molecular systems biology

A map of cell type-specific auxin responses

Bastiaan O. R. Bargmann, Steffen Vanneste, Gabriel Krouk, Tal Nawy, Idan Efroni, Eilon Shani, Goh Choe, Jiří Friml, Dominique C Bergmann, Mark Estelle, Kenneth D. Birnbaum

Corresponding author: Bastiaan OR Bargmann, UCSD

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1st Editorial Decision 19 April 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which should be convincingly addressed in a revision of the manuscript.

Overall, the reviewers appreciate the presented approach and acknowledge that your work could be of interest for plant and developmental biologists. However, they also raise a number of major points, which should be carefully addressed. Among the more fundamental issues are the following:
- Additional experimental evidence should be provided to better demonstrate the quality of the tissue-specific datasets.
- On several occasions the interpretation of the results remains rather vague. As such, it is essential to provide rigorous and precise discussion of the results, leading to substantiated conclusions.
- Additional clarifications need to be provided regarding the analysis of longitudinal spatial expression and the use of the dataset of Brady et al., 2007.

On a more editorial level, I would kindly ask you to deposit the microarray datasets in the appropriate public databases. (Additional information is available in the "Guide for Authors" section in our website at <http://www.nature.com/msb/authors/index.html#a3.5.2>) Furthermore, I would like to ask you to include the links and accession numbers in the "Data Availability" section of your manuscript.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

REFEREE REPORTS

Reviewer #1 :

This manuscript by Bargmann and colleagues describe the outcome of a transcriptomic analysis of tissue-specific responses to the plant hormone auxin, a central morphogenetic regulator in plants. While many studies have analyzed the auxin-regulated transcriptome at the level of whole tissues or whole plants, the authors used here cell-sorting of lines expressing a GFP marker in specific tissues (epidermis, stele, mature pericycle and columella) of the root tip followed by microarray analysis to identify the early auxin-regulated transcriptome from these tissues. Analysis of these different transcriptomes (with or without a 3h auxin treatment on the different tissues) through the identification of dominant patterns of expression and GO analysis allow them to highlight trends such as repression of trichoblast identity in the epidermis. Data on the analysis of promoters of the auxin-regulated genes (500 bp upstream of start) are also presented, leading to identification of overrepresented motifs. The generation of a database of genes enriched in specific tissues of the root tip (using published datasets obtained by cell sorting and microarray analysis by some of the authors -Birnbaum et al. Science 2003- and by Brady et al. Science 2007) allows the author to show different behaviors in response to auxin in different tissues (e.g. up-regulation in quiescent center and meristematic xylem cells, down-regulation in cortex, maturing xylem and trichoblasts). Concerning the xylem, the authors highlight the fact that auxin induces transcription in meristematic cells and represses it in differentiating cells. This interesting observation clearly show that auxin can regulate transcription differentially in a given tissue depending on the differentiation status. Expanding this observation, the authors crossed their data on the tissue-specific auxin-induced transcriptome with other publicly available datasets (from the same publications as above) that sampled the transcriptome along the longitudinal axis of the root (thus allowing to follow cell differentiation from the stem cell niche in the meristem to fully differentiated cells). Using this approach, they reveal anti-correlated patterns of gene expression along the longitudinal axis for the auxin-induced and auxin-repressed gene that might result from auxin distribution.

Understanding how tissues respond differently to a morphogenetic question is a central question in biology. The approaches presented in the manuscript are both elegant and pertinent. I think this work has the potential to be of broad interest to the plant science community and more generally to developmental biologists.

However I feel the analyses need to be strengthen and that important clarifications are required prior to publication. On several occasions it seems that the authors did not go to the end of their exploration and provide rather vague interpretations of highly complex results. I am providing below several comments (that I think the authors should be able to address) that could help improving the manuscript.

1- My first concern is with the experimental design and the choice of the GFP lines used to analyze tissue-specific transcriptomes. While covering most of the tissue of the root, the lines chosen do not allow analysis of the ground tissue and QC (while GFP markers are available). But these tissues are included when the results of the authors are crossed with the available datasets. This raises the question of the gain in sensitivity achieved through the tissue-specific analysis, a rather central point that is not clearly discussed in the manuscript. This corresponds to the last paragraph of the first section of the results (P8). Is it really better to do the tissue specific analysis? Can the gain be estimated for the different tissues? Is it similar between the tissues that have been analyzed? This could maybe be done considering specifically the tissue specific genes and relatively to the number of these genes for a given tissue (for ponderation). And then this should be a point included in the discussion.
If the gain is very important then there is risk that a significant bias is introduced when comparing with published data from tissues that are not included in the tissue-specific analysis performed by the authors. The corresponding dataset should then be added to the manuscript. If the authors think that it is not the case, they should provide a careful justification in the manuscript.

2- In the first result section 2nd paragraph P7, p-value thresholds are given when indicating how many genes were found. I first thought that the statistics had not been corrected for multiple testing but found in the Methods that this correction was done by calculating q-values. What are this p-values then? What is the number of false positives expected, what is the stringency?
Concerning the statistics, P11: "Fifty-six of 57 auxin-regulated developing-xylem-enriched genes in the stele sample were induced by auxin (t-test $p<0.01$, 2-test $p=5.66e-11$) and 63 out of 77 auxin-regulated maturing-xylem-enriched genes were repressed (2-test $p=7.59e-11$)."
Why is there first a t-test and and chi-squared test and then just a chi-squared test?
3- P8/Fig. 2: Providing expression for 2 genes in the root (that were already known) to claim that the tissue-specific datasets are of good quality is not very convincing. Further reporter lines and/or qPCR need to be added.

4- Fig. 3A is completely cryptic.

5- P8 2nd paragraph: the authors note that Auxin-related GO terms are overrepresented in clusters "containing genes with relatively uniform up-regulation of expression". Is it the case with other clusters or not at all? This paragraph is an example of a description of the results that stays relatively vague without highlighting specific conclusions. There are several other examples like that in the text: - P8 in the previous paragraph: "Many genes are significantly regulated in only one cell type and many others in a subset of two or three of the assayed cell types. These findings demonstrate a pervasive tissue-specific amplitude modulation of auxin responses."; P9 3rd paragraph: "Several auxin response clusters representing a localized spatial pattern of induction or repression showed overrepresentation of specific functions. A number of these were related to development."; P10 1st paragraph: "The enrichments of different GO-term associations and promoter elements among genes with similar spatial responses to auxin may give indications of the regulation of cell type-specific gene expression and cellular development." etc.
The authors need to carefully check the entire manuscript. This is not doing justice to the work. For example, it would be interesting to have an idea of which are the over-presented motifs in promoters. Does this bring any important knowledge?

6- P11 1st phrase: how genes are found in this overlap?

7- P11 2nd paragraph: TMO5 - needs references. Citation are indeed often missing (e.g. the cell sorting datasets). This need to be checked.

8- P11 Basal meristem needs to be defined.

9- The analysis of the longitudinal spatial expression. This part needs important clarifications: - why not using here the stringent list of genes induced by auxin?; - for Fig 5B on one side and 5C&D the meristematic elongation and maturation zones do not cover the same slices in the root; - the author claim that Fig 5B shows "a strong link between auxin responsiveness and spatial expression". This is not really convincing; it rather shows a drop of expression that depends on whether the genes are induced or repressed; - I do not really understand how the authors obtained Fig 5C&D. The SEM is surprisingly low when considering the variability seen in Fig 5B. It is also unclear to me how the authors really decided on the four groups of genes. They say that the genes can be "broadly subdivided into 4 categories" (P13). I am puzzled why they have not used a clustering approach for example rather than a qualitative subdivision. The response in Fig 5C and 5D seems then to show similar trends, mostly with different amplitudes. This would rather tend to say that there is broadly 2 groups, the repressed and the activated one.

10- The authors state on several occasion (e.g. Introduction-P3 2nd paragraph-, p16 -"endogenous auxin gradient"- and in the discussion) that there is a proximo-distal gradient of auxin in the root tip i.e. that auxin concentration diminishes with elongation and differentiation of the cells. In the introduction they cite Brunoud et al. 2012 to justify this statement. However Brunoud et al. demonstrate that the distribution of auxin in the root tip is not simply a proximo-distal gradient of auxin. They observe that, while auxin levels are high around the QC and in the xylem axis, auxin is

lower in the other tissues of the meristem and start increasing closer to the start of the elongation zone. This has been also pointed out by Santuari et al. Curr Biol 2011. This is an important point for the discussion of results of this manuscript because these publications show that auxin concentrations is distributed differently between the different tissues of the root. The authors should make use of these recent data and not oversimplify what is now known on auxin distribution in the root. Petersson et al. 2009 is an important study but the auxin measurements do not have a cellular distribution and the map of auxin proposed in this publication is an interpretation compatible with the tissular measurement that were performed in this paper.

