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The MADS transcription factor XAL2/AGL14 modulates auxin transport during Arabidopsis root development by regulating PIN expression

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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.)

Editor:

1st Editorial Decision 30 May 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

The referees appreciate the analysis, but also find that additional experiments are needed to fully support the conclusions drawn. Some of the issues raised concern the need for a better analysis of the root phenotype in XAL2 mutants, that the expression of XAL2 needs to be further clarified as well as additional support for a direct regulation of PIN1 and PIN4 by XAL2. Should you be able to address the raised concerns in full then we would consider a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Work of Garay-Arroyo et al., reveals that XAL2/AGL14 transcription factor of the MADS box gene family directly transcriptionally regulate expression of several members of auxin efflux family. Authors thoroughly analyzed root phenotype of xal2 mutants and observed developmental abnormalities (reduce size of roots and root meristem; defects in QC specification and collumela differentiation, modulated DR5 auxin reporter expression) indicative of defects in the polar auxin transport . Direct measurements of shootward and rootward auxin transport confirmed reduction in auxin transport through the root. Expression analysis of PIN genes in xal2 mutant demonstrate that XAL2 is involved in the regulation of PIN1 and PIN4 expression, and no significant effect on PIN2 and PIN7 expression was observed. Direct transcriptional regulation of PIN1 and PIN4 auxin efflux carriers by XAL2/AGL14 was shown using chromatin immunoprecipitation approach.
The work reveals that MADS-box gene XAANTAL XAL2/AGL14 transcriptionally regulates two members of auxin efflux carrier family PIN1 and PIN4. The work is technically well performed, clearly presented and discussed. The phenotype analysis of xal2 loss of function mutant and expression analysis of PIN auxin efflux carriers in xal2 clearly demonstrate auxin transport defects.
My main concern is related to evidence on the direct regulation of PIN1 and PIN4 by XAL2. As this is one of the core messages of the work the direct transcriptional regulation of PIN should be confirmed by other technic such as EMSA or Y-1H assays. Eventually, it would be useful to test whether other part of PIN1 promoter sequence interact with XAL2 and thus examine specificity of XAL2 binding to CArG box fragments containing promoter parts.
Related question to specificity of the XAL2 interaction with PIN1 and PIN4 promoters. Did authors check the presence of the CArG box fragments in PIN2 and PIN7 genes, which do not appear to be regulated by XAL2 transcription factor?

Other comments:

1. Do overexpression of 35S::XAL2 enhances PIN1 and PIN4 expression?

2. It would be useful, to present detailed XAL2::GUS expression analysis (higher magnification then presented at Figure 6C) in root meristem to confirm in situ hybridization results (Figure 1A).

3. Discrepancy in description figure S3A versus Figure legend

4. Figure 3 and Figure 4A might be fused into one panel and part of data presented in panel 4A (showing defect of QC specification in xal2 mutant using several different reporters) might be transferred to supplementary material

5. I have impression that some of presented figures might be distorted probably due to scale adjustment (Fig. 4A WTx QC25, xal2-2xQC25-:GUS; supplement Figure S1)

Referee #2

In the manuscript, the authors report that a MADS-box gene XAL2/AGL14 regulates expression of PIN1, PIN3 and PIN4 in Arabidopsis roots and thus it is important for maintain auxin gradients along the roots.
Major comments:
-My major concern is about the expression patterns of XAL2. If XAL2 regulates the transcription of PINs, one would expect to see the overlapping of their expression domains in roots. However, there is discrepancy in current expression data, i.e, in situ RT-PCR data (Figure 1) and GUS reporter data (Figure 6). The authors stated that by in situ experiments, XAL2 is expressed in lateral root cap, epidermis and columella, as well as in the vascular cylinder in primary root (Figures 1C-I). However, in the XAL2:GUS line as shown in Figure 6C, no strong expression of XAL2 in root can be observed without IAA treatment. Also, please specify was this a transcriptional or translational reporter line. Please explain the discrepancy.
- As another evidence besides altered expression of the PIN genes the authors used 35S::XAL2-GFP

in complementation and ChIP experiments. When used under 35S and given the discrepancy above, it is difficult to judge the value of this data. Also, why didn't the authors use the endogenous promoter?
- The root phenotypes of xal2 should be characterized more in details, for example, is the size of stele reduced in the mutant? (Optical) cross sections.
- For me PIN1 data is not very clear. It looks like pPIN1::GFP is reduced in xal2-2, however, this can be also explained by the reduced size of stele where the PIN1 is expressed in xal2-2. I think more quantitative analysis should be done to compare PIN1 expression levels, such as qRT-PCR comparing PIN1 mRNA levels in WT and xal2-2 (as there is no band detected on the gel), also fluorescent signal quantification in WT and xal2-2. Optical cross sections might be informative as well.
- The authors claim that XAL2 is a negative regulator of PIN3, but the evidence is not solid. The only data supporting this claim was the qRT-PCR data in Figure 5D, however, it's a bit confusing since the data was normalized to WT. The authors should explain the data in the text. In addition, the authors mentioned that they obtained the PIN3::GUS from J. Friml, but no data was shown in this line. This is important as PIN3 is included in the gene regulatory network proposed by authors.
- Statistical analysis should be done for all qRT-PCR data. Moreover, although the authors mentioned in the Experimental procedures that the qRT-PCR data in Figure 5D and Figure S2 was normalized to WT, it would be more informative to specify this also in Figure legend. On the other hand, the authors stated that auxin responsive markers were similarly induced by auxin treatment in WT and xal2-2 (Figure 5D), however, it's impossible to confirm the auxin responsiveness of these gene in WT since the data was normalized to WT. From this point of view, I suggest the authors present the data for WT as well.
-The authors claimed that SCR:GFP is not changed in xal2-2, however, it seemed like the expression of SCR is obviously decreased in QC (Figure 3B). Please interpret this.
-The authors stated that in xal2-2, a reduction in rootward IAA transport similar to levels seen in pin1, however, no data for pin1 is shown. Please also show data for pin1 to compare the level of reduction.
-What are the unspecific but strong bands in Figure 6A, lower panel?
-It is hard to read the text sometimes. The authors should revise the text by using shorter sentences and simplifying some parts of the manuscript (especially in the Summary section).

