The auxin signalling network translates dynamic input into robust patterning at the shoot apex

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 February 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two of the three referees whom we asked to evaluate your manuscript, and have decided to render a decision now to avoid any further delay. As you will see from the reports below, the referees generally acknowledged that this manuscript represented an large body of work, and they found the topic of potential interest. Nonetheless, they both raised substantial concerns, which, I am afraid to say, preclude publication of your work in its present form.

The second reviewer's concerns were the most fundamental. This reviewer had significant reservations regarding the key assumption that auxin receptor activity was relatively uniform across the SAM (especially given the higher expression of TIR1 in the inner meristem core), and felt that differences in auxin receptor activity could significantly influence the read-out from the DII-Venus reporter. While this reviewer suggested that additional discussion and more cautious interpretations may be helpful, s/he indicated clearly that this issue needed to be addressed convincingly before this work would be appropriate for publication, and additional experiments may be needed.

The first reviewer had some important, but more general, concerns regarding the presentation of this work. S/he felt that the Results and Methods sections should be expanded to include greater detail, in particular regarding the mathematical modeling. This reviewer also notes that standard deviation

tends to be unreliable with only two replicates -- the editor recommends showing either both data points or an error bar representing the range in Figure 1.

With your revised manuscript, we would like to ask that you provide machine readable versions of your mathematical model as supplementary material. Moreover, we strongly encourage authors, when appropriate, to provide models in the SBML format, and to deposit their models in a public database like BioModels or JWS Online.

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.

In addition, I would like to acknowledge that the review process took longer than normal in this case, in part because we had hoped to receive a report from a third reviewer who ultimately could not return their comments in a timely manner. I am sorry for the delay.

PLEASE NOTE As part of the EMBO Publications transparent editorial process initiative (see http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology now publishes online a Review Process File with each accepted manuscript. Please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this file, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office (msb@embo.org).

Yours sincerely,

Editor

Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

Vernoux et al. submit a manuscript that more or less comprehensively describes the expression patterns and protein-protein interactions within the auxin signalling network in the shoot meristem. Briefly, from a set of yeast two hybrid assays and complementing in planta interaction assays, the authors determine the possible interactions among auxin response factors (ARFs) and their co-suppressors (AUX/IAAs). They demonstrate that this interaction matrix is more specific than probably most in the field would have expected. They then map the possible interactions onto the shoot apical meristem based on the region-specific in situ expression patterns determined for a considerable number of ARFs and AUX/IAAs. The data are complemented by expression patterns determined for auxin receptors (which degrade AUX/IAAs upon auxin binding) and an exciting novel tool for relative in situ measurement of local auxin concentration. The interactions are then fed into a set of ODEs, which form regulatory networks whose response to auxin stimulus can be simulated. By matching these simulations with the expression patterns, the authors show that the differential ARF and AUX/IAA expression translates the dynamic input of changing auxin concentration patterns into a buffered, robust patterning.

To start with, I must admit that this manuscript presents an astounding volume of experimental data on the auxin perception network. Although not completely comprehensive, in part for technical reasons, this data set will be immensely useful to others and represents a major achievement in the plant hormone field. The conclusions drawn from the derived models are interesting and reconcile a few paradoxical observations, maybe most outstanding they provide an explanation why auxin concentrations and auxin response do not, and do not have to, always match.

The paper is accompanied by extensive supplemental information, and partly because of this, partly because of the format chosen, difficult to access. In my opinion, the authors should have presented this story as a full length paper, because in the present format, too many details and procedures remain hidden. For example, the whole modelling part is immensely cryptic to any outsider, probably even to colleagues who have some network modelling experience. Particularly one aspect that is not well explained in the paper is how the ODE models developed relate to the spatial lay-out of the shoot meristem, and in turn the experimentally observed local variations in auxin response/concentration.

Thus, the paper would benefit from being extended, from moving various key figures from the supplemental information into the "real" figures, maybe most important from providing an overview figure that outlines the process of how the ODE models and the different types of experiments come together (some type of workflow diagram).