11- Again on the interpretation/discussion of the differences in auxin-induced responses between tissues, the authors propose at the end of the discussion (P18) that the distribution of auxin-inducible genes in the root is likely under the control of both auxin distribution and auxin signaling capacities of the cell. This is a sensible hypothesis that fits with the complex distribution of Aux/IAA and ARF in the root that is illustrated in Fig. S1 (and is compatible with a number of publications: Brunoud et al. Nature 2012, Rademacher et al Dev Cell 2012, Vernoux et al. MSB 2011, Rademacher et al. Plant J 2011 for the most recent and others notably from Weijers and colleagues). However the results hidden in Fig. S1 are in my view underexploited. They should be moved in the main Figs. and discussed in details (notably in relation to the work of Rademacher where all the ARFs where mapped) in order to present a clear vision of the distribution of the effectors of auxin-induced gene transcription. The analysis should also be extended by including the data on longitudinal expression of these signaling effectors in order to provide a map of their distribution in the root tip. This would seriously strengthen the paper and possibly (will it be the case?) provide support to statement like "the xylem lineage is uniquely tuned to respond to auxin" (P16). This is of particular interest in the light of the recent demonstration of an auxin maximum in the xylem instrumental for vascular tissue patterning (Bishopp et al. Curr Biol 2011). It would also open the possibility of an interesting discussion on auxin signaling effector distribution in different tissues (shoot, embryo from the papers cited above).

Reviewer #2 :

This manuscript is based on an experimentally generated dataset that profiles gene expression in response to auxin in four distinct tissues of the Arabidopsis root. Auxin is an extremely important plant hormone that plays a role in most plant developmental processes. Auxin receptors and their downstream transcription factors and repressors are often expressed in a cell type-specific manner thus confounding interpretation of two available auxin response reporters (DR5 and DII:VENUS). This dataset is used in conjunction with other publically available datasets to place auxin response readout in the context of distinct cell types and developmental stages. Important observations include the responsiveness of different cell types based on their developmental stages, and the clear separation between preferential expression of genes that are important in cell specification in response to auxin, vs. genes that are more important in morphogenesis. Since auxin is an extremely important hormone and the root is an ideal model organ to dissect spatiotemporal development, I imagine that this dataset and its analysis would be of great use to the Arabidopsis community and to those interested in an organ's response to an instructive hormone.

Major points:

1) How auxin responsive are the markers used? If these markers change their response by expanding into other cell types, this could definitely change the interpretation of their results.

2) The analysis from page 12 onwards refers to the longitudinal root dataset as a spatial dataset. This can get quite confusing as the first portion of the results refers to the tissue-specific response as a spatial response. I would be much more clear with respect to the longitudinal dataset as a temporal dataset. Also, it is unclear how for the Brady et al. 2007 dataset how data from all 6 sections in the meristem or all 4 sections in the maturation zone were considered. Were they considered as a composite or individually?

Minor notes:

Page 7 - a description of significant induction of Aux/IAA genes and GH3s is given. The statement is a little misleading - there is statistical significance for most of these genes on a gene by gene basis, but is there statistical significance for the entire group? This would be a simple hypergeometric distribution test and would be able to distinguish between both of these possibilities.

Page 8 - a statement is made "although genes are often reciprocally induced or repressed in different cell types" - I don't really see this in Figure 3A. In fact, I would say that only a small proportion of genes are reciprocally induced or repressed.

Page 17 - the Moreneo-Risueno paper shows that it is a TF that is driving the oscillation, not auxin.

Figure S1 - what is the distance metric for the hierarchical clustering shown?

Figure S4B - why are there 3 boxplots shown for each tissue type? This isn't explained in the figure legend.

Reviewer #3 :

Hormone regulated plant growth and development represents an area likely to be of great interest to the MSB readership. Whilst great strides have been made characterizing the core hormone response and transport machinery for the plant hormone auxin by several of the co-authors, their spatio-temporal output (in terms of expression of downstream targets) has only been monitored at the level of individual genes through use of reporter genes.

The current study explores the spatio-temporal output of the auxin response pathway in individual root tissues employing cell sorting and Affy arrays to profile global changes in transcript abundance in response to auxin treatment. The authors compared transcriptional auxin responses between various tissues: stele, xp pericycle, epidermis and columella with whole root tissue. I was puzzled why they selected a 3 hour time point following auxin treatment to sample after they showed every root tissue exhibited an auxin response after 30 minutes hormone treatment (based on degradation of the DII-VENUS marker). Irrespective, they identify 5097 genes that show differential expression after auxin treatment and between different tissues. As well as providing a very useful list of auxin responsive genes in these individual tissues, the study reveals that many cell-type specific auxin responsive genes were not detected in the past due to other researchers sampling at organ/ismal scales.

The authors describe several biologically interesting examples of uniformly and spatially distinct classes of auxin up and down responsive genes. They also note the enrichment of AuxRE elements in promoters of the former class of up regulated genes, but it was not clear whether this was also the case for auxin down regulated genes? Next, the authors correlate the expression patterns of those genes with root stage and cell specific transcriptomic datasets from Brady et al (2007) and Birnbaum et al (2003). Interestingly, this revealed cell types specific effects of auxin and also stage specific effects such as enrichedment of auxin up and down regulated genes in developing and maturing xylem cells, respectively.

In summary, the manuscript is well written and the biological significance of the authors observations made much clearer with the inclusion of selected examples. In addition, the datasets will provide a very useful resource to researchers.

1st Revision - authors' response 14 June 2013

Reviewer #1 (Remarks to the Author):

1- **My first concern is with the experimental design and the choice of the GFP lines used to analyze tissue-specific transcriptomes. While covering most of the tissue of the root, the lines chosen do not allow analysis of the ground tissue and QC (while GFP markers are available). But these tissues are included when the results of the authors are crossed with the available datasets. This raises the question of the gain in sensitivity achieved through the tissue-specific analysis, a rather central point that is not clearly discussed in the manuscript. This corresponds to the last paragraph of the first section of the results (P8). Is it really better to do the tissue specific analysis? Can the gain be estimated for the different tissues? Is it similar between the tissues that have been analyzed? This could maybe be done considering specifically the tissue specific genes and relatively to the number of these genes for a given tissue (for ponderation). And then this should be a point included in the discussion.**

If the gain is very important then there is risk that a significant bias is introduced when comparing with published data from tissues that are not included in the tissue-specific analysis performed by the authors. The corresponding dataset should then be added to the manuscript. If the authors think that it is not the case, they should provide a careful justification in the manuscript.

The primary advantage of cell sorting was the ability to detect differences in the auxin response among the tissues. This point is independent of the sensitivity issue. The points on gain in sensitivity were based on the analysis of the overlap between genes found to be responsive in the intact root and genes found to be responsive in specific tissues (Fig 2C). In that analysis, we stated that the cell type-specific analysis may give increased sensitivity because genes whose response in intact root analysis may be too dilute to detect can be detected when analysis is conducted at a cellular resolution. Figure 2C provides the best estimation of the gain. We now clarify the interpretation of figure with respect to the statement.

page 9 2nd paragraph:

"In Figure 2C, the heatmap overlaid on the Venn diagram shows the gain in sensitivity for detecting cell-type specific auxin responses. Genes whose regulation by auxin were found in only one tissue show the least amount of overlap with responses in the intact root (1411, 445, 600, and 363 for stele, xp pericycle, epidermis, and columella, respectively). Transcripts whose response was detected in higher numbers of cells show the highest overlap with those detected in the intact root."

With respect to whether sensitivity might be higher in certain tissues compared to the intact root, in Figure 2C, the proportional overlap in the different tissues analyzed can be seen to be fairly equal, with no strong bias towards any specific tissue.