Minor comments:
-What are the expression patterns of auxin biosynthesis genes in xal2?
-Does xal2 display flowering phenotype?
- I wonder why didn't the authors use qRT-PCR instead of RT-PCR in Figure 5C and Figure 6B?
- In Experimental Procedures, there are mistakes indicating the concentration of NPA and 2,4D used for experiments (10mM NPA or 10mM 2,4-D?). Probably the authors used micromolar concentrations of these compounds. Please correct accordingly.
- In Figure 1I, is the positive sense control from the same stage of lateral root primordia development as the antisense?
- Higher resolution images in Figure 4A as well as in Figure S2A is required to state that "two tiers of columella initials were observed in xal2-2 (white arrows), which was also detected at the position of the wild-type QC (white arrowhead)".
- Figure legends and labeling in Figures should be checked carefully and corrected. What does the white arrowhead stand for in Figure 3? . What is presented in Figure 3C - PLT1::GUS in xal2-2 or COL148 promoter trap line (as indicated in the legend)? Please correct accordingly. What was the right concentration of 2,4D used in this experiment in Figure 6B legend? Probably it was not 10mM 2,4-D.
- Could the authors explain the use of COL148 (plt1-1) line? It is mentioned in Experimental procedures section, but there is no data or explanation in the text.
- Please reformulate the sentence "untreated roots of wild-type and mutant. . .with or without an auxin treatment" in figure legend for Figure 5A and 5C.
- Figure 5D and Figure S3B. Standard error bars for some genes are missing.

Referee #3

In this manuscript the authors propose the XAL2 MADS-box gene as a regulator of several PIN genes thus controlling root development.

The authors start with a detailed description of the root phenotype of two alleles of the XAL2 mutant. While it is clear that the mutant has a decreased meristem and root length I quite disagree with the fact that in the in the stem cell niche there is a supernumerary of culumella stem cell. The pictures presented in this paper do not support this conclusion and actually I would conclude the opposite. This is also supported by the observation that SCR is not express in the QC FIG 3B(while the author conclude that the expression pattern of the gene is not altered) and SCR is needed it the QC to sustain stem cell activities. I suggest to do double staining with the QC marker and lugol on the same mutant root to visualize at the same time the two cell type and see how often (and If) the phenotype is observed compared to wild type root. The author should also analyse the root grow in time to see if the meristem can sustain root grow. Also the pictures of the in situ experiments are very poor and I do not see expression of the gene in the vascular tissues of the root meristem or in the stem cell niche were PIN1 And PIN4 are respectively expressed. This is also confirmed by the analysis of the XAL2:GUS reporter line where no expression of the gene is observed in the root meristem not even after auxin treatment. This is fundamental to sustain the idea that XAL2 controls directly the activities of these PIN genes in the root meristem tissues.
The authors conclude that the root phenotype of XAL2 depends on the lack of PIN4 and PIN1 expression. I doubt that since the double pin1,pin4 mutant does not have such sever phenotype and therefore the lack of PIN4 expression and the attenuation of PIN1 probably contributes to it but it is not the main cause.
In conclusion I think that this is potential a very interesting paper but the authors should provide further evidence to support their conclusions.

1st Revision - authors' response 31 January 2013

Referee #1

Work of Garay-Arroyo et al., reveals that XAL2/AGL14 transcription factor of the MADS box gene family directly transcriptionally regulate expression of several members of auxin efflux family. Authors thoroughly analyzed root phenotype of xal2 mutants and observed developmental abnormalities (reduce size of roots and root meristem; defects in QC specification and collumela differentiation, modulated DR5 auxin reporter expression) indicative of defects in the polar auxin transport . Direct measurements of shootward and rootward auxin transport confirmed reduction in auxin transport through the root. Expression analysis of PIN genes in xal2 mutant demonstrate that XAL2 is involved in the regulation of PIN1 and PIN4 expression, and no significant effect on PIN2 and PIN7 expression was observed. Direct transcriptional regulation of PIN1 and PIN4 auxin efflux carriers by XAL2/AGL14 was shown using chromatin immunoprecipitation approach.
The work reveals that MADS-box gene XAANTAL XAL2/AGL14 transcriptionally regulates two members of auxin efflux carrier family PIN1 and PIN4. The work is technically well performed, clearly presented and discussed. The phenotype analysis of xal2 loss of function mutant and expression analysis of PIN auxin efflux carriers in xal2 clearly demonstrate auxin transport defects. My main concern is related to evidence on the direct regulation of PIN1 and PIN4 by XAL2. As this is one of the core messages of the work the direct transcriptional regulation of PIN should be confirmed by other technic such as EMSA or Y-1H assays.
ANSWER: We agree with the reviewer that it is always good to have independent experiments to support a conclusion. In our case, we already have: the phenotypes concerning altered auxin transport, the RT-PCR and the CHIP experiments which support the conclusion that the XAL2 transcription factor regulates *PIN1* and *PIN4*, and it seems to exert such regulation by directly binding to some of the CArG boxes in their promoter regions. However, the MADS-domain transcriptional factors do not act alone or in homodimers, but generally form heterodimers. For that reason, when we first planned our experiments we decided to use a CHIP assay to test if XAL2 directly bound *in vivo* to the regulatory genes that encode for the PIN transporters; and hence we chose to use a CHIP assay, rather than an *in vitro* or an approach that uses heterologous systems. We had hypothesized that the EMSA experiment would not work. In response to the reviewer recommendation, however, we embarked in doing EMSA assays and, as expected they did not show binding *in vitro*. This is not surprising because we know that XAL2 exerts its transcription factor activity in hetero-dimers (De Folter et al., 2005). We are currently analyzing the several dimers formed between XAL2 and other MADS-domain proteins and searching for their targets. However, this is beyond the scope of the paper that has been sent to the EMBO Journal. Nonetheless, we decided to repeat the

CHIP assays for PIN1 and PIN4 and the new repetition has confirmed our results: XAL2 directly binds to some of the CArG boxes of the promoters of the *PIN1* and *PIN4* genes. Indeed, nowadays CHIP experiments are the most popular assays and best choice for *in vivo* localization of promoter region´s DNA-protein interactions.

Eventually, it would be useful to test whether other part of PIN1 promoter sequence interact with XAL2 and thus examine specificity of XAL2 binding to CArG box fragments containing promoter parts.
ANSWER: Yes, indeed, we completely agree with the reviewer, and we will continue to pursue the downstream genes regulated by XAL2. However, this is beyond the scope of the ms under consideration.

Related question to specificity of the XAL2 interaction with PIN1 and PIN4 promoters. Did authors check the presence of the CArG box fragments in PIN2 and PIN7 genes, which do not appear to be regulated by XAL2 transcription factor?
ANSWER: We found three CArG boxes in PIN1 and PIN4 regulatory regions (in the promoter and in one intron) and five in PIN2 and PIN7 regulatory regions. Although PIN2 has more CArG boxes than PIN1 and PIN4, it is not regulated by XAL2: we tried RT-PCR (data not shown) and qRT-PCR for *PIN2* (see Figure 4D) and do not see any difference in the expression levels of this *PIN2* between *xal2-2* and WT. Some of the CArG boxes of the *PIN1* (two) and *PIN4* (one) are not recognized by XAL2 in our CHIP assays.