On the model itself, it is not clear to me how this model was run and how the output was obtained (software environment)? Like many details, this might simply be lost in the wealth of supplemental information. Again, I think the authors should make an effort to make their work more easily accessible to the non-specialist. Also, I believe the authors should clearly line out the assumptions of the model (supplemental information 1, page 1) in the main text. Where possible, additional evidence/citation should be added. For example, has cooperativity of ARF dimers been demonstrated experimentally? Do auxin-responsive genes in fact contain multiple auxin-responsive elements to indicate that ARF dimerization is the "standard"? Also, are we confident that all ARFs are themselves not auxin-inducible? How do the models react to parameter variation in the assumptions (not tested for most)? etc.

Beyond the modelling part, the paper would benefit from some more experimentation/clarification. For example, the extensive yeast two hybrid matrix is excellent, but did the authors verify that all baits and preys are indeed stably expressed? That is, are some interactions missing because the respective fusion protein(s) are not expressed? One interesting aspect is the largely absent interactions between repressive ARFs and AUX/IAAs. Given that repressive ARFs still contain the conserved domains mediating AUX/IAA interaction, could the authors pinpoint, maybe with a few site-directed mutants, why the interaction does not take place? Can they identify the discriminating amino acids? Minor: regarding the qPCR experiments, it does not make sense to add a standard deviation if there were only two replicates.

Other comments:

I trust the authors on the characteristics of the DII-Venus auxin reporter based on the submitted, accompanying paper. Still, I believe if the latter paper is not in press, this manuscript cannot yet be published.

Reviewer #2 (Remarks to the Author):

Ms titled "The auxin signalling network translates dynamic input into robust patterning at the shoot apex" by Teva Vernoux and co-workers.

With the objective of addressing the relative contribution of auxin distribution and auxin signal transduction pathway for patterning in the SAM, authors have analyzed TIR1/AFB1, 2, 3 F-box proteins and the network of Aux/IAA and ARF transcriptional regulators in the SAM. The manuscript results from a tremendous amount of work. Update and exhaustive view of possible interactions between members of ARF and Aux/IAA families is provided however there is no clear evidence that defined pairs co-localize in cells of the SAM. in situ hybridizations for both ARF and Aux/IAA genes are provided on SAM sections, however not with a cellular resolution. A mathematical model integrating Aux/IAA and ARF network data is also proposed. Two predictions emerging from the model are investigated.

The original conclusion is that the signaling network (meaning here transduction of the auxin signal not auxin distribution) is essential to create robust patterns at the SAM.

Several issues impair a strong output of the manuscript:

Point 1: Homogenous or non-homogenous TIR1/AFB expression in the SAM?

Page 3: " TIR1 was weakly expressed, most obviously in the inner core of the meristem,..."

Based on the provided results (figure 1), expression of TIR1 is not that weak and interestingly seems to exhibit differences of expression across the SAM, as an increase in the outer layer of the center and at the periphery both in outer and inner layers of primordia initiation sites.

This point is critical as interpretation of the data within the whole manuscript is based on the "assertion" that there is no difference of expression of F-box proteins targeting the degradation of Aux/IAA throughout the SAM. TIR1 pattern of expression suggests that there are significant differences. Even subtle changes of TIR1 might influence the responsiveness. In addition, figure S10 reveals a significant discrepancy between TIR1 and ABF1 interaction with DII:VENUS whatever the auxin concentration used in the assay, suggesting a difference of relative affinity for Aux/IAA substrates. It can make a huge difference in vivo even if AFB1 is more abundant in the meristem. Parry et al. (PNAS 2009) reported already a strong expression of AFB1 in seedlings in comparison with TIR1 and other members of the family (AFB2, AFB3), differences likely to be due to the absence of regulation of that F-box by miRNA. The same manuscript also reported that "Both TIR1 and AFB2 interact with each Aux/IAA protein "(namely IAA3 and IAA7)" in an auxin-dependent way . In contrast, Aux/IAA binding to AFB1 and AFB3 was low or undetectable

with this assay. These results suggest that AFB1and AFB3 may have a reduced affinity for Aux/IAA proteins compared to TIR1 and AFB2." Here, the authors assumed that degradation of Aux/IAAs at the SAM mainly results from AFB1 because of its high expression. The contribution of AFB1 in the degradation of Aux/IAA substrates is not that clear. One would expect to detect alterations of SAM development in afb1 mutants if that particular F-box is so critical, it is not the case. Expression of other AFBs should be investigated to provide a complete picture of TIR1/AFB expression in SAM. In addition, if there are high and low affinity TIR1/AFB and Aux/IAA pairs, even a low level of expression of one of the F-box will change the capacity of the response to auxin.