With respect to the comparison of our tissue-specific auxin responses and cell type-specifically enriched (CTSE) genes derived from public expression data, a similar proportion of cell type-specifically enriched transcripts can be seen to be responsive in the different individual tissues analyzed as well as in the intact root (all around 20%; data extracted from Table S2). This is not significantly more than expected, given the relative quantity of identified cell type-specifically enriched transcripts genome-wide (3416 out of 21119 non-ambiguous probesets on the chip). We don't believe this issue speaks to sensitivity to detect auxin responses, as the presented list is of genes with enriched expression in specific cell types; many of these genes may also be expressed (and putatively responsive) in other tissues. Therefore, although we did not cover all the tissues used to make the cell type-specifically enriched list of the template matching

analysis, we believe we can draw conclusions from the auxin response of these genes as well as the spatial specificity of that response within our dataset. Moreover, we have conducted additional analysis addressing and further validating the cell type-specific data. This analysis (Supplementary Figure 2B) displays the expression of the 3416 cell type-specifically enriched genes in the four marker lines used in our study. It demonstrates that the RNA isolated from our various markers is differentially enriched for the transcripts that would be expected to be found in the domains covered by these markers. This data, analysis and discussion have been added to the revised manuscript.

page 7 2nd paragraph, Supplementary Figure 2B, Supplementary Table 2:

"In order to establish that the tissue-specific expression profiles gathered here were consistent with the previously published root expression data (Birnbaum et al, 2003; Brady et al, 2007; Lee et al, 2006; Levesque et al, 2006), we generated a list of cell type-specifically enriched (CTSE) genes using the public data and visualized their expression in our dataset. This CTSE list was based on expression profile template matching in a select dataset of 13 non-overlapping cell type-specific expression profiles of sorted GFP marker lines (Supplementary Figure 2B, Supplementary Table 2). This procedure yielded a total of 3416 genes whose expression is enriched in one specific cell type (maturing xylem, developing xylem, xp pericycle, phloem-pole pericycle, phloem element, phloem companion cell, quiescent center, endodermis, cortex, trichoblast, atrichoblast, lateral root cap or columella) or in two related cell types (xylem, pericycle, phloem, ground tissue, epidermis or root cap). The relative expression of the CTSE genes in the tissue-specific data generated in this study is differentially enriched in a manner that fits with the domains covered by the different markers used here (Supplementary Figure 2B). These results indicate a successful isolation of the transcriptomes of distinct cell types and show that the enrichment in specific tissues is consistent across the datasets."

2- In the first result section 2nd paragraph P7, p-value thresholds are given when indicating how many genes were found. I first thought that the statistics had not been corrected for multiple testing but found in the Methods that this correction was done by calculating q-values. What are this p-values then? What is the number of false positives expected, what is the stringency?

One of the priorities of the analysis was having a consistent cutoff for auxin regulation across the four tissues tested, to allow some comparison of the strength of responses in each tissue. Our rationale was that choosing a cutoff based on the type I error for positive cases represented a benchmark directly related to response and variation. Of course, accounting for multiple testing was also a concern. To address these issues, we imposed a p-val of 0.01 and showed that this led to acceptable and consistent false discovery rates. One could choose other approaches but we believe this method is consistent with the features of data we want to identify. We have elaborated on this implementation in the manuscript. Furthermore, we have included the q-values for each probeset individually in Supplementary Table 2.

page 26 2nd paragraph, Supplementary Table 2:

"False discovery rates (FDR) were calculated based on p-value distribution (Q-Value). For the comparison between statistical tests in separate tissues, equal cutoffs were set at $p<0.01$. FDR at this cutoff and q-values for the individual probesets are reported in Supplementary Table 2. Additionally, a fold-change cutoff of >1.5 was set for the tissue-specific and intact root t-tests. To generate the stringent list of 2846 auxin responders, genes had to pass the ANOVA for treatment or interaction ($p<0.01$) and at least one of the tissue-specific t-tests ($p<0.01$, fold change>1.5)."

Concerning the statistics, P11: "Fifty-six of 57 auxin-regulated developing-xylem-enriched genes in the stele sample were induced by auxin (t-test p<0.01, χ2-test p=5.66e-11) and 63 out of 77 auxin- regulated maturing-xylem-enriched genes were repressed (χ2-test p=7.59e-11)."
Why is there first a t-test and and chi-squared test and then just a chi-squared test?

The t-test was referring to the significant auxin-regulation in the stele and the χ2-tests to the distribution of induced and repressed genes in the developing and maturing xylem. The reference to the t-test may have been confusing and was superfluous, therefore "t-test" has been removed from the text.

page 13 2nd paragraph:
"Fifty-six of 57 auxin-regulated developing-xylem-enriched genes in the stele sample were induced by auxin (χ2-test p=5.66e-11) and 63 out of 77 auxin-regulated maturing-xylem-enriched genes were repressed (χ2-test p=7.59e-11)."

3- P8/Fig. 2: Providing expression for 2 genes in the root (that were already known) to claim that the tissue-specific datasets are of good quality is not very convincing. Further reporter lines and/or qPCR need to be added.

We have added supplemental experimental data, including additional reporter lines, to support the validation of the data. In addition to the two lines shown previously, we have included confocal analysis of three other reporter lines, pTMO6:GFP, pGH3.5::GFP and pIAA5::GUS, that match the microarray data and exhibit both more complex tissue-specific expression (TMO6) as well as induction throughout the root (GH3.5 and IAA5; Supplementary Figure 3A-H). This adds to the corroboration previously presented for LBD33 and ATHB-8. In addition, in Figure S5, we note that a third reporter line had been presented, pTMO5:GFP. This gene was identified as a marker for developing xylem identity by our template matching analysis of publicly available cell type-specific data (Supplementary Table 2), shows stele-specific induction in our microarray data and matching xylem-specific increase of GFP expression in the reporter line (Supplementary Figure 5B-C). The increase in GFP expression was further quantified by cytometry, showing a significant induction of GFP intensity and the percentage of GFP-positive cells (Supplementary Figure 5D). This provides a total of six reporter lines for validation. Additionally, a new cross-reference of the identified auxin-responsive genes with previously published cell type-specific data (Supplementary Figure 2B) shows that genes with enriched expression in specific tissues, according to public data, match the expected tissue-specific expression in our data. This further corroborates the consistency of techniques in this manuscript compared to previous sorting data from multiple labs. In addition, we note that the quality of the tissue-specific datasets is also supported by other evidence, including the finding that known auxin-responsive gene families can be seen to be identified as significantly regulated across the different tissues (Supplementary Figure 2C-F, Supplementary Table 2), activation of several LBD genes specifically in the pericycle sample (Supplementary Figure 2E).

page 9 1st paragraph, Figure 2E-H, Supplementary Figure 2B-F, Supplementary Figure 3A-H:
"Measured auxin responses were corroborated on two levels. First, we observed the significant regulation of known auxin-responsive genes in the cell type-specific dataset. This includes significant regulation of 22 members of the Aux/IAA family of auxin co-receptors (Calderon-Villalobos et al, 2010), 14 GH3 auxin conjugases (Hagen et al, 1991), 18 SAURs (Hagen & Guilfoyle, 2002) and 7 LATERAL ORGAN BOUNDARY DOMAIN CONTAINING PROTEIN (LBD) transcription factors (Shuai et al, 2002) (Supplementary Figure 2C-F, Supplementary

Table 2). Several of the responsive LBD genes, that are known to be involved in lateral root initiation (Okushima et al, 2007), displayed dramatic up-regulation specifically in the xp pericycle, the tissue where lateral roots originate (Supplementary Figure 2E). These results indicate the robust induction of known auxin responsive transcripts in the four cell types sampled in this work. Second, we confirmed that tissue-specific transcript level measurements matched auxin induction patterns in transcriptional reporter lines. This included xp pericycle-specific induction of pLBD33::GUS and pTMO6::GFP (TARGET OF MONOPTEROS 6) as well as stele-specific induction of pATHB-8::GFP (ARABIDOPSIS THALIANA HOMEOBOX GENE 8) and ubiquitous induction of pGH3.5::GFP and pIAA5::GUS (Figure 2E-H and Supplementary Figure 3; (Kang & Dengler, 2002; Lee et al, 2006; Okushima et al, 2007; Schlereth et al, 2010))."

page 14 1st paragraph, Supplementary Figure 5B-D:

"In planta, the expression of developing-xylem identity marker pTMO5::GFP (Schlereth et al, 2010)) intensifies and expands from the apical meristem further into the basal (shootward) meristem upon auxin treatment (Supplementary Figure 5B-D), corroborating the transcriptomic data and showing that this increase in expression seen in the stele sample takes place exclusively within the xylem lineage."

4- Fig. 3A is completely cryptic.