Other comments:

1. Do overexpression of 35S::XAL2 enhances PIN1 and PIN4 expression?
ANSWER: No, it does not increase *PIN1* or *PIN4* expression. We did the crosses between the over-expression line (35S::XAL2genomic) and *PIN1::GFP* or *PIN4::GUS* and did not observe any differences between these lines and corresponding control roots. Again, this is not surprising due to: a) the lack of XAL2 partners over-expressed together with XAL2, and b) the fact that the effect of XAL2 over *PIN1* and *PIN4* levels may not be linear. Indeed the over-expression of *XAL2* cDNA and genomic constructs in wt plants yields a short root! We are currently analyzing these phenotypes, as well as co-overexpression lines which have augmented levels of mRNA of several MADS together with *XAL2*, in order to further explore the types and functions of transcriptional complexes in which XAL2 participates, as well as their targets and conditional responses. However, all of this is ongoing research that goes beyond the scope of this paper.

2. It would be useful, to present detailed XAL2::GUS expression analysis (higher magnification then presented at Figure 6C) in root meristem to confirm in situ hybridization results (Figure 1A).

ANSWER: Yes, we agree that further analyses on this were necessary, and concordantly, we have now analyzed plants at 4 and 7 days after sowing and have also repeated the assays for the GUS reaction, and observed it for a longer time (see Figures 5D and S6C). We also repeated the IAA induction experiment for another independent line, and we have obtained the same result. The expression pattern of *XAL2* is now clearer and more concordant with the conclusions and expectations concerning this aspect of the paper. Thank you!

3. Discrepancy in description figure S3A versus Figure legend.
ANSWER: Thank you, this was a mistake that has now been corrected.

4. Figure 3 and Figure 4A might be fused into one panel and part of data presented in panel 4A (showing defect of QC specification in xal2 mutant using several different reporters) might be transferred to supplementary material.
ANSWER: Thank you, we have moved the crosses in which no effect is observed in the *xal2* mutant with respect to wt to supplementary material, and have integrated the rest in one new figure.

5. I have impression that some of presented figures might be distorted probably due to scale adjustment (Fig. 4A WTx QC25, xal2-2xQC25-:GUS; supplement Figure S1).

ANSWER: Thank you, we have gone over all the figures to make sure that no distortion is observed due to changes in amplification or scale. We have particularly corrected the ones noted by the reviewer.

Referee #2

In the manuscript, the authors report that a MADS-box gene XAL2/AGL14 regulates expression of PIN1, PIN3 and PIN4 in Arabidopsis roots and thus it is important for maintain auxin gradients along the roots.
Major comments:
-My major concern is about the expression patterns of XAL2. If XAL2 regulates the transcription of PINs, one would expect to see the overlapping of their expression domains in roots. However, there is discrepancy in current expression data, i.e, in situ RT-PCR data (Figure 1) and GUS reporter data (Figure 6). The authors stated that by in situ experiments, XAL2 is expressed in lateral root cap, epidermis and columella, as well as in the vascular cylinder in primary root (Figures 1C-I). However, in the XAL2:GUS line as shown in Figure 6C, no strong expression of XAL2 in root can be observed without IAA treatment. Also, please specify was this a transcriptional or translational reporter line. Please explain the discrepancy.
ANSWER: This is an important point. First, we clarify that the line that we used is a transcriptional reporter line with 1Kb of the promoter of the XAL2 gene. However in several reporter lines we do see expression of *XAL2* promoter in the root meristem, and this becomes very clear when left for longer staining times, in coincidence with what we observe in the *in situ* data. And in both cases, the expression domain overlaps with that of the *PIN* genes, that we have claimed to be regulated by *XAL2*. We have included now (Figure 5D) a photograph of plants with this construction that were stained for GUS for a longer period (Figure S6C). As it can be seen, there is expression of XAL2 in the root meristem as in the in situ. Maybe these lines are missing some regulatory motifs upstream of the promoter fragment used, or in the introns, and the GUS is observed in some places where the *in situ* does not show expression. Also, since *XAL2* is a transcriptional factor with very low expression levels, it is probable that in the "*in situ*" hybridization experiment we have missed some sites of expression, but both experiments coincide overall. Finally, Birnbaum et al., 2003 reported the presence of *XAL2* in many parts of the root: mainly in columella but also in the QC and in the vascular bundle. We have now included the additional data, and clarified all this in the ms.

- As another evidence besides altered expression of the PIN genes the authors used 35S::XAL2-GFP in complementation and ChIP experiments. When used under 35S and given the discrepancy above, it is difficult to judge the value of this data. Also, why didn't the authors use the endogenous promoter?
ANSWER: Since we have not been able to clone the complete promoter, we decided to use the 35S line as it has been done previously in many other recent papers. The line used for CHIP was chosen because it had a clear expression in all the root meristem, thus making sure that the cDNA would be expressed in the sites where *XAL2* is normally expressed. Also, since this gene exerts its function only in those cells where the MADS partners are found, we are confident that the CHIP assays done with a 35S would yield functionally meaningful data. Moreover, given that XAL2 functions as a heterodimer with other MADS, the binding will only occur in the cells where it normally exerts its function as a transcription factor. We have now several biological repetitions of the CHIP assays with the same results, together with the RT-PCR experiments, and the phenotypic analyses, constitute a solid evidence for the direct regulation of *PIN1* and *PIN4* by XAL2. In contrast, we found that it does not bind other CArG boxes in these *PIN genes.*

- The root phenotypes of xal2 should be characterized more in details, for example, is the size of stele reduced in the mutant? (Optical) cross sections.
ANSWER: We agree that this is important, and we have proceeded as suggested by the reviewer. We show now a cross section of *xal2-2* roots indicating the altered cellular patterns observed in comparison to wt (Figure S7A). We have also measured the stele in both wt and mutant plants and did not find a significant difference in terms of width (see Figure S7B for quantification).

- For me PIN1 data is not very clear. It looks like pPIN1::GFP is reduced in xal2-2, however, this can be also explained by the reduced size of stele where the PIN1 is expressed in xal2-2. I think more quantitative analysis should be done to compare PIN1 expression levels, such as qRT-PCR comparing PIN1 mRNA levels in WT and xal2-2 (as there is no band detected on the gel), also fluorescent signal quantification in WT and xal2-2. Optical cross sections might be informative as well.
ANSWER: The quantitative qRT-PCR data for PIN1 (Fig. 4D) also confirms our previous conclusion, and we now also provide quantitative data on the fluorescence signal between these two lines and show that there is a difference between the wt and *xal2-2* roots (Figure S6B). Furthermore, we now have measured the stele of wt and the mutant, and we did not find a significant difference (Figure S7B).