A non homogenous expression of even one of these F-box, here at least TIR1, completely modifies the interpretation of the data. An homogenous expression of AFB1 was the starting point of the manuscript and differences for TIR1 were neglected. Previous report and experimental data in this manuscript indicate that there are differences in TIR1/AFB expression and differences of affinity between TIR1/AFB and Aux/IAAs; thus, authors must reconsider their interpretation.

For similar reasons, it is not possible to interpret DII:VENUS changes as the reflect of auxin distribution.

Point 2: SAM reduced to center and periphery?

P4: "...signalling components albeit at different concentrations"

Authors distinguish only between the center and the periphery to discuss about the expression of ARFs and Aux/IAAs whereas a much more subtle spatial distribution would have to be taken into account. Based on figures 1, S1 and S2 rather obvious differences can be observed in terms of relative abundance AND expression pattern for both ARFs and Aux/IAAs. Previous reports, including by some of the authors, brought evidences supporting that pairs of Aux/IAA -ARFs can differ from one cell to the other. The resolution at the level of center versus periphery is an approximation that biases the interpretation of the data. It is not clear whether it can be more precise based on the available in situ hybridization data, but authors can at least in the discussion introduce the limits of their observations.

P4: "...the presence of ARFs together with elevated auxin at the periphery likely explain the observed distribution of Aux/IAAs."

Can the authors be more precise? The periphery is not an homogenous zone, spatial variations correlating with primordia initiation have been shown to be essential, including by the authors themselves and others. Coming back to the periphery as a whole is confusing.

Point 3: interpretation of connectivity profiles

P5: "...(iii) ARF repressors do not interact with other proteins in the network."

In the clustering analysis, authors conclude that ARF repressors do not interact with other proteins in the network, it might to true considering cluster III as a whole, however low connectivity for cluster III can also be interpreted as the reflect of more specific interactions of proteins of this cluster (ARF-) with a limited number of Aux/IAAs. Such more specific interaction can be of significant importance, affecting cellular responses of given cells and not of others. Once again the analysis favors the basis of a uniform response capacity instead of a more versatile model where for example combination of various repressors would affect Aux/IAA availability or recruitment by TIR1/AFB and thus will affect gene expression. Alternative interpretation should be discussed.

Point 4: DII:VENUS

P7: "By design, these fusion proteins monitor local degradation of Aux/IAAs, which corresponds to the input of the signaling pathway and depends on auxin levels and/or on local differences in auxin perception"

...corresponds to local differences in auxin perception - this is true actually, authors should thus be more cautious when discussing DII:VENUS data. Its degradation results from two distinct parameters and one is not directly linked to the auxin content.

P8: " Taken together, our results suggest that DII-VENUS reports auxin distribution in the SAM."

Taken into account what is discussed before (point1), this sentence is definitely too strong. There is no doubts that DII:VENUS degradation is enhanced by auxin and auxin changes in vivo, however this is also clearly not the only parameter influencing its accumulation or degradation. The risk with such strong assessment is also that further use will be made as "DII:VENUS is an auxin concentration reporter". DII:VENUS cannot be considered as something else than an auxin response reporter. It does not weaken the interest for this novel reporter as it is much faster and potentially more sensitive than DR5 but in terms of interpretation in this manuscript it has to be reconsidered.

Other points:

Abstract: "... to visualize dynamically auxin distribution..."

As a consequence of points discussed before, distribution should be replaced by responses

P3: "A complex ensemble of 29 Aux/IAAs and 23 ARFs regulates gene transcription in

response to auxin".

Authors should mention that additional regulators are also involved as the Topless repressor for example

P3: "TIR1 F-box of the SCF complex (or its AFB homologs) that acts as an auxin receptor"

It was titled as such in the original publications but the binding of the TIR1 F-box to auxin requires an Aux/IAA F-box substrate, it would be more precise to mention that they act as co-receptors.

P4: "...suggesting an unexpected cooperative function".

This wording "cooperative" has an ambiguous meaning: is it really what the author means? context. Co-expression of activators and repressors is not sufficient to evoke a cooperative function that corresponds to a precise biochemical mechanism.

P8 : "This demonstrated important physiological variations in overall auxin levels"

What physiological variations mean here? is it closely related to the process of primordia initiation or considering the time lapse something more global as circadian changes? Why considering that it is only auxin levels? is there anything known on TIR1/AFB or ARF... expression in response to these "physiological variations" ?