Figure 3A presents a heatmap of the gene expression in control and treated cell types for genes that pass a t test and ANOVA filter. The lack of clarity may have arisen from the description of the filtering steps and the column arrangement in the legend. The legend has been appended to clarify the heatmap composition.

page 10 2nd paragraph, Figure 3A:

"Using the stringent list of (2846) auxin-responsive genes, expression patterns were ordered hierarchically by pairwise correlation. A heatmap of gene regulation patterns shows how almost all auxin responsive genes exhibited some type of spatial bias in their regulation (Figure 3A). Although genes are most often regulated in the same direction (induced or repressed) in different cell types, the response is usually stronger in a subset of samples. These findings demonstrate a pervasive tissue-specific amplitude modulation of auxin responses and suggest most auxin-controlled genes have context-dependent aspects to their transcriptional regulation."

page 35 legend Figure 3A:

"Spatial auxin-response patterns arranged by hierarchical clustering (pairwise Pearson correlation). 2846 genes significantly regulated in the ANOVA for treatment or for the interaction between treatment and cell-type ($p<0.01$) and in at least one t-test of the four separate tissues assayed ($p<0.01$, fold change>1.5). The heatmap consists of row-normalized gene expression in rows and cell type +/-treatment in columns; blue (low) to yellow (high) color-code indicates standard deviations from the row mean."

5- P8 2nd paragraph: the authors note that Auxin-related GO terms are overrepresented in clusters "containing genes with relatively uniform up-regulation of expression". Is it the case with other clusters or not at all?

There are other, more cell type-specific response clusters that show overrepresentation of certain auxin-associated GO terms. However, the bulk of the overlap with auxin-GO-term genes is found in these uniform clusters. This can be seen in Supplementary Figure 4B and

Supplementary Table 3. This has been emphasized in the revised text and is discussed in the light of the specific genes associated with these GO terms in the different clusters.

page 11 1st paragraph:

"Extending the trend noted above for genes significantly regulated in all tissues, genes previously associated with the response to auxin stimulus as well as auxin mediated signaling and auxin homeostasis were mainly overrepresented in clusters containing genes with relatively uniform up-regulation of expression, these included 10 Aux/IAAs and 4 GH3s (Figure 3B and Supplementary Figure 4 clusters 15 and 16; Supplementary Table 3). Four IAAs and GH3.3 were included in a cluster that showed relatively stronger induction in the stele and the induction of GH3.6/DWARF IN LIGHT 1 was strongest in the columella. Two genes previously associated with the response to auxin, LATE ELONGATING HYPOCOTYL and a uncharacterized homeodomain transcription factor (At1g74840), were found in a cluster of genes with strong down-regulation in the pericycle. PIN-FORMED 7, NO VEIN and ACAULIS 5 are linked to the auxin-transport GO term found to be overrepresented in a cluster with relatively strong induction in the stele and pericycle. Genes associated with auxin biosynthesis were overrepresented in a cluster of uniformly down-regulated genes. These enrichments show that, although most genes previously associated with the auxin response display universal induction, there are cell type-specific expression biases to the transcriptional regulation by auxin among genes that influence its own perception, metabolism and transport."

This paragraph is an example of a description of the results that stays relatively vague without highlighting specific conclusions. There are several other examples like that in the text: - P8 in the previous paragraph: "Many genes are significantly regulated in only one cell type and many others in a subset of two or three of the assayed cell types. These findings demonstrate a pervasive tissue-specific amplitude modulation of auxin responses.";

The manuscript has been revised to address this point.

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"Using the stringent list of (2846) auxin-responsive genes, expression patterns were ordered hierarchically by pairwise correlation. A heatmap of gene regulation patterns shows how almost all auxin responsive genes exhibited some type of spatial bias in their regulation (Figure 3A). Although genes are most often regulated in the same direction (induced or repressed) in different cell types, the response is usually stronger in a subset of samples. These findings demonstrate a pervasive tissue-specific amplitude modulation of auxin responses and suggest most auxin-controlled genes have context-dependent aspects to their transcriptional regulation."

P9 3rd paragraph: "Several auxin response clusters representing a localized spatial pattern of induction or repression showed overrepresentation of specific functions. A number of these were related to development.";

The manuscript has been revised to address this point.

page 11 2nd paragraph:

"Several auxin response clusters representing a localized spatial pattern of induction or repression showed overrepresentation of functions linked to growth processes known to be regulated by auxin. For example, clusters of genes that showed epidermis-specific down-

regulation by auxin (e.g. cluster 37) had statistically overrepresented GO terms for trichoblast maturation…"

P10 1st paragraph: "The enrichments of different GO-term associations and promoter elements among genes with similar spatial responses to auxin may give indications of the regulation of cell type-specific gene expression and cellular development." etc. The authors need to carefully check the entire manuscript. This is not doing justice to the work. For example, it would be interesting to have an idea of which are the over-presented motifs in promoters. Does this bring any important knowledge?

We have conducted a new promoter analysis to further explore overrepresented motifs in the different expression patterns, focusing on the auxin response element and related elements. This analysis revealed that this element is overrepresented mostly in clusters showing uniform up-regulation. This intriguing and novel finding is addressed further in the Discussion.

page 12 2nd paragraph:

"Promoter analysis of the cell type-specific auxin-response clusters was conducted to look for overrepresentation of the canonical auxin response element TGTCTC (Liu et al, 1994). Clusters 15 and 16, which show relatively uniform up-regulation of gene-expression across tissues (Supplementary Figure 4A), contain significantly more genes with this element in the 500 bp upstream of their transcription start site than expected by chance (hypergeometric distribution analysis; Supplementary Table 3). Additionally, the occurrence of the generic TGTCNC and the individual -A-, -C- and -G- variants was examined and found that TGTCAC, TGTCCC and TGTCNC were also overrepresented in the promoters of the uniformly up-regulated genes assigned to dominant expression patterns 15 and 16. Furthermore, TGTCAC was overrepresented in the promoters of genes assigned to pattern 34, which shows down-regulation in all tissues that is strongest in the stele (Supplementary Figure 4A). None of these elements were significantly enriched in any other up- or down-regulated clusters. These results suggest that direct targets of auxin signaling through the auxin response promoter element are generally uniformly induced across tissues of the root and that variants of the canonical element may also participate in auxin regulation of transcript levels."

page 23 1st paragraph:

"Interestingly, the ARF binding site (the auxin response element TGTCTC) and several variants thereof (TGTCNC) are only found to be overrepresented among genes with a relatively uniform up-regulation (Supplementary Figure 4, Supplementary Table 3). This could indicate that, if the more spatially distinct responses and auxin-regulated gene repression are in part directly mediated by binding of particular ARF isoforms, a more complex DNA binding-site recognition may be involved in the target-specificity of different ARF isoforms. Alternatively, the spatially distinct responses could be composed more of indirect target genes that are regulated by secondary activators or repressors. Lastly, signal transduction outside of the TIR1/AFB-Aux/IAA-ARF auxin response pathway may also account for cell type-specific responses."

6- P11 1st phrase: how genes are found in this overlap?

This was referring to the literal overlap between the stringent list of 2846 auxin responsive genes and the list of 3416 cell type-specifically enriched genes (which can be further subdivided into groups of genes with expression enriched in the different tissues). The text has been altered to clarify.

page 13 1st paragraph:
"To explore the influence of auxin on cellular development in the root in more depth, the CTSE sets of cell identity markers (Supplementary Figure 2A, Supplementary Table 2) were used to analyze the effect of auxin on tissue-enriched genes in our cell type-specific dataset. The overlap between the stringent list of 2846 auxin-responsive genes and the 3416-gene CTSE list enabled us to assess whether auxin had a positive or inhibitory overall effect on transcriptional cell identity."

7- P11 2nd paragraph: TMO5 - needs references. Citation are indeed often missing (e.g. the cell sorting datasets). This need to be checked.

We have inserted additional references here and in several other places.

page 14 1st paragraph:
"the expression of developing-xylem identity marker pTMO5::GFP (Lee et al, 2006; Schlereth et al, 2010) intensifies and expands from the apical meristem further into the basal (shootward) meristem upon auxin treatment"

8- P11 Basal meristem needs to be defined.

We show the region we define as the basal meristem in Figure 1A. In addition, we have also added a definition in the text.

page 14 1st paragraph:
"the expression of developing-xylem identity marker pTMO5::GFP (Lee et al, 2006; Schlereth et al, 2010) intensifies and expands from the apical meristem further into the basal (shootward) meristem upon auxin treatment"

9- The analysis of the longitudinal spatial expression. This part needs important clarifications: - why not using here the stringent list of genes induced by auxin?