- The authors claim that XAL2 is a negative regulator of PIN3, but the evidence is not solid. The only data supporting this claim was the qRT-PCR data in Figure 5D, however, it's a bit confusing since the data was normalized to WT. The authors should explain the data in the text. In addition, the authors mentioned that they obtained the PIN3::GUS from J. Friml, but no data was shown in this line. This is important as PIN3 is included in the gene regulatory network proposed by authors.
ANSWER: In order to confirm our interpretation about PIN3 regulation by XAL2 we did the cross between PIN3::GUS and *xal2-2*. However, we did not observe the same regulation of XAL2 over PIN3 as we had for the qRT-PCR experiment; actually we obtained the opposite. Since we still lack an explanation for such apparently contradictory data, and this does not affect our central point in this paper, we have decided to leave this data out of this paper and we will further pursue this aspect for future publications.

- Statistical analysis should be done for all qRT-PCR data. Moreover, although the authors mentioned in the Experimental procedures that the qRT-PCR data in Figure 5D and Figure S2 was normalized to WT, it would be more informative to specify this also in Figure legend. On the other hand, the authors stated that auxin responsive markers were similarly induced by auxin treatment in WT and xal2-2 (Figure 5D), however, it's impossible to confirm the auxin responsiveness of these gene in WT since the data was normalized to WT. From this point of view, I suggest the authors present the data for WT as well.
ANSWER: We agree with the reviewer and we have now done as suggested and include complete data analyses in the new version of the paper (see Figure S6A).

-The authors claimed that SCR:GFP is not changed in xal2-2, however, it seemed like the expression of SCR is obviously decreased in QC (Figure 3B). Please interpret this.
ANSWER: In order to confirm our interpretation we analyzed 40 plants of a new cross and observed them under the confocal microscope. We did not observe a significant difference in the expression level of SCR between the wt and the *xal2-2* roots. We have now provided an example that is more representative of what we observe.

-The authors stated that in xal2-2, a reduction in rootward IAA transport similar to levels seen in pin1, however, no data for pin1 is shown. Please also show data for pin1 to compare the level of reduction.
ANSWER: The rootward transport data in our ms was obtained in A Murphy´s laboratory at which the published data that we have used for comparison was also obtained. The growth conditions and procedures were identical and hence we consider that such controls, besides the other internal ones used in the new experiments, are valid. We now clarify this now in the paper (please see Page 10).

-What are the unspecific but strong bands in Figure 6A, lower panel?
ANSWER: We have observed these bands in CHIP experiments even though we used different primers.

-It is hard to read the text sometimes. The authors should revise the text by using shorter sentences and simplifying some parts of the manuscript (especially in the Summary section).
ANSWER: We have sent the manuscript to proofreading and have improved the English in the whole ms to improve clarity.

Minor comments:
-What are the expression patterns of auxin biosynthesis genes in xal2?
ANSWER: We do not have this data and only presented auxin responsive markers in both lines: WT and *xal2-2* (see Fig. S5B).

-Does xal2 display flowering phenotype?
ANSWER: Yes, this mutant allele has a clear flowering time phenotype. This is reported in a different ms that will be submitted shortly.

- I wonder why didn't the authors use qRT-PCR instead of RT-PCR in Figure 5C and Figure 6B?
ANSWER: Our data are semi-quantitative and we obtained several biological repetitions that were consistent. In our hands, such types of experiments are as reliable, and sometimes more reliable, than quantitative RT-PCR´s.

- In Experimental Procedures, there are mistakes indicating the concentration of NPA and 2,4D used for experiments (10mM NPA or 10mM 2,4-D?). Probably the authors used micromolar concentrations of these compounds. Please correct accordingly.
ANSWER: Indeed! This was a mistake, thank you! We have corrected this. We used micromolar concentrations, and have also gone over all our procedures carefully to make sure that no additional mistakes remained.

- In Figure 1I, is the positive sense control from the same stage of lateral root primordia development as the antisense?
ANSWER: It is smaller in the control, but we did not see expression in any stage of the control lateral root primordia, and observed expression of *XAL2* anti-sense in emerged lateral root primordia of different sizes. But since the experimental results are ephemeral, it is not easy to gather many pictures, and we do not have additional shots. However, we have provided new photographs of "in situ" hybridization of lateral roots at different developmental stages with a positive sense control in a more advanced developmental stage (Figure S1).

- Higher resolution images in Figure 4A as well as in Figure S2A is required to state that "two tiers of columella initials were observed in xal2-2 (white arrows), which was also detected at the position of the wild-type QC (white arrowhead)".
ANSWER: OK, we have now provided a better picture with higher resolution, but we have actually quantified the presence of the J2341 marker in one or two tiers of cells in the wt vs the mutant. We now provide quantitative data and a table showing the proportion of one and two tiers of columella initials in wt and *xal2-2* roots. The difference is very clear (Figure S4).

- Figure legends and labeling in Figures should be checked carefully and corrected. What does the white arrowhead stand for in Figure 3? .
ANSWER: Yes, indeed. We have now gone over all Figure legends and corrected any remaining errors.

What is presented in Figure 3C - PLT1::GUS in xal2-2 or COL148 promoter trap line (as indicated in the legend)? Please correct accordingly. - Could the authors explain the use of COL148 (plt1-1) line? It is mentioned in Experimental procedures section, but there is no data or explanation in the text.
ANSWER: We apologize for the confusion. Is the same line, a promoter trap line that reports the expression of *PLT1*; now we specify this in Figure S3. We have now clarified this in the methods, as well.

What was the right concentration of 2,4D used in this experiment in Figure 6B legend? Probably it was not 10mM 2,4-D.
ANSWER: Yes, indeed. We have also corrected this; it is micromolar. Thank you!

- Please reformulate the sentence "untreated roots of wild-type and mutant. . .with or without an auxin treatment" in figure legend for Figure 5A and 5C.
ANSWER: Ok, we have re-written this as suggested.

- Figure 5D and Figure S3B. Standard error bars for some genes are missing.
ANSWER: We have corrected or clarified this in the Figure legends and experimental procedures accordingly.

Referee #3

In this manuscript the authors propose the XAL2 MADS-box gene as a regulator of several PIN genes thus controlling root development.
The authors start with a detailed description of the root phenotype of two alleles of the XAL2 mutant. While it is clear that the mutant has a decreased meristem and root length I quite disagree with the fact that in the stem cell niche there is a supernumerary of columella stem cell. The pictures presented in this paper do not support this conclusion and actually I would conclude the opposite. This is also supported by the observation that SCR is not express in the QC FIG 3B (while the author conclude that the expression pattern of the gene is not altered) and SCR is needed it the QC to sustain stem cell activities. I suggest to do double staining with the QC marker and lugol on the same mutant root to visualize at the same time the two cell type and see how often (and If) the phenotype is observed compared to wild type root.
ANSWER: We thank the reviewer for providing an alternative interpretation of our data. In order to evaluate both interpretations, we have gone back to analyze the histological patterns of the root apical meristem of the *xal2-2* mutant plants and have quantified the data concerning the number of initial columella cells. We have analyzed LUGOL stained roots, and additional photographs for the GUS lines that are neater and conclude that our original interpretation is supported (see Figure S4). He Concerning the expression pattern of *SCR* in the *xal2-2* background, we have looked at another cross of *xal2-2 X SCR::GFP* and we have not found a significant difference between the expression pattern in the *xal2-2* in comparison to wt (Figure S3). We have observed at 40 additional roots of this cross and did not detect a clear difference between the wt and *xal2-2* roots. Hence, we reinforce our original interpretation concerning the role of *XAL2* in QC identity. We now provide another picture of the cited cross that is more representative of the population of plants that we have analyzed (Figure S3).