P8: The fact that DII:VENUS and DR5:VENUS do not exhibit "reciprocal" changes might also reflect that DR5 is a poor reporter of auxin responsiveness (auxin and signaling). Repeats of auxin response elements within the DR5 promoter might be responsible for the observed " buffering" effect.

p9: "We thus demonstrate that the rather simple distribution..."

instead of "demonstrate" , provide evidence would be more appropriate

In conclusion, the issues have to be addressed and fairly more balanced interpretations have to be considered. It will reinforce the original data of the manuscript that is to provide evidence supporting that the signaling network (means not only auxin distribution) is essential to create robust patterns at the SAM. Suppressing some dogmatic assertions from the manuscript will also help to gain into quality. The work supporting the manuscript is rather impressive and merits future consideration however it is impaired by over-interpretation or mis-interpretation. Despite the general interest for the manuscript and the consideration for the impressive work what was achieved, I cannot recommend consideration of the present manuscript but would encourage resubmission of a substantially revised manuscript.

1st Revision - authors' response

We would like to submit a revised version of our manuscript entitled "The auxin signalling network translates dynamic input into robust patterning at the shoot apex" by Vernoux et al. for consideration as an article in Molecular Systems Biology.

We are grateful for the positive comments and useful suggestions of the reviewers. In response to these suggestions and with your agreement, the manuscript has been now presented as a full-length article.

We have also addressed all the specific concerns of the reviewers in the following way:

Reviewer 1:

The reviewer 1 raised the following points:

1- *The presentation of the work:*
-- Reviewer comments: "The paper is accompanied by extensive supplemental information, and partly because of this, partly because of the format chosen, difficult to access. In my opinion, the authors should have presented this story as a full length paper, because in the present format, too many details and procedures remain hidden. For example, the whole modelling part is immensely cryptic to any outsider, probably even to colleagues who have some network modelling experience. Particularly one aspect that is not well explained in the paper is how the ODE models developed relate to the spatial lay-out of the shoot meristem, and in turn the experimentally observed local variations in auxin response/concentration. Thus, the paper would benefit from being extended, from moving various key figures from the supplemental information into the "real" figures, maybe most important from providing an overview figure that outlines the process of how the ODE models and the different types of experiments come together (some type of workflow diagram)." --

We agree that it was important to make the manuscript more accessible and the paper might have been difficult to access in part due to the choice of a short format. To answer this point and with the agreement of the editor, the manuscript is now presented as a full-length article.

To explain better how the different parts of the work are connected (and notably the use of the modelling to interpret the biological results), we have also followed the reviewer suggestion and added a flowchart showing the logic of our work presented in a new Figure 1.

In addition, we have used the extra place to clarify in the text how we used the model to analyze the functional implications of the spatial distribution of Aux/IAAs and ARFs in the meristem. We notably state clearly that this is based on a simplified interpretation of the distribution of the genes in the SAM corresponding to the general trend identified by our in situ hybridisation analysis (Page 12 3rd paragraph). The model and simulations are now in an independent figure (Figure 4) in which we have added a 3D plot (Figure 4C) that allows visualizing the effects of both the ARF activator-to-repressor ratio and the total ARF concentration. This 3D plot clarifies how we can predict a higher sensitivity to auxin at the periphery despite the fact that both ARF activators and repressors are expressed at high levels in this part of the meristem (this is explained Page 12 4th paragraph). We have also explained in more details in the text why we study the effect of oscillations and how we relate this to the situation in the meristem (Page 13 2nd paragraph). For the sake of simplicity, we have chosen not to add extra illustrations on that aspect in Figure 4 and refer the reader to the Note S1, where we now have put figures representing the effect of the different parameters of the model on its behaviour.

2- The presentation of the model:

-- Reviewer comments: "On the model itself, it is not clear to me how this model was run and how the output was obtained (software environment)? Like many details, this might simply be lost in the wealth of supplemental information. Again, I think the authors should make an effort to make their work more easily accessible to the non-specialist. Also, I believe the authors should clearly line out the assumptions of the model (supplemental information 1, page 1) in the main text. Where possible, additional evidence/citation should be added. For example, has cooperativity of ARF dimers been demonstrated experimentally? Do auxin-responsive genes in fact contain multiple auxin-responsive elements to indicate that ARF dimerization is the

"standard"? Also, are we confident that all ARFs are themselves not auxin-inducible? How do the models react to parameter variation in the assumptions (not tested for most)? etc." –

We have now stated in the Material and Methods section (Page 34) which software environment was used to build the model and to run the simulations. We are also providing with the manuscript a SBML format version of the model that will also be deposited in the Biomodels database.