We use the broader list of auxin-responsive genes to demonstrate the pervasiveness of the correlation. However, the correlation can also be observed with the stringent list of auxin regulated genes. This data has now been added to Supplementary Figure 6F and is discussed in the text.

page 16 2nd paragraph, Supplementary Figure 6F, Supplementary Table 5:
"correlation was also evident with auxin responses measured in individual tissues and with auxin responses in the intact root as well as the stringent list of 2846 auxin-responsive genes (Supplementary Figure 6B-K; Supplementary Table 5)."

- for Fig 5B on one side and 5C&D the meristematic elongation and maturation zones do not cover the same slices in the root;

The heatmap in figure 5B depicts each slice of the dataset with a column of equal width. The schematic above the heatmap is meant to depict the position of the slices in the root. The meristematic zone reaches from the columella slice to slice 6, the elongation zone from 7 to 8

and the maturation from 9-12 in figure 5B and C&D. In figure 5C&D the slices are sized according to actual size. We have added explanation of the representation in the figure legend.

page 37 legend Figure 5:

"B. Heatmap of the spatial expression of auxin-responsive genes (ANOVA treatment p<0.01, 5097 genes) in the 13-slice longitudinal dataset (root1; Brady et al, 2007). Genes were ordered by fold-change response to auxin treatment; blue (low) to yellow (high) color-code indicates standard deviations from the row mean. Up- and down-regulated genes were further subdivided into groups 1-4 based on relative induction or repression and broad differences in longitudinal expression (red and green color coding). C-D. Average normalized longitudinal expression patterns of auxin-responsive genes, +/- SEM (groups 1-4 in B.). The relative spatial separation of the 13-slice dataset (Brady et al, 2007) is represented on the x-axis and the standard deviations from the row mean on the y-axis. C. Longitudinal expression of archetypal auxin-responsive genes (groups 1 and 3 in B.), consisting of the top 1000 induced and the first 1000 repressed genes. The quiescent center (QC), oscillation zone and first lateral root primordium (LRP) are indicated. D. Longitudinal expression of graded auxin-responsive genes (groups 2 and 4 in B.), consisting of the remaining 1842 induced and 1255 repressed genes."

- the author claim that Fig 5B shows "a strong link between auxin responsiveness and spatial expression". This is not really convincing; it rather shows a drop of expression that depends on whether the genes are induced or repressed;

The longitudinal correlation with response to auxin results not only from induction or repression by auxin but also trends within these groups, as shown in the heatmap. The correlation of 0.58 for thousands of genes is quite high for a global pattern. Of course, the word "strong" is subjective, so we removed it in the revision of the manuscript.

page 15 2nd paragraph:

"This representation again revealed a link between relative responsiveness to auxin and spatial expression along the longitudinal axis of the root."

- I do not really understand how the authors obtained Fig 5C&D. The SEM is surprisingly low when considering the variability seen in Fig 5B.

Figures 5C&D represent the average row-normalized (standard deviations from the row mean) expression for the genes in groups 1-4 in the different slices of the Brady et al. root data. These groups consist of 1000 or more genes each, which contributes to the error of means (stdev/sqrt(n)). These figures have been described more clearly in the legend.

page 37 legend Figure 5:

"C-D. Average normalized longitudinal expression patterns of auxin-responsive genes, +/- SEM (groups 1-4 in B.). The relative spatial separation of the 13-slice dataset (Brady et al, 2007) is represented on the x-axis and the standard deviations from the row mean on the y-axis. C. Longitudinal expression of archetypal auxin-responsive genes (groups 1 and 3 in B.), consisting of the top 1000 induced and the first 1000 repressed genes. The quiescent center (QC), oscillation zone and first lateral root primordium (LRP) are indicated. D. Longitudinal expression of graded auxin-responsive genes (groups 2 and 4 in B.), consisting of the remaining 1842 induced and 1255 repressed genes."

It is also unclear to me how the authors really decided on the four groups of genes. They say that the genes can be "broadly subdivided into 4 categories" (P13). I am puzzled why they have not used a clustering approach for example rather than a qualitative subdivision.

The main point was that it was possible to find a cutoff along the continuum of fold induction that subdivided two groups of genes that showed coherent trends along the longitudinal axis. Without more information, optimizing the partitioning of these groups would have had its own subjectivity in choosing the method and would have been subject to over fitting. It seemed most appropriate to simply represent the cutoff as subjective. In brief, these groups were devised to visualize the relation between relative induction or repression and spatial expression along the longitudinal axis of the root and based on the trends seen in heatmap of Figure 5B (colored panels next to the heatmap). The figure legend has been modified to clarify.

page 37 legend Figure 5:

"Genes were ordered by fold-change response to auxin treatment; blue (low) to yellow (high) color-code indicates standard deviations from the row mean. Up- and down-regulated genes were further subdivided into groups 1-4 based on relative induction or repression and broad differences in longitudinal expression (red and green color coding). C-D. Average normalized longitudinal expression patterns of auxin-responsive genes, +/- SEM (groups 1-4 in B.)."

The response in Fig 5C and 5D seems then to show similar trends, mostly with different amplitudes. This would rather tend to say that there is broadly 2 groups, the repressed and the activated one.

We agree that the most apparent trend in the data is longitudinal expression of induced vs repressed genes. We further point to the observation that the relative strength of the up- or down-regulation also seems to have bearing on longitudinal expression. There is a difference in the longitudinal expression of strongly induced auxin-responsive genes compared to weakly induced auxin-responsive genes (e.g., green trend peak), and the same holds for repressed genes. We believe that this difference is noteworthy and may give us further understanding of the putative regulation of spatial expression by local gradients, as we discuss in the manuscript.

page 20 2nd paragraph:

"The auxin response can be seen to be bipartite; consisting of genes with an archetypal expression, including high expression in the root tip and secondary shootward peaks (group1 and complementary group3), and genes with a graded meristematic expression pattern (group2 and group4; Figure 5B-D). The archetypal response resembles the expression of the DR5 reporter (Supplementary Figure 1B), for which oscillating expression has also been observed in more shootward portions of the root that correspond to the regions of pre-branch site specification and lateral root primordia (De Smet et al, 2007; Dubrovsky et al, 2008; Moreno-Risueno et al, 2010). The graded response, however, more closely matches measured auxin concentrations (Petersson et al, 2009) and slopes along with the cellular-maturation gradient in the apical and basal meristem (Figure 1A, 5B-D). One speculation is that the archetypal response is directly under control of the auxin signal transduction machinery, including the negative feedback regulation, which could explain why these genes do not mirror the measured concentration gradient. The graded response may be under non-canonical or indirect regulation, conceivably through auxin-responsive master-regulator transcription factors that do reflect the developmental- and auxin-concentration gradient in the meristem (such as the PLTs (Galinha et al, 2007))."

10- The authors state on several occasion (e.g. Introduction-P3 2nd paragraph-, p16 - "endogenous auxin gradient"- and in the discussion) that there is a proximo-distal gradient of auxin in the root tip i.e. that auxin concentration diminishes with elongation and differentiation of the cells. In the introduction they cite Brunoud et al. 2012 to justify this statement. However Brunoud et al. demonstrate that the distribution of auxin in the root tip is not simply a proximo-distal gradient of auxin. They observe that, while auxin levels are high around the QC and in the xylem axis, auxin is lower in the other tissues of the meristem and start increasing closer to the start of the elongation zone. This has been also pointed out by Santuari et al. Curr Biol 2011. This is an important point for the discussion of results of this manuscript because these publications show that auxin concentrations is distributed differently between the different tissues of the root. The authors should
make use of these recent data and not oversimplify what is now known on auxin distribution in the root. Petersson et al. 2009 is an important study but the auxin measurements do not have a cellular distribution and the map of auxin proposed in this publication is an interpretation compatible with the tissular measurement that were performed in this paper.

We have added a section in the discussion addressing our findings in relation to tissue-specific gradients in more detail.

page 21 1st paragraph:
"It is important to note that there is likely a cell-lineage-specific aspect to the interpretation (or maintenance) of auxin gradients. This can be observed with the auxin signaling reporters DR5::3xVenus and DII-Venus (Supplementary Figure 1; (Brunoud et al, 2012; Santuari et al, 2011)). The correlation seen between the auxin response and the whole-root longitudinal data (Birnbaum et al, 2003; Brady et al, 2007), therefore, represents a global response-gradient that may consist of several distinct cell type-specific gradients. The availability of longitudinally separated markers of the same cell-lineage in the xylem (Figure 4C) makes possible the visualization of a response gradient in this tissue specifically that is consistent with mass spectrometric auxin measurements in the stele (Petersson et al, 2009). It will be interesting to see if similar gradients can be seen in other cell types, as more specific marker lines become available; especially in the epidermis where DR5::3xVenus and DII-Venus reporters indicate a putative inverted gradient (Supplementary Figure 1A-B)."