The author should also analyse the root grow in time to see if the meristem can sustain root grow.
ANSWER: Once again, we appreciate this additional idea in order to confirm our interpretation or a possible alternative interpretation of the phenotypes. We observed that *xal2-2* is able to continue growing, and this is concordant with our hypothesis that it is not necessary for QC identity. We now provide a growth kinetics curve of WT and xal2 mutant plants (Figure 1L). It is observed that the mutant grows at a clearly slower rate than wt roots, but both show sustained growth.

Also the pictures of the in situ experiments are very poor and I do not see expression of the gene in the vascular tissues of the root meristem or in the stem cell niche were PIN1 And PIN4 are respectively expressed. This is also confirmed by the analysis of the XAL2:GUS reporter line where no expression of the gene is observed in the root meristem not even after auxin treatment.
ANSWER: We did several *in situ* experiments and although it is hard to recover integral sections of the most basal part of the root, where the vascular tissue starts to differentiate, we were able to clearly observe the expression of *XAL2* in these tissues, this was also clearly observed in the *in situ* PCR (Please see now: Figure 1C and 1E). Moreover, now we provide additional GUS staining experiments and we summarize these with additional photographs (Figures 5D and S6C) where XAL2 GUS staining is clearly observed in the root meristem, including the QC, and it is also observed in the vascular tissue as in the *in situ* PCR (shown in Figures 1C and 1E). Please, also see Figure 5D in which we provide a zoom of the root apex. Additionally, Birnbaum et al., 2003 reported the presence of *XAL2* in many parts of the root: mainly in columella but also in the QC and in the vascular bundle. Hence, we conclude that the expression data provided for *XAL2* substantiates that it is expressed in tissues that coincide with the sites of expression of the *PIN* genes that we claim are regulated by this MADS.

This is fundamental to sustain the idea that XAL2 controls directly the activities of these PIN genes in the root meristem tissues.

ANSWER: Indeed, we consider that the data provided on the expression pattern, together with the RT-PCR and CHIP data, strongly support our conclusions.

The authors conclude that the root phenotype of XAL2 depends on the lack of PIN4 and PIN1 expression. I doubt that since the double pin1,pin4 mutant does not have such sever phenotype and therefore the lack of PIN4 expression and the attenuation of PIN1 probably contributes to it but it is not the main cause.
ANSWER: Yes, we agree with the reviewer and we clarify now in the paper, that the phenotype of the mutant is explained only partially by the lack of PIN4 and attenuation of PIN1, and that XAL2 likely regulates other important genes involved in root development. We are aware of this and are presently pursuing this issue for another paper.

In conclusion I think that this is potential a very interesting paper but the authors should provide further evidence to support their conclusions.
ANSWER: We appreciate this positive view, and hope that the additional data that we now provide, together with the previous data, is found sufficient to support our conclusions.

2nd Editorial Decision 14 March 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referees #1 and 2. Referee #3 was not available to review the revised version. I have now received the comments from the referees and as you can see significant concerns still remain.

Both referees find that many of the initial raised points have not been adequately resolved. The referees still have issues that need to be resolved before the manuscript can be consider for publication here. Given this, both referees are not able to strongly recommend publication here. As you know, we normally allow only one major round of revision. In this case I can offer a second round of revision, but you will have to add additional data to address the concerns in order for us to consider the revision. Should you be unable to address the remaining concerns then it is in your best interest to seek publication elsewhere at this stage. I should also add that that I need strong endorsement from the referees to move forward with the paper and that at this stage this is a bit uncertain.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

I hope that you find the comments helpful.

REFEREE REPORTS

Referee #1

I appreciate the efforts of the authors to address referee comments. The revised manuscript clearly improved over the original version. However, I have to say that authors did not address several of the key requests of the reviewers satisfactorily such as clarification of the partially non-overlapping expression patterns and the verification of direct action on the PIN promotors besides the CHIP experiments. I appreciate the difficulties and authors provide quite plausible explanation why they did not performed the required experiments but I still believe they might have done a bit more effort. Also the English formulations, though better than originally, can be further improved for sake of clarity and readability.

Referee #2

In the revised manuscript, the authors have done some experiments to confirm and strengthen their

statements. Some experiments are well performed while there are still some aspects which need to be revised.

1. My major concern is still on the expression patterns of XAL2. In the current version, the authors were able to show the strong expression of XAL2 by longer GUS staining times (Fig. 5D). However, the authors mentioned they use different reporter lines to see the expression. I would like the authors to show statistically how many GUS lines have been analyzed and how variable the expression patterns are? Moreover, the authors only observed the GUS with longer staining time, to me, the images in Fig. 5D might be over stained, it may be worth to show GUS staining images with time gradients. On the other hand, the authors also showed the in situ RT-PCR to demonstrate the expression domains of XAL2 in root, since the authors have the GUS lines, I suggest they also show the GUS expression more in details to confirm the expression domain of XAL2. In addition, please show a better image of root tip in Fig. 5D, current images are not focused. Did the authors use some labeling for in situ in Fig. 1 and Fig. 1S? Did the authors show same data from the same line in Fig. 5C and 5D?

2. In Fig. 5C, the authors claimed that XAL2:GUS expression is enhanced upon IAA treatment and it's dosage dependent. However, I don't see any expression in root tip meristematic region even with high IAA concentration treatment. If with longer GUS staining time, the GUS can be observed in meristem, I demand the authors change the images in Fig. 5C to stronger GUS staining ones. This is important since PIN1 and PIN4 are expressed in root meristemtic region.

3. The authors should present more solid data for marker expression. For example, the authors used J2341 as a maker for columella initial cells. However, the expression pattern of J2341 is variable. Sometimes one can see expression in QC and columella initial (Sabatine et al. 2003, Genes and Development), and sometime one don't see expression in QC. Also, signals in endodermis and vascular cell can be observed sometimes. Therefore, I would like the authors to describe more about the variation of the expression patterns of this marker. Another example is DR5:GUS which is also variable in root tip in WT. To me, the GUS result in WT without IAA that the authors presented in Fig. 3B is under average. Could the authors confirm that this is a representative image?

4. In the previous round, I suggest the authors show WT expression levels of auxin responsiveness genes in (current) Fig. 4D. The authors claimed that they have revised accordingly, however, I don't see the changes.