A large part of the description of the model has now been included as a Result section (starting on Page 9 3rd paragraph). We clearly line out all the assumptions used for the model and we have also added references as requested to justify these (Page 10). To ease the reading, the details of the equations have been put in the Material and Methods (starting Page 31 2nd Paragraph). However, we highlight in the results that the most important aspect of the model is that it allows analysing the transcriptional output as a function of auxin concentration and more generally of the input into the pathway (Page 11 2nd Paragraph).

Concerning the cooperativity of ARF activator dimers (assumption (v) on Page 10), this is suggested by the work of Ulmasov et al. 1999. We have not used this as the standard but rather considered that a cooperative effect can occur when 2 ARF activators are bound on the promoters that in our model contain two AuxREs (assumption (ii) on Page 10).

Concerning the number of AuxREs in promoters, we are referring the reader to several references (Page 10 1st Paragraph) and notably the one of Goda et al 2004 that suggests a majority of promoters with one to two AuxREs.

We also state Page 10 1st Paragraph that only 2 amongst the 23 ARFs are induced by auxin (notably based on Paponov et al. 2008 that analyzed various published microarray datasets in addition to the one they generated), an observation that is in support of assumption (iv) on Page 10.

Concerning the effects of parameters on the model, we have extended the Note S1 in order to show the effect of varying these parameters on the model behaviour. This analysis led to the conclusion that the model is very robust to parameter variations ((ii) on Page 11 3rd Paragraph, continued on Page 12).

3- Clarification on the yeast two hybrid dataset:

-- Reviewer comments: "For example, the extensive yeast two hybrid matrix is excellent, but did the authors verify that all baits and preys are indeed stably expressed? That is, are some interactions missing because the respective fusion protein(s) are not expressed?" --

We have verified expression for all baits and preys using Western blots. We have detected expression for all the fusions to the GAL4 binding domain. Expression was also confirmed for all the fusions to GAL4 activation domain except for 5 of them. This is now pointed out in the Material and Methods on Page 27 at the end of the first paragraph:

"We confirmed expression for all the Gal4-BD fusions but a few Gal4-AD fusion were very weak or could not be detected indicating that the number of interactors might be underestimated for ARF9, ARF17, IAA11, IAA20 and IAA30."

This observation thus validates the quality of our dataset, although these results suggest that some interactions might be missing for these 5 genes.

4- On the absence of interactions between ARF repressors and Aux/IAAs:

-- Reviewer comments: "One interesting aspect is the largely absent interactions between repressive ARFs and AUX/IAAs. Given that repressive ARFs still contain the conserved domains mediating AUX/IAA interaction, could the authors pinpoint, maybe with a few site-directed mutants, why the interaction does not take place? Can they identify the discriminating amino acids?" --

We agree that this is a very interesting question raised by our results. To answer it, we have attempted to identify elements in the sequence that could explain this interaction behaviour. We used sequence alignments of domain III and IV and reconstruction of phylogenetic trees using the most conserved amino-acids in these domains. This analysis did not allow pinpointing any obvious candidate amino-acid changes that would explain this absence of interactions. This suggests that the reason might be structural and not obviously understandable from the raw DNA sequences. Answering this specific point would thus require work that we believe is out of the scope of the manuscript. Also, we have not added in the paper the analysis explained above given the large amount of data already presented in it.

5- Presentation of qPCR data on Figure 2 ZE:

-- Reviewer comments: "Minor: regarding the qPCR experiments, it does not make sense to add a standard deviation if there were only two replicates." –

The error bars now represent the range of values and not the standard deviation.

Reviewer 2:

The reviewer asked us on several occasions to discuss the limit of our observations/interpretations and alternative hypotheses. To do so, the manuscript is now presented as a full-length article and the discussion starting Page 18 was written in order to address this criticism. We will precise this below more specifically while explaining how we have answered the different issues raised by the reviewer.