11- Again on the interpretation/discussion of the differences in auxin-induced responses between tissues, the authors propose at the end of the discussion (P18) that the distribution of auxin-inducible genes in the root is likely under the control of both auxin distribution and auxin signaling capacities of the cell. This is a sensible hypothesis that fits with the complex distribution of Aux/IAA and ARF in the root that is illustrated in Fig. S1 (and is compatible with a number of publications: Brunoud et al. Nature 2012, Rademacher et al Dev Cell 2012, Vernoux et al. MSB 2011, Rademacher et al. Plant J 2011 for the most recent and others notably from Weijers and colleagues). However the results hidden in Fig. S1 are in my view underexploited. They should be moved in the main Figs. and discussed in details (notably in relation to the work of Rademacher where all the ARFs where mapped) in order to present a clear vision of the distribution of the effectors of auxin-induced gene
transcription.

Supplementary *Figure 1 is a novel presentation of published results. We believe that juxtaposing these potential auxin signaling mechanisms with our global responses is premature right now, as the connection between the two datasets is intriguing but the specificity of the signaling mechanisms is speculative. This figure is meant as part of the background information given in the introduction and was therefore not given a prominent placement. We have added to the discussion addressing our findings in relation to cell type-specific auxin-response machinery.*

page 21-23:

"**Cellular competence for a unique auxin response**
Cells perceive auxin as though selectively processed…"

The analysis should also be extended by including the data on longitudinal expression of these signaling effectors in order to provide a map of their distribution in the root tip. This would seriously strengthen the paper and possibly (will it be the case?) provide support to statement like "the xylem lineage is uniquely tuned to respond to auxin" (P16). This is of particular interest in the light of the recent demonstration of an auxin maximum in the xylem instrumental for vascular tissue patterning (Bishopp et al. Curr Biol 2011). It would also open the possibility of an interesting discussion on auxin signaling effector distribution in different tissues (shoot, embryo from the papers cited above).

The longitudinal expression of the Aux/IAAs, ARFs and AFBs has been added to Supplementary Figure 1C and Supplementary Table 1. A reference to auxin's role in xylem determination has been added to the discussion.

Supplementary Figure 1C, Supplementary Table 1, page 18 2nd paragraph:

"Analysis of radial patterning in the stele of the RAM has indicated cross-talk between auxin and cytokinin modulates a PIN-driven high-auxin domain in the xylem that mediates cell specification (Bishopp et al, 2011). Consistent with high auxin levels in the xylem lineage, a subset of strong auxin-induced genes (including Aux/IAA6, 8, 19 and 29) shows high basal expression throughout the xylem (enriched in both the developing and maturing xylem; Supplementary Figure 1 and Figure 4B-C; Supplementary Table 4). The auxin-responsive xylem-enriched transcriptome can be used to investigate xylem specification by looking for potential auxin-responsive "master-regulators" of development."

Reviewer #2 (Remarks to the Author):

Major points:

1) How auxin responsive are the markers used? If these markers change their response by expanding into other cell types, this could definitely change the interpretation of their results.

Microscopic analysis of the used reporters after auxin treatment has been added (Supplementary Figure 2A). The results show that there is no apparent ectopic expression of the GFP marker expression upon auxin treatment conditions within the three hour treatment period. This has been noted in the text.

page 7 1st paragraph, Supplementary Figure 2A:

"Expression of the markers used was stable within the treatment period (Supplementary Figure 2A)."

2) The analysis from page 12 onwards refers to the longitudinal root dataset as a spatial dataset. This can get quite confusing as the first portion of the results refers to the tissue-specific response as a spatial response. I would be much more clear with respect to the longitudinal dataset as a temporal dataset.

We feel that to refer to this dataset as temporal may be also be confusing, as there is no time dimension to the sampling protocol. We have replaced "spatial" with "longitudinal" in reference to this dataset. For example,

page 15 1st paragraph:

"...and indicates that, for thousands of genes, auxin-sensitivity predicts longitudinal expression."

Also, it is unclear how for the Brady et al. 2007 dataset how data from all 6 sections in the meristem or all 4 sections in the maturation zone were considered. Were they considered as a composite or individually?

The separate slices composing the different zones of the root were visualized individually in Figure 5A and Supplementary Figure 6A. The meristematic and maturation zones in Supplementary Figure 6B-J refer to the independent longitudinal expression dataset by Birnbaum et al. (2003). The use of the Birnbaum dataset has been noted in the text.

page 15 1st paragraph:

"To quantify the relationship between auxin response and spatial expression along the longitudinal root axis, we examined the overlap of the 6850 genes with differential expression between the meristematic and maturation zone (t-test $p<0.01$; Supplementary Table 5 (Birnbaum et al, 2003)) and our extensive list of 5097 auxin responsive genes according to the ANOVA treatment factor. The two lists yielded an intersection of 2437 genes, for which fold-change of the auxin response (averaged over four tissues) was plotted against the fold-change in expression between the meristematic and maturation zone (Figure 5A)."

Minor notes:

Page 7 - a description of significant induction of Aux/IAA genes and GH3s is given. The statement is a little misleading - there is statistical significance for most of these genes on a gene by gene basis, but is there statistical significance for the entire group? This would be a simple hypergeometric distribution test and would be able to distinguish between both of these possibilities.

Although the proportional response of the Aux/IAAs, GH3s and SAURs is higher than expected by chance for a random sampling of genes, the enrichment of genes significantly responsive in our dataset among known auxin-responsive gene families does not necessarily speak to the validity of the measured response. These families are based sequence homology, the genes within each family do not necessarily all respond to auxin. Therefore, it is not the number of genes within these families that respond but that fact that we find numerous members of these families being induced, as such, that indicates that we have successfully activated the auxin response in our separate samples. The reference to the proportion of genes represented on the microarray to be seen responding within each of these families was therefore irrelevant and has been removed. We have changed the text (removing the misleading statement that we see significant regulation of gene families) and have added a concluding statement after the mention of these findings in the text.

page 9 1st paragraph:

"we observed the significant regulation of known auxin-responsive genes in the cell type-specific dataset. This includes significant regulation of 22 members of the Aux/IAA family of auxin co-receptors (Calderon-Villalobos et al, 2010), 14 GH3 auxin conjugases (Hagen et al, 1991), 18 SAURs (Hagen & Guilfoyle, 2002) and 7 LATERAL ORGAN BOUNDARY DOMAIN CONTAINING PROTEIN (LBD) transcription factors (Shuai et al, 2002) (Supplementary Figure 2C-F, Supplementary Table 2). Several of the responsive LBD genes, that are known to be involved in lateral root initiation (Okushima et al, 2007), displayed dramatic up-regulation specifically in the xp pericycle, the tissue where lateral roots originate (Supplementary Figure 2E). These results indicate the robust induction of known auxin responsive transcripts in the four cell types sampled in this work."

Page 8 - a statement is made "although genes are often reciprocally induced or repressed in different cell types" - I don't really see this in Figure 3A. In fact, I would say that only a small proportion of genes are reciprocally induced or repressed.

We meant reciprocally as a group: that if a gene is induced in one cell type, it will likely be induced (rather than repressed) in other cell types, not that they necessarily have the same strength of induction or repression. We see that the wording might have been confusing and have changed the text.

page 10 2nd paragraph:

"Although genes are most often regulated in the same direction (induced or repressed) in different cell types, the response is usually stronger in a subset of samples."

Page 17 - the Moreneo-Risueno paper shows that it is a TF that is driving the oscillation, not auxin.