5. In my opinion, Fig. S6D could be moved to the main part of the manuscript because it is showing the quantitative data on the fluorescence signal of PIN1::GFP construction between wild-type plants and xal2-2 roots, which is more representative than Figure 4C showing RT-PCR results with no band for PIN1. Also authors should explain what "107.35" and "78.75" mean in the Figure legend. I am also wondering how authors have done this procedure and which region they took for quantification. This should be shortly mentioned in the Figure legend. Have the authors tried to do the optical cross sections for PIN1::GFP?

6. In Fig. S7A, the authors showed the cross sections of WT and xal2 to observe the stele phenotypes and they state that the cellular patterns are altered. However, I am not clear what patterns are changed? Also the difference may be due to some sectioning technical issues as the presented cross section of xal2-2 seems squeezed. I would like the authors show a better section image or multiple sections to show the real phenotypes. In addition, how old are the plants used for sectioning and which part of the root was sectioned? I would like the authors to show the cross sections just above QC where PIN1 and PIN4 are expressed.

7. The authors claim that pSCR::GFP is not changed in the new cross they did between pSCR::GFP and xal2. I wonder how many independent lines they have checked. Is this variable between different crosses?

Minor points:
1. In Fig.4D, error bars are missing for PIN4. During the first version, there was a large bar for PIN4.
2. In Fig. 5A, correct the labeling about "ATG".
3. For construct names the authors use both forms like "pPIN4:GUS" and "DR5:GUS". As they are

both promoter GUS lines, there should be same system for typing both (like pPIN4:GUS and pDR5:GUS).
4. "in situ" should be in italic. This is repeating throughout the text.

2nd Revision - authors' response 15 July 2013

Referee #1

I appreciate the efforts of the authors to address referee comments. The revised manuscript clearly improved over the original version. However, I have to say that authors did not address several of the key requests of the reviewers satisfactorily such as clarification of the partially non-overlapping expression patterns
ANSWER: As we have summarized above, the data at hand show that the three genes (*XAL2*, *PIN1* and *PIN4*) are strongly expressed and overlap in the vascular tissues of the root and in other regions. *PIN1* was shown to be induced in the root stele (Gälweiler et al., 1998; Michniewicz et al., 2007; this work Fig. S7 in the differentiation zone) and *XAL2* and *PIN4* (this work) are expressed in this zone too (see Fig. S7). In these experiments, we grew the plants for 3 days (but also repeated the experiment with plants for 4, 5 and 7 days and all gave the same results). We also looked at the expression patterns of *pPIN4-GUS* and *p1KbXAL2:XAL2-GUS* at the same time to see the overlapping zone of expression. As can be seen in Fig. S7, the pattern of expression of *pPIN4-GUS* is in the central root meristem as reported (Friml et al, 2002) and in the vascular tissues. With the construction of XAL2 (*p1KbXAL2:XAL2-GUS*) we saw a strong expression in the vascular tissue and in the lateral roots consistent with the "*in situ*" data (see Figs. S1, S2 and S9). The GUS expression (unless left for several days) in the columela or lateral root cap, and other cells of the root tip and meristem is weak, but we observe it in some of the lines (see Fig. 1). This could be explained by the fact that we only have 1Kb of the promoter and maybe these lines are missing some enhancer motifs upstream of the promoter fragment used. In the previous version, we showed only the strong GUS expression in the meristem but this was obtained when the roots were stained for up to six days. We now show the expression pattern after a few hours only because the longer times may lead to artifactual patters. In any case, the *in situ* PCR the dig-*in situ* data, and the published results, together with the GUS assays we have performed, confirm that *XAL2* has overlapping regions of expression with *PIN1* and *PIN4*.

and the verification of direct action on the PIN promotors besides the CHIP experiments.
ANSWER: We agree with the reviewer that it is always good to have independent experiments to support a conclusion. In our case, we already have: the phenotypes of *XAL2* concerning altered auxin transport and also similar cellurar patterns around the QC as *pin4* mutants, the crosses of *xal2-2* loss of function mutants with *PIN1/4:GUS/GFP,* the RT-PCR and several biological repetitions of the CHIP experiments which support the conclusion that the XAL2 transcription factor regulates *PIN1* and *PIN4*, and it seems to exert such regulation by directly binding to some of the CArG boxes in their regulatory regions. In contrast, XAL2 does not bind other CArG boxes in the promoter or intronic regions of these *PINs*, or any of the CArG boxes of *PIN2* and *PIN7*. We now make all these corroboratory data clearer.

In addition, and in response to the reviewer´s recommendation, we embarked in doing EMSA assays for a second time, although we were expecting problems for reasons explained above. We are attaching a power point document with all the steps that we did in order to obtain the purified protein. First of all, we cloned XAL2 in and entry vector (TOPO T/A) to recombine it with a destiny vector, pDEST24 (gateway plasmid with GST). When XAL2 was fused with GST, the bacteria grew very slowly and we obtained very little protein; however, we were able to see the fusion protein (see Figures 1 and 2). Afterwards we induced with IPTG under different incubation times and temperatures; we established that the best condition was 0.5mM IPTG/ 1,30 hrs at 30ºC. As the protein was insoluble and remained in the pellet we tested Betaine HCL and betaine OH to solubilize it (see Figure 3). The first condition was able to solubilize the protein but the pH of the medium was very low and we could not purify the protein. Subsequently we tried manitol without success and a mixture of sarkosyl and chaps (1%, 30mM) that helped solubilizing the protein, but, once again, it was not purifyable (Figure 4). In Figure 5, we show the EMSA assays with soluble total extracts from bacterial

transformed with PGEX (GST) and PDEST24-AGL14 (AGL14-GST) using primers labeled with 5- biotin with CArG boxes from region -3 and region +1 of PIN1 gene (and with those of PIN4 in Figure 6). As competitors we used the same primers unlabeled (Comp) or labeled but with mutations on putative CArG boxes (Mcomp). Unfortunately, we could not obtain any retardation bands.

Hence, we decided to make additional biological repetitions of the CHIP assays, as well as the RT-PCR assays. Both confirmed our results.

We are now starting several functional genomic, transcriptomic and proteomic assays to further pursue the target genes of XAL2, but these efforts are beyond the scope of the paper under consideration.

I appreciate the difficulties and authors provide quite plausible explanation why they did not performed the required experiments but I still believe they might have done a bit more effort. Also the English formulations, though better than originally, can be further improved for sake of clarity and readability.
Yes, thank you. We have personally carefully reviewed the manuscript for English grammatical and spelling mistakes. Afterwards, Dr. Virginia Walbot reviewed our paper thoroughly and marked several additional points that could be improved in terms of English usage and also to clarify arguments. We think that the paper has greatly benefited from this.