The reviewer 2 raised the following main points:

1- *The distribution of TIR1/AFBs in the meristem and its interpretation:*
-- Reviewer comments: " Homogenous or non-homogenous TIR1/AFB expression in the SAM?"
"A non homogenous expression of even one of these F-box, here at least TIR1, completely modifies the interpretation of the data. An homogenous expression of AFB1 was the starting point of the manuscript and differences for TIR1 were neglected. Previous report and experimental data in this manuscript indicate that there are differences in TIR1/AFB expression and differences of affinity between TIR1/AFB and Aux/IAAs; thus, authors must reconsider their interpretation." --

We fully agree with the analysis of the reviewer and with the fact that the distribution of the TIR1/AFB co-receptors had to be carefully described and taken into account in the interpretation of both the role of the ARF-Aux/IAA pathway and of the patterns of DII-VENUS fluorescence (see also point 4 below).

To answer this point, we have first performed in situ hybridisations with the AFB4 and AFB5 to provide a complete vision of the expression of TIR1/AFBs in the SAM as requested by the reviewer (Fig 2D, Supp Fig 1D,E). We have also provided serial sections in Supp Fig1 for all of the TIR1/AFB (only one section when there was no expression) to allow easier visualisation of the spatial pattern. The conclusion from this analysis is now that TIR1, AFB1 and AFB5 control auxin-dependent degradation of Aux/IAAs in the SAM, since we could not detect AFB4. Note that we still say that TIR1 has a weak expression in the meristem (Page 6 second Paragraph) compared to AFB1. This is not based on the observation of the pattern in the meristem but on the time needed to detect the GUS staining in the inflorescence tissue. As illustrated directly on Figure 2 and stressed in the legend, AFB1 staining is obtained after 8 h while TIR1 staining required 48 h.

In the main text (Page 6 second Paragraph) we also describe in details the expression patterns. We state clearly that TIR1 is lower in the L2 and L3 layers at the centre of the meristem. AFB5 although weakly expressed also showed a differential expression with a higher expression in the internal tissue of the organ primordia. We stress several time in the manuscript (Results: Page 7 second Paragraph, Page 13 middle of first Paragraph, Discussion: Page 19 2nd Paragraph) that this differential regulation of F-box receptors might create a differential in the auxin-induced degradation capacity of Aux/IAAs that would be lower in the internal tissues at the centre and higher in the internal tissues at the periphery (although we say in the discussion that further experiments will be needed to definitely prove this). This follows the general trend observed in the expression of Aux/IAA and ARF (lower expression at meristem centre and higher expression at meristem periphery) and we thus expect it to participate to creating the differential sensitivity predicted by our model and confirmed by using DII-VENUS and DR5::VENUS together.

Also, to clarify how changes in perception can be accounted for in the model, we state in the description of the model (that is now largely included in the main text) that the parameter "x" representing auxin modulates Aux/IAA degradation rate as a function of both auxin concentration and auxin perception (Page 11 2nd Paragraph). A change in this degradation rate can thus be obtained either upon a variation in auxin level or a variation in auxin perception, and these two parameters play a perfectly symmetric role in the function. The parameter "x" can thus be viewed more generally as representing the "auxin signalling input". This thus allows taking into account the variations of the TIR1/AFBs in our interpretation of the role of auxin signalling in the SAM.

The interpretation we propose for the differential expression of TIR1/AFBs is the simplest one given our current knowledge. We are discussing this point Page 19 2nd Paragraph and, as was pointed out by the reviewer, we stress the fact that TIR1 and AFB1 show significantly different relative affinity to IAA28, similarly to what has been observed for IAA3 and 7. Despite their difference in expression level, they are thus both expected to be important for Aux/IAA degradation in the SAM as was pointed out by the reviewer. Although further experiments will be needed, we discuss that the differential distribution of TIR1/AFB could globally affect the capacity to degrade Aux/IAAs and participate to establishing a differential sensitivity to auxin in the SAM.

2- The reduction of the SAM to centre and periphery:

To address this point we have described in more details the different expression patterns of Aux/IAAs and ARFs (Page 6 third Paragraph continuing on Page 7). Since the goal of our analysis was to conduct a global analysis of the role of auxin signalling (using the systems biology approach illustrated now by the flowchart in Figure 1), we clearly state that we have chosen to focus on a general trend in expression that appears to be true for a majority (but not all) of the genes (Page 7 2nd Paragraph). We however highlight the fact that the differential expression between the centre and the periphery of the SAM is often accompanied by an even higher expression in the organ primordia (notably Page 7 2nd Paragraph). We also clearly state Page 12 3rd Paragraph that this is a simplified view of the meristem that was used for the interpretation.

