary or tertiary activated factor which again itself could regulate other factors in its zone of expression. The requirement for the mobility of NARS1 is in my opinion not given in the provided data and the lack of a detectable protein underlines this point. Higher order mutants or different combinations of the ectopically expressed proteins could, as detailed above, help to understand the role of NARS1 as a downstream element in SHR-mediated control of cell proliferation.

RESPONSE: Thank you very much for constructive and detailed comments on this manuscript. Several of the experiments you suggested could not be performed because of the time constraints and prioritization of experiments to collectively address the major issues of this manuscript. We wish that you find the new data and molecular mechanism in this revision is now acceptable. Your comments greatly helped us to improve our understanding of SHR-mediated phloem development.

TPC2019-00455-RAR1 2nd Editorial decision – *accept with minor revision* January 24, 2020

We have received reviews of your manuscript entitled "SHORTROOT mediated intercellular signals that coordinate the phloem development in Arabidopsis roots." 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 following:

Reviewer 4 thought that the quantitation of phloem cell numbers in Figure S2A should have a statistical test applied, and should also be extended to the *snd2* and *nars1* mutant genotypes. This should be possible simply by counting cells in various micrographs generated in the course of the work. You may consider whether it would be simpler to present such data as numbers of cell types rather than as the distribution into different phenotypic classes as presented currently. Reviewers 2 and 4 also suggested to move some or all of this data to the main text, as it helps readers to judge the importance of the pathway. These two Reviewers also mentioned several other minor points that you can also consider in your revisions. You also might want to make Figure 4B more compact, as the current width of the graphs in that panel is unnecessarily wide and it leaves a lot of empty white space in the rest of the figure.

TPC2019-00455-RAR2 2nd Revision received February 5, 2020

Reviewer comments and **author responses**:

Reviewer #2:

I am very happy with the revised version of the manuscript, in which all of my major concerns have been addressed. It is nice work and should be published!

I still would like to raise some minor points, which may slightly improve the quality of the manuscript.

Point 1. In my previous review, I and the other reviewers have asked for the quantification of the data / phenotypes presented. Numbers are now included and can be found in the supplemental data. Personally, I would prefer to see them in the text.

RESPONSE: Following your comment and recommendations by reviewer #4 and editors, we revised Supplemental Figure 2 by adding an additional panel, Supplemental Figure 2B. This is a graph summarizing scoring results of combined phloem sieve elements, marked with statistical significance (more information related to analysis is available in Supplemental Table 3). In this, we included *nars1*, *snd2*, and *nars1 snd2*. Because Supplemental Figure 2 covers a broad range of genotypes in this study, we could not find an ideal place to fit in a main text. Thus, we decided to keep this as Supplemental data.

Point 2. I still think that a small discussion about the observed broadened expression of phloem markers in *nars*—as now provided in the response to the reviewers—would also benefit the readers of the manuscript.

RESPONSE: We further discussed on this issue in lines 332-339 as follows. "This phenomenon was not due to the misexpression of *S32* itself given that the expression pattern of *pS32::erGFP* was not affected in *nars1* (Figures 6G-I). *pS32::erGFP* begins to undergo GFP expression broadly in the procambial cell right above the QC and is then restricted to the phloem precursors and protophloem SE. In *pS32::H2B:GFP*, stable H2B-GFP would be transferred along the cell lineage as the cells divide. Thus, it may be that the change in the division patterns of the procambial cells in *nars1* resulted in the spread of H2B-GFP to the inner part of the phloem pole."

Point 3. I totally understand the argument that language editing will only be performed once the manuscript will be in its final form. The manuscript is really well written, but should still go for a language check, in particular the use of articles is a bit random.

RESPONSE: The manuscript was extensively edited for grammatical corrections and consistencies in wording and formats. We hope you find that the manuscript is better read now.

Reviewer #3:

The authors have made an effort to improve the paper, and although not all of the concerns I raised could be fully addressed. I concur with the authors that for various reasons, technically and/or biologically, this is as good as it gets at this point, and further delay of this otherwise important manuscript is not justified.

Reviewer #4:

The manuscript by Kim et al describes the role of SHR in phloem cell specification. The authors provide evidence that SHR targets NARS1 and SND2. There are some excellent sections to this manuscript. In particular, the gene expression analysis that was used to identify putative SHR target genes is clear and the data easy to understand. Similarly, the gene expression relationships are well defined by ChIP and reporter analyses. However, I have one major concern, which is shared with other reviewers in the previous round of review regarding statistical analysis of the phenotypes. This data is only partially present, but it is essential that it is presented in full in the primary manuscript in a clear manner with the number of individuals indicated and the statistical tests marked. Without this data, the reader is left guessing as to the importance of these findings for cell type specification and ACD.

Point 1. The distribution of phenotypes in figure S2 is helpful, and it should be in the main manuscript. However, this data is limited in that no statistical test is applied. Determining the total number of cells in the stele is useful background, but to my mind it does not address the central issue, which is phloem cell number. To address this we really need to see a graph showing the number of SE's and the number of CC's in each of the genotypes tested for differences with an appropriate statistical test. This analysis should be performed not just for *shr* genetic combinations, but also for *snd2 nars1* mutants.

RESPONSE: To address this comment, we added Supplemental Figure 2B, which shows the number of phloem SEs in each genotype including *snd2*, *nars1*, and *snd2 nars1* mutants. The graph shows the statistical significance of phloem SE number in each genotype in comparison to the wild type Col-0. The ANOVA table is available as Supplemental Table 3. We did not place this graph in the main manuscript though because some genotypes in the graph are commented in the later part of the manuscript and because this bar graph also relies on the information available in panel A.

TPC2019-00455-RAR2 3rd Editorial decision – *acceptance pending* **February 7, 2020**

We are pleased to inform you that your paper entitled "SHORTROOT-mediated intercellular signals that coordinate the phloem development process in Arabidopsis roots" 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 its presentation of scientific content, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **February 25, 2020**