Referee #2

In the revised manuscript, the authors have done some experiments to confirm and strengthen their statements. Some experiments are well performed while there are still some aspects which need to be revised.
1. My major concern is still on the expression patterns of XAL2. In the current version, the authors were able to show the strong expression of XAL2 by longer GUS staining times (Fig. 5D). However, the authors mentioned they use different reporter lines to see the expression. I would like the authors to show statistically how many GUS lines have been analyzed and how variable the expression patterns are?
ANSWER: Yes, this was also a concern of the first reviewer and we have addressed this point in this version and we argue about this point both in the general response letter and in the response to the previous reviewer. Please see above.

Moreover, the authors only observed the GUS with longer staining time, to me, the images in Fig. 5D might be over stained, it may be worth to show GUS staining images with time gradients.

ANSWER: Yes, thank you. This is a good point, as well. So now we show the GUS staining at shorter times only, because the longer period may show artifactual patterns. Indeed, it seems that at shorter times GUS is faintly observed in the root tip and root meristem, besides bein strongly expressed in tissues that coincide with those in which the *PIN1* and *PIN4* are expressed or those observed in the two types of *in situ* assays that we have performed. We scanty expression of GUS in the *XAL2* promoter line may be due to the fact that the GUS construct only has 1 kb or upstream regulatory sequences for this MADS and some enhancers might be missing (Figs. S2 and S7). But as we argue above, we consider that the ms has sufficient data to substantiate the overlap of expression of *XAL2* and *PIN1/2*. In light of this, we have decided to leave the GUS patterns for short times and argue that we see broader stronger expression at longer times (data not shown). The restricted patterns might be due to the fact that we only have 1 kb of the promoter.

On the other hand, the authors also showed the in situ RT-PCR to demonstrate the expression domains of XAL2 in root, since the authors have the GUS lines, I suggest they also show the GUS expression more in details to confirm the expression domain of XAL2.

ANSWER: Yes, we have repeated the GUS stains and confirm that there is overlap with the *in situ* patterns but when stained during short times, the expression in the meristem is very faint and scanty (see Fig. S7). Hence, we have reviewed data from cell-sorting analyses and now

show that this published information also confirms the expression pattern of *XAL2* and its overlap with *PIN1* and *PIN4*.

In addition, please show a better image of root tip in Fig. 5D, current images are not focused. Did the authors use some labeling for in situ in Fig. 1 and Fig. 1S? Did the authors show same data from the same line in Fig. 5C and 5D?

ANSWER: We have repeated the experiment and show better images. All the experimental procedures for the "in situ" experiments (dig and PCR) are in Materials and Methods now clearly explained.

2. In Fig. 5C, the authors claimed that XAL2:GUS expression is enhanced upon IAA treatment and it's dosage dependent. However, I don't see any expression in root tip meristematic region even with high IAA concentration treatment. If with longer GUS staining time, the GUS can be observed in meristem, I demand the authors change the images in Fig. 5C to stronger GUS staining ones. This is important since PIN1 and PIN4 are expressed in root meristematic region.

ANSWER: The reviewer is right in pointing out this contradiction. Thank you very much. In the previous revision we had included a GUS staining after several days. But this could be artifactual and as explained above, have relied on the *in situ* assays and the published data to infer the more complete pattern of expression of *XAL2*. So we have used the GUS stainings at short times for the induction experiments, as well. Indeed, at these times, we recover a faint and scanty expression in the meristem and a strong expression in the vascular tissue, where the strong response to auxins is apparent. Again, the lack of clear response in the root tip and meristem, might be due to lack of enhancer motifs in the promoter region that we have. But in the areas in which the GUS is clearly expressed the difference is also very clear when induced. In addition, the induction of *XAL2* by auxin is confirmed with qRT-PCR experiments.

3. The authors should present more solid data for marker expression. For example, the authors used J2341 as a maker for columella initial cells. However, the expression pattern of J2341 is variable. Sometimes one can see expression in QC and columella initial (Sabatine et al. 2003, Genes and Development), and sometime one don't see expression in QC. Also, signals in endodermis and vascular cell can be observed sometimes. Therefore, I would like the authors to describe more about the variation of the expression patterns of this marker.

ANSWER: Yes, we found that this marker has a variable expression but we did not find expression in the QC in wild-type plants. In both lines (wild-type and *xal2-2*; n=30) we found expression in the vascular tissues (20%) and in endodermal (10%) and epidermal cells (5%).
Another example is DR5:GUS which is also variable in root tip in WT. To me, the GUS result in WT without IAA that the authors presented in Fig. 3B is under average. Could the authors confirm that this is a representative image?
ANSWER: Yes, it is variable, but we show a representative case under our conditions for both the wild-type and the *xal2-2* mutant backgrounds. In any case, we now show a power point document (only for the reviewer to see, that is called *pDR5-GUS*) with three independent experiments with different times of GUS reaction for the wild-type and the *xal2-2* mutant to substantiate the clear differences found between these two lines. In the paper we summarize these observations by showing the picture of a representative case, but also mentioning the number of lines observed.

4. In the previous round, I suggest the authors show WT expression levels of auxin responsiveness genes in (current) Fig. 4D. The authors claimed that they have revised accordingly, however, I don't see the changes.

ANSWER: Thank you. We have corrected this and now we have added wild-type data with the data for the *xal2-2* mutant in the main text (Fig. 4D). In the previous version, we showed the wild-type control in a supplementary figure (S6A).

5. In my opinion, Fig. S6D could be moved to the main part of the manuscript because it is showing the quantitative data on the fluorescence signal of PIN1::GFP construction between wild-type plants and xal2-2 roots, which is more representative than Figure 4C showing RT-PCR results with

no band for PIN1. Also authors should explain what "107.35" and "78.75" mean in the Figure legend. I am also wondering how authors have done this procedure and which region they took for quantification. This should be shortly mentioned in the Figure legend.

ANSWER: We agree with the reviewer and as suggested by the reviewer, we have now moved this quantitative data to the main part of the manuscript (see Fig. 4C). Also, we have now explained what these figures mean in the legend. In addition, in the Material and Methods section we have explained the procedure used.

Have the authors tried to do the optical cross sections for PIN1::GFP?

ANSWER: Yes, we have now done cross sections for PIN1::GFP for wild-type and *xal2-2* plants and did not find any differences. To make the transverse sections, Z-scanning was performed at the level of the QC, and at the level of the 1st and 5th cortical cell, including cortex/endoderm initial cells. Images were acquired using 63x C-Apochromate water immersion objective and a sequential scanning, first in GFP channel and then in PI channel. Analysis of pixel density was performed using Image J software. To measure the influorescence signal we established a region of interest (ROI) that comprised an area of the provascular tissues above the QC, 2-3 cells thick and 4-5 cells of height. As in this experiment no QC markers were used, it was impossible to identify the QC cells unambiguously on the transverse sections. Therefore, average pixel density of the green channel was measured in all root tissues of the section made at the QC level. At the levels of the first and fifth cortex cells, average pixel density of the green signal was measured only within the provascular tissues, including the pericycle. For this, the provascular area was manually outlined using LSM (Zeiss) program, then the red channel was turned off and an image with outlined area and green signals were analyzed for mean pixel density. In all cases, we used n = 8. We now explain all of this in detail in the paper and include a Supplemental figure with all of this. We further argue that while the difference in fluorescence is clear for *PIN1* and *PIN4*, when comparing the wild-type and the *xal2-2* mutant, we did not see any difference in fluorescence for other *PINs*.