We are aware that it has been reported that specific combinations of Aux/IAAs and ARFs could be the basis for the specificity of auxin responses and refer to it at the beginning of the discussion (Page 18 3rd Paragraph). However in the SAM, we uncovered a much more simple situation than what could have been expected with 12 ARFs and 13 Aux/IAAs expressed in the structure, as highlighted Page 18 3rd Paragraph. To show the limits of our interpretation, we also discuss (Page 21 first Paragraph) that there is exceptions to this general principle and focus on the example of the patterns of IAA20 and 30, two non-canonical Aux/IAAs, which might generate different signalling capacities in the pro-vasculature.

3- The interpretation of connectivity profiles

-- Reviewer comments: "Once again the analysis favors the basis of a uniform response capacity instead of a more versatile model where for example combination of various repressors would affect Aux/IAA availability or recruitment by TIR1/AFB and thus will affect gene expression. Alternative interpretation should be discussed." --

As discussed above, we have focused throughout this systems biology work on the general characteristics of the system rather than considering exhaustively its specificities. Although taken together our data support our interpretation, we agree that it was important to stress that alternative interpretations are likely relevant. We thus now discuss several specificities in the interaction profiles that could play a role in providing different characteristics to the signalling network, in order to highlight the limits of our interpretation (Page 20 2nd Paragraph).

4- The interpretation of DII-VENUS as a direct tool for visualization of auxin:

-- Reviewer comments: "For similar reasons, it is not possible to interpret DII:VENUS changes as the reflect of auxin distribution."

"Taken into account what is discussed before (point1), this sentence is definitely too strong. There is no doubts that DII:VENUS degradation is enhanced by auxin and auxin changes in vivo, however this is also clearly not the only parameter influencing its accumulation or degradation. The risk with such strong assessment is also that further use will be made as "DII:VENUS is an auxin concentration reporter". DII:VENUS cannot be considered as something else than an auxin response reporter. It does not weaken the interest for this novel reporter as it is much faster and potentially more sensitive than DR5 but in terms of interpretation in this manuscript it has to be reconsidered." --

We fully agree that DII-VENUS is not a bona fide auxin sensor and we have been careful to call it an "auxin signalling input sensor" throughout the manuscript (starting with the abstract). We have added in the manuscript data showing a weak auxin-induced increase in binding of AFB5 to the domain II of IAA28 (Fig 5E). This indicates that the 3 co-receptors present in the SAM regulate DII-VENUS degradation. We have also removed from the Results section the conclusion that it allowed to visualize directly auxin distribution in the SAM and now stress that it depends on both auxin and perception (Page 16 end of 1st Paragraph). The rest of the text has also been carefully edited along the same line.

We however discuss (Page 21 second Paragraph) the fact that DII-VENUS can provide information on auxin distribution if the spatial expression of the F-box co-receptors is known. At the centre of the SAM, the lower expression of TIR1 is expected to lower DII-VENUS degradation capacities and to lead to an underestimation of the auxin parameter. The fact that DII-VENUS is efficiently degraded at the centre despite the lower TIR1 expression is thus compatible with high auxin concentrations. Similarly AFB5 shows a higher expression in the internal tissue of the organs where DII-VENUS is generally not detectable. This might lead to a more efficient degradation of DII-VENUS in these tissues but the apparent low affinity of AFB5 to domain II of IAA28 allows again predicting high auxin concentrations. However we state clearly that these are estimates of relative auxin concentrations (Page 21 beginning of second Paragraph).

Other points:

-5: -- Reviewer comments: "Authors should mention that additional regulators are also involved as the Topless repressor for example" –

We have now mentioned the role of Topless in the introduction, Page 4 second paragraph.

-6: -- Reviewer comments: "P3: "TIR1 F-box of the SCF complex (or its AFB homologs) that acts as an auxin receptor". It was titled as such in the original publications but the binding of the TIR1 F-box to auxin requires an Aux/IAA F-box substrate, it would be more precise to mention that they act as co-receptors." –

As proposed, TIR1/AFBs are now called co-receptors and not receptors in the text.