We agree that the oscillations seen in the Moreno-Risueno et al. study are likely not due to oscillations of the local auxin concentration itself. The data presented here does not argue for or against this notion and does not provide evidence either way. We feel it is noteworthy that "archetypal" auxin responders also show expression peaks in the zone denoted by Moreno-Risueno et al and other studies (De Smet et al., Dubrovsky et al.) and is similar to the DR5 expression seen in this region. We have removed the word "oscillating" to avoid the impression that we are making definitive statements about the putative role of auxin in this oscillation.

page 20 2nd paragraph:

"The auxin response can be seen to be bipartite; consisting of genes with an archetypal expression, including high expression in the root tip and secondary shootward peaks (group1 and complementary group3), and genes with a graded meristematic expression pattern (group2 and group4; Figure 5B-D). The archetypal response resembles the expression of the DR5 reporter (Supplementary Figure 1B), for which expression has also been observed in more shootward portions of the root, similar to the secondary peaks of group1 (Figure 5C). For DR5, these shootward peaks of expression have been shown to correspond to the regions of pre-branch site specification and lateral root (De Smet et al, 2007; Dubrovsky et al, 2008; Moreno-Risueno et al, 2010)."

Figure S1 - what is the distance metric for the hierarchical clustering shown?

The metric used was Pearson correlation, this information has been added to the legend and methods.

page 38 legend Supplementary Figure 1:

"Genes were ordered based on hierarchical clustering (Pearson correlation) of the tissue-specific dataset"

Figure S4B - why are there 3 boxplots shown for each tissue type? This isn't explained in the figure legend.

The cell type-specific analysis consists of data from 13 marker lines. Template matching was used to identify genes enriched in each individual cell type or genes enriched in two marker lines of related cell types. For example, in the xylem there are three groups of genes: genes enriched in the maturing xylem (marker S18 compared to the 12 other marker lines), genes enriched in the developing xylem (marker S4 compared to the 12 other marker lines), and genes enriched in both (S18 and S4 compared to the 11 other marker lines). We have changed the legend text to clarify this.

page 7 2nd paragraph:

"This CTSE list was based on expression profile template matching in a select dataset of 13 non-overlapping cell type-specific expression profiles of sorted GFP marker lines (Supplementary Figure 2B, Supplementary Table 2). This procedure yielded a total of 3416 genes whose expression is enriched in one specific cell type (maturing xylem, developing xylem, xp pericycle, phloem-pole pericycle, phloem element, phloem companion cell, quiescent center, endodermis, cortex, trichoblast, atrichoblast, lateral root cap or columella) or in two related cell types (xylem, pericycle, phloem, ground tissue, epidermis or root cap)."

Supplementary Figure 2B, page 39 legend Supplementary Figure 2B:

"B. 3416 genes retrieved by template matching for tissue-specific expression in 13 non-overlapping GFP marker lines (Pavlidis algorithm, R>0.8) for genes enriched in one or two closely related cell types (see Supplementary Table 4) displayed in two heatmaps of row-normalized gene expression; blue (low) to yellow (high) color-code indicates standard deviations from the row mean. The heatmap on the left shows the expression in the 13 GFP marker lines used for the template matching : S18-maturing xylem, S4-developing xylem, J0121-xp pericycle, S17-pp pericycle, APL-phloem, SUC2-phloem companion cells, AGL42-quiescent center (QC), SCR-endodermis, C1-cortex, COBL9-trichoblasts, GL2-atrichoblasts, J3411-lateral root cap (LRC), PET111-columella. The heatmap on the right shows the average expression of these genes in the 6 samples (3 mock and 3 treated) gathered for each of the four GFP markers used in this study."

Supplementary Figure 5A, page 41 legend Supplementary Figure 5A:

"A. Boxplot representation of the fold-change distribution of cell-identity markers (CTSE, see Supplementary Figure 2B) that significantly respond to auxin treatment"

Reviewer #3 (Remarks to the Author):

I was puzzled why they selected a 3 hour time point following auxin treatment to sample after they showed every root tissue exhibited an auxin response after 30 minutes hormone treatment (based on degradation of the DII-VENUS marker).

The choice for this duration is elaborated on in the Materials and Methods section.

page 24 1st paragraph:

"Duration of treatment in was chosen to obtain a relatively early yet robust representation of responses to auxin in the root; before morphological effects, such as cell division, could be observed but late enough to include secondary/indirect target genes."

The authors describe several biologically interesting examples of uniformly and spatially distinct classes of auxin up and down responsive genes. They also note the enrichment of AuxRE elements in promoters of the former class of up regulated genes, but it was not clear whether this was also the case for auxin down regulated genes?

The AuxRE was not found to be enriched in any of the down-regulated clusters. We did find the GTGCAC motif overrepresented in a cluster of down-regulated genes in an additional promoter analysis included in the revised manuscript. This has been noted and discussed in the text.

page 12 2nd paragraph:

"Promoter analysis of the cell type-specific auxin-response clusters was conducted to look for overrepresentation of the canonical auxin response element TGTCTC (Liu et al, 1994). Clusters 15 and 16, which show relatively uniform up-regulation of gene-expression across tissues (Supplementary Figure 4A), contain significantly more genes with this element in the 500 bp upstream of their transcription start site than expected by chance (hypergeometric distribution analysis; Supplementary Table 3). Additionally, the occurrence of the generic TGTCNC and the individual -A-, -C- and -G- variants was examined and found that TGTCAC, TGTCCC and TGTCNC were also overrepresented in the promoters of the uniformly up-regulated genes assigned to dominant expression patterns 15 and 16. Furthermore, TGTCAC was overrepresented in the promoters of genes assigned to pattern 34, which shows down-regulation in all tissues that is strongest in the stele (Supplementary Figure 4A). None of these elements were significantly enriched in any other up- or down-regulated clusters. These results suggest that direct targets of auxin signaling through the auxin response promoter element are generally uniformly induced across tissues of the root and that variants of the canonical element may also participate in auxin regulation of transcript levels."

page 23 1st paragraph:

"Interestingly, the ARF binding site (the auxin response element TGTCTC) and several variants thereof (TGTCNC) are only found to be overrepresented among genes with a relatively uniform up-regulation (Supplementary Figure 4, Supplementary Table 3). This could indicate that, if the more spatially distinct responses and auxin-regulated gene repression are in part directly mediated by binding of particular ARF isoforms, a more complex DNA binding-site recognition may be involved in the target-specificity of different ARF isoforms. Alternatively, the spatially distinct responses could be composed more of indirect target genes that are regulated by secondary activators or repressors. Lastly, signal transduction outside of the TIR1/AFB-Aux/IAA-ARF auxin response pathway may also account for cell type-specific responses."

2nd Editorial Decision 08 July 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the reviewers acknowledge that you have satisfactorily addressed most of their major concerns. However, Reviewer #2 lists a number of issues that should be addressed and makes suggestions for modifications, which we would ask you to carefully address in a revision of the manuscript. Overall, this reviewer's comments mostly refer to the need to cautiously interpret and discuss the presented results in order to avoid overstatements.

As a matter of course, please make sure that you have correctly followed the instructions for authors as given on the submission website.

Thank you for submitting this paper to Molecular Systems Biology.

REFEREE REPORTS

Reviewer #1 :

The authors have adequately addressed most of the concerns I have raised. The revised version of the manuscript has been significantly strengthened by clarifications in the text and addition of new data. Notably the new analysis on the frequency of occurrence of ARF binding sites in the promoters of genes whose expression is modified by auxin treatment leads to very interesting conclusions and perspectives. The resulting manuscript represents an important addition to our knowledge in providing an exhaustive genome-level analysis of tissue-specific responses to a morphogenetic regulator.

Reviewer #2 :

Re-review of "A map of cell type-specific auxin responses"

Important conclusions of this manuscript are the same:

1. Different cell types respond to auxin differently
2. Auxin sensitivity of transcripts is associated with the promotion of meristematic state and repression of differentiation
3. There are two groups of auxin responses - the graded response and the archetypal response which correlates with two observed patterns: a. the DR5 marker gene and b. the reported cell type-specific auxin levels

All of my previous comments were sufficiently addressed. Some other minor issues need to be addressed.

Page 16 - bottom paragraph - the Vanneste et al. studies are described as being "non-meristematic root tissues" . This dataset represents newly initiated lateral root primordia and are therefore meristematic tissue.

Figure 5 and description of the archetypal and graded auxin response gene classes: It is unclear how these patterns were identified. Given that there was good use of clustering and significance changes to identify cell type-specific genes and auxin responses, it is unclear to me why the authors chose this subjective definition of gene groups along the longitudinal axis. Some sort of clustering method should be applied to more definitively call these gene groups. Otherwise the authors are likely missing on potentially interesting patterns and are choosing gene groups based on prior information.

Discussion:

Page 17: "auxin promotes or inhibits transcriptional cell identities differentially in the separate tissue

samples analyzed here". The authors are using molecular markers as a proxy for cell identity. However, the molecular markers described are also transcripts whose expression changes in response to auxin levels. This seems like a circular argument. The true experiment to demonstrate this would be enhanced auxin signaling in a single cell type and demonstration of an earlier adoption or inhibition of one of these markers.