-7: -- Reviewer comments: " P4: "...suggesting an unexpected cooperative function".

This wording "cooperative" has an ambiguous meaning: is it really what the author means? context. Co-expression of activators and repressors is not sufficient to evoke a cooperative function that corresponds to a precise biochemical mechanism." –

This phrase has been removed to avoid confusion (Page 8 end of first paragraph) and replaced by one stating simply that the expression patterns suggest a role for the co-expression of ARF activators and repressors.

-8: -- Reviewer comments: " P8 : "This demonstrated important physiological variations in overall auxin levels". What physiological variations mean here? is it closely related to the process of primordia initiation or considering the time lapse something more global as circadian changes? Why considering that it is only auxin levels? is there anything known on TIR1/AFB or ARF... expression in response to these "physiological variations" ? " --

We have removed the word "physiological" from the text (Page 19 second Paragraph). Understanding the origin and the role of these fluctuations in the SAM would require a large body of analysis that we believe is beyond the scope of the present manuscript. We simply conclude the discussion (Page 21 2nd Paragraph) by stressing the fact that the role of these fluctuations will need to be explored.

-9: -- Reviewer comments: "The fact that DII:VENUS and DR5:VENUS do not exhibit "reciprocal" changes might also reflect that DR5 is a poor reporter of auxin responsiveness (auxin and signaling). Repeats of auxin response elements within the DR5 promoter might be responsible for the observed " buffering" effect." --

The available data indicates that DR5 is the best available tool for analysing auxin transcriptional responses and we refer to these in the text (page 14 2nd Paragraph). A large number of publications show that it responds well to auxin treatments in a large range of conditions and made it a tool of choice for our analysis. Its design with 9 AuxREs in its promoter is expected given our current knowledge to make it very sensitive to changes in the signalling input by binding of multiple ARFs. This is consistent with the high amplitude of its activation upon auxin treatments in root tissues and with the activation of DR5 upon auxin treatment at the periphery of the meristem (as shown notably in de Reuille et al. PNAS, 2006 and Smith et al. PNAS, 2006). It has also been used successfully for analysing developmental processes involving changes in auxin concentration, such as gravitropism.

A parameter that could have strongly buffered responses would have been a high stability of the VENUS protein. However, we provide evidence (Supplementary Figure 9) that VENUS has a turn-over in the SAM that is shorter than the time-scale of the variations in DII-VENUS we are observing. This important control and the considerations above strongly support our conclusions.

-10: -- Reviewer comments: "In conclusion, the issues have to be addressed and fairly more balanced interpretations have to be considered. It will reinforce the original data of the manuscript that is to provide evidence supporting that the signaling network (means not only auxin distribution) is essential to create robust patterns at the SAM. Suppressing some dogmatic assertions from the manuscript will also help to gain into quality. The work supporting the manuscript is rather impressive and merits future consideration however it is impaired by over-interpretation or mis-interpretation. Despite the general interest for the manuscript and the consideration for the impressive work what was achieved, I cannot recommend consideration of the present manuscript but would encourage resubmission of a substantially revised manuscript." --

We have extensively revised the manuscript to answer the different issues that were raised. We believe that this version of the manuscript presents more balanced interpretations and that we have removed over- or mis-interpretations as well as dogmatic assertions.

PS: I would also like to mention that a revised version of our manuscript providing further characterization of the DII-VENUS auxin reporter in root tissues is currently being resubmitted and is thus following the same timeline.

Acceptance letter

18 May 2011

Thank you again for sending us your revised manuscript. As you will see below, the reviewers indicated that they are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor

Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

The authors have submitted a revised version of their paper and made a serious effort to address my earlier criticisms. For example, the format has been changed to the longer version and has been rewritten, making the paper much more accessible. Expression of preys and baits in yeast two hybrid has now been verified by Western analysis, etc. I concur with the authors that some experiments asked, like point mutants to determine why certain ARFs cannot interact with AU/IAAs, are going a bit beyond the scope of this study. So all in all, I am very satisfied with the revisions.

Reviewer #2 (Remarks to the Author):

My comments are short and positive:

The revised manuscript is significantly improved and the novel format is fully justified. Most of the points that I raised in the first review have been addressed properly either by providing additional and original data or by improving writing of both results and discussion. I feel the manuscript merits now to be considered for publication.