Page 18: second-last paragraph. There is a description of genes that are strongly induced in both the developing and mature xylem and which are strongly auxin-induced. The authors suggest that these could be potential auxin-responsive "master regulators" of development. However, there is no evidence to even suggest this - wouldn't the genes that are expressed in meristematic xylem quite early on be better candidates for "master regulators" (i.e both necessary and sufficient to drive cell identity).

Page 19: "This correlation provides evidence than an endogenous auxin gradient directly influences the global transcriptional state of cells along this dimension to regulate maturation" - I disagree with the word "directly" - correlation does not equal causation - again, the authors would have to disrupt auxin signaling in a cell type-specific manner to show this.

2nd Revision - authors' response 18 July 2013

Reviewer #2 comments:

Page 16 - bottom paragraph - the Vanneste et al. studies are described as being "non-meristematic root tissues" . This dataset represents newly initiated lateral root primordia and are therefore meristematic tissue.

The text has been revised to address this issue. We want to emphasize that this analysis was conducted on the proximal portion of seedling roots, excluding the RAM.

Supplementary Figure 6, page 43 legend Supplementary Figure 6, page 16 2nd paragraph:
"In addition, correlation was observed between longitudinal expression and previously published data of auxin responses in proximal root tissues above the primary meristem (excluding the RAM; (Vanneste et al, 2005))."

Figure 5 and description of the archetypal and graded auxin response gene classes: It is unclear how these patterns were identified. Given that there was good use of clustering and significance changes to identify cell type-specific genes and auxin responses, it is unclear to me why the authors chose this subjective definition of gene groups along the longitudinal axis. Some sort of clustering method should be applied to more definitively call these gene groups. Otherwise the authors are likely missing on potentially interesting patterns and are choosing gene groups based on prior information.

The up- and down-regulated genes were grouped based on the intensity of the auxin response (fold-change) and the relation between intensity of the response and longitudinal expression. It is a one dimensional criterium so there was no opportunity to cluster. As the reviewer pointed out, the clustering on the larger dataset was already done and the subpatterns are observable in that analysis. The point of this analysis is to show that the relative strength of the response has bearing on the longitudinal expression. In Figure 5B, genes are ordered (from top to bottom) by the fold-change of their response to auxin treatment; it can be seen that the longitudinal expression of strongly vs. weakly induced and strongly vs. weakly repressed genes varies notably. The patterns observable in Figure 5B are extrapolated in Figure 5C&D and displayed in relation to the actual distance/size of the sections gathered by Brady et al. 2007. There may be subdivisions that reveal further details on the patterns but, without additional information, that

seemed to have the danger of overfitting the patterns. Choosing a precise cutoff did not seem justified and we felt it was best to present it as subjective

page 16 1st paragraph:

"Within the groups of induced and repressed genes, there were also notable differences in longitudinal expression patterns that were associated with relative sensitivity to auxin treatment. Using induction *versus* repression and relative fold-change of the response to auxin treatment to subdivide response strength, the 5097 auxin-responsive genes could be broadly subdivided into four categories"

Page 17: "auxin promotes or inhibits transcriptional cell identities differentially in the separate tissue samples analyzed here". The authors are using molecular markers as a proxy for cell identity. However, the molecular markers described are also transcripts whose expression changes in response to auxin levels. This seems like a circular argument. The true experiment to demonstrate this would be enhanced auxin signaling in a single cell type and demonstration of an earlier adoption or inhibition of one of these markers.

To clarify, we did not mean to imply that auxin necessarily acts at an early stage of development or has an exclusive role in specification, rather we observe here that it enhances the level of expression of cell-specific markers. We have changed the wording of the sentence to clarify this important distinction.

page 18 1st paragraph:

"auxin can promote or inhibit cell character by enhancing or repressing the expression level of cell-specific markers differentially in the separate tissue samples analyzed here"

Speaking to the first point of the reviewer, we note that, in Supplementary Figure 2B, we did track the response of cell specific markers in different cell types. While there was no a priori constraint that a marker respond to auxin in the cell type in which it endogenously enriched, we do find this pattern; meaning that these genes are competent to respond to auxin in some cells but not in others. We think this is an important point and this observation has now been emphasized in the revised text and is further illustrated by the inclusion of Venn diagrams in

Supplementary Figure 5B that show that these groups of auxin-responsive CTSE genes have skewed distributions of the tissue-specific auxin response as assayed by the individual t-tests in the different tissues analyzed here.

Supplementary Figure 5B, page 13 1st paragraph:

"Furthermore, auxin-responsive tissue-enriched gene clusters show cell type-specific auxin sensitivity. For example, auxin-responsive genes enriched in the developing xylem are predominantly induced in the stele and the majority of auxin-responsive genes enriched in trichoblasts are repressed in the epidermis (as judged by relative expression levels as well as the tissue-specific t-tests; Figure 4A, Supplementary Figure 5B)."

Page 18: second-last paragraph. There is a description of genes that are strongly induced in both the developing and mature xylem and which are strongly auxin-induced. The authors suggest that these could be potential auxin-responsive "master regulators" of development. However, there is no evidence to even suggest this - wouldn't the genes that are expressed in meristematic xylem quite early on be better candidates for "master regulators" (i.e both necessary and sufficient to drive cell identity).

To clarify, we did not mean to say that such regulators are to be found among the genes described above (i.e. "a subset of strong auxin-induced genes (including Aux/IAA6, 8, 19 and 29) [that] shows high basal expression throughout the xylem (enriched in both the developing and maturing xylem")*. Rather, we were referring to the xylem analysis as a whole; we might expect to find these among the auxin-responsive developing- or maturing-xylem-enriched genes. The text has been changed to avoid misconception.*

page 18 3rd paragraph:

"The auxin-responsive, developing- or maturing-xylem-enriched transcriptomes can be used to investigate xylem specification by looking for potential auxin-responsive high-order regulators of development."

Page 19: "This correlation provides evidence than an endogenous auxin gradient directly influences the global transcriptional state of cells along this dimension to regulate

maturation" - I disagree with the word "directly" - correlation does not equal causation - again, the authors would have to disrupt auxin signaling in a cell type-specific manner to show this.

The evidence that the effect of auxin signaling on the transcriptional state of the cells is direct (and not caused by, for instance, the redistribution of a secondary signaling molecule within the root) can be found in the analysis of transcriptional responses to gain-of-function Aux/IAA expression in protoplasts, where an inverse correlation with longitudinal expression in the root is observed upon the disruption of auxin signaling in dissociated cells (Supplementary Figure 6J). We have clarified this reasoning in the revised manuscript.

page 16-17:

"The relation between auxin-response and longitudinal expression could be recapitulated using independent auxin-response datasets collected in the root, in this study and by others (Bargmann & Birnbaum, 2009; Vanneste et al, 2005). The correlation for the various lists of genes regulated by auxin signaling was quantified by cross-referencing with the meristematic versus maturation dataset (Supplementary Figure 6B-K; Supplementary Table 5; (Birnbaum et al, 2003)). First, correlation was also evident with auxin responses measured in individual tissues and with auxin responses in the intact root as well as the stringent list of 2846 auxin-responsive genes (Supplementary Figure 6B-G; Supplementary Table 5). In addition, correlation was observed between longitudinal expression and previously published data of auxin responses in basal root tissues (excluding the RAM; (Vanneste et al, 2005)), indicating this correlation is not restricted to responses in the root apex (Supplementary Figure 6H). However, no strong correlation of auxin responses to expression in the root emerged from auxin-response profiles measured in whole seedlings (Okushima et al, 2007), suggesting that responses outside the root do not correlate with expression in the root tip (Supplementary Figure 6I). Lastly, an inverse correlation could be observed between longitudinal expression and effect of transient expression of gain-of-function Aux/IAA repressors of the transcriptional auxin response in root epidermal protoplasts (Bargmann & Birnbaum, 2009). Here, genes repressed by the expression of Aux/IAA19mII repressor (Tiwari et al, 2001) displayed relatively high meristematic expression whereas genes induced by the expression of a gain-of-function Aux/IAA repressor showed low expression in the meristem (Supplementary Figure 6J). Consequently, as a correlation between auxin-signaling and longitudinal expression is recapitulated by the manipulation of the canonical auxin signal transduction pathway in dissociated cells, the observed correlation can be attributed to a direct cellular response to auxin signaling."