6. In Fig. S7A, the authors showed the cross sections of WT and xal2 to observe the stele phenotypes and they state that the cellular patterns are altered. However, I am not clear what patterns are changed? Also the difference may be due to some sectioning technical issues as the presented cross section of xal2-2 seems squeezed. I would like the authors show a better section image or multiple sections to show the real phenotypes. In addition, how old are the plants used for sectioning and which part of the root was sectioned? I would like the authors to show the cross sections just above QC where PIN1 and PIN4 are expressed.

ANSWER: Yes, thank you. This and the previous point were very important to confirm that the difference between the *xal2* and the wild-type backgrounds was due to the loss of funtion of *XAL2*, rather than by the fact that *XAL2* could have an overall thinner vasculature. But the additional observations have also helped clarify the role of *XAL2* in radial patterning. We have also changed the figure and have made additional experiments to substantiate this part better. We performed cross sections at the level of the 1st and 5th cortical (including initial) cells above the QC. As can be seen in the new figure (Fig. S4A-E) XAL2 mutation affects the stele development. This gene loss of function mutant shows clear alterations in the anticlinal radial division of the pericycle cells; in normal development, the diameter of stele (provascular tissues) is increasing at the level of the fifth cortical cell of the root meristem compared to that at the level of the first cortical cell above the QC. This increase was by 70% in wild-type and by 48% in the *xal2-2*, however, no statistical difference in diameter between wild-type and *xal2-2* was found (P>0.05). The latter is important, because this shows that the difference in fluorescence that we observed is due to the mutation and not to the thinner root in the *xal2* mutant. Moreover, we also measured root and stele width at the differentiation zone, at the level of the first hair, and found no differences between the root and stele width between wild-type and *xal2-2* plants (n=29 in both cases; see Fig. S4G). The recovery of the width of the stele in *xal2-2* could be explained either if there are additional divisions in the vascular tissue (above the 5th cell) in *xal2-2* plants and/or if the cells widthwise grow more in *xal2-2* in comparison to wild-type. We will continue to pursue the role of *XAL2* in cell growth in

different parts of the root, but additional detailed analyses of this aspect of the mutant is beyond the scope of the ms under consideration.

7. The authors claim that pSCR::GFP is not changed in the new cross they did between pSCR::GFP and xal2. I wonder how many independent lines they have checked. Is this variable between different crosses?

ANSWER: Indeed, pSCR::GFP did not show a signifcant difference between the lines in wild-type and mutant backgrounds; we checked five independent lines of two independent crosses; in two of them (the first cross), we found a rare expression of pSCR::GFP in both, wild-type and *xal2-2* plants and we performed another cross with another pSCR::GFP wild type plant. In this new cross we checked three independent lines and did not detected any difference in expression between wild-type and *xal2-2* plants.

Minor points:
1. In Fig.4D, error bars are missing for PIN4. During the first version, there was a large bar for PIN4.
ANSWER: Thank you, we now included the bar for PIN4.

2. In Fig. 5A, correct the labeling about "ATG".
ANSWER: Thank you, we now corrected this.
3. For construct names the authors use both forms like "pPIN4:GUS" and "DR5:GUS". As they are both promoter GUS lines, there should be same system for typing both (like pPIN4:GUS and pDR5:GUS).
ANSWER: Thank you; we have made reference to the constructs consistently.
4. "in situ" should be in italic. This is repeating throughout the text.
ANSWER: Thank you, this was a mistake that has now been corrected throughout.

3rd Editorial Decision 07 August 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #2 and I am happy to say that the referee find the analysis significantly improved. The referee has a few remaining points that should be clarified before final acceptance here. Most of these points should be relative easily addressed, if not then please contact me and we can discuss it further by email.

Thank you for the opportunity to consider your work for publication. I look forward to seeing the final version!!

REFEREE REPORT

Referee #2

In the current version, the authors addressed all the comments from the reviewers and they have improved the statistical analysis for almost all the data. But I think there are still a few points must be
clarified.

Please see my comments in details.

Major points:

- Both reviewers pointed out the partially non-overlapping expression domain of XAL2 compared with PIN1 and PIN4 in the previous version of the manuscript. One of the most striking phenotypes of xal2 is the two layers of columella initials just below QC, however, from the in situ PCR data, there is almost no expression of XAL2 at the columella initials. Moreover, PIN1 is not expressed in that region either

(Figure 4C), only PIN4 is strongly expressed there. All in all, current data are not solid enough to prove the overlapping expression domain of XAL2 with PIN1 and PIN4 in meristem. I would like to ask the
authors to clarify this aspect by either provide better in situ data OR construct longer promoter version perhaps using fluorescent reporter rather than GUS. On the other hand, they can also try to complement the mutant phenotypes with the existed construct p1Kb::XAL2::gXAL2-GUS to prove if the 1kb promoter is sufficient. On the other hand, I suggest the authors move some GUS/GFP data presented in Figure S7 to the main text. Also I think Figure 1G and H are redundant, one of them can be removed.

- The authors claimed that only PIN1 and PIN4 (not PIN2 and PIN7) expression are affected in xal2, however, from the data presented in Figure S8, it's quite obvious to me that the expression domain of PIN7 is expanded to xylem axis. The authors also respond to the reviewer about the radial pattern of PIN1 is not changed in the mutant but there is no data to support the statement. Therefore, I would like to ask the authors to provide optical cross sections for PIN1 and PIN7 to confirm the phenotypes.

- I suggest the authors put the information about the variation of the expression patterns of some marker genes such as J2341 in the text as they responded to the reviewer. On the other hand, in the figure legend of Figure 3, it is written "specific enhancer trap J2341 expression (25%; n=30)", I wonder what is the data for other 75% plants? Please clarify this.

- Concerning about the qRT-PCR data in Fig. 4, I appreciate that the authors add Col+IAA data, I think it will make the statement stronger if they can also add Col (without IAA) data. Also, please indicate if the increase of ABCB19 is significant or not.

Minor point:

- I would like the authors to modify the description of mutant phenotypes regarding to radial patterning in xal2 in the text on page 8. It is not informative to say "the radial cellular structure of xal2-2 roots was altered with rounded cells in 20% of 30 plants analyzed (Figure S4F)". At least, I can see the pericycle cell division is altered.

- There is no data for PLT1 but there is data for COL148 in Figure S5. Please make the description consistent in the text and figure.

3rd Revision - authors' response 26 August 2013

Referee #2

In the current version, the authors addressed all the comments from the reviewers and they have improved the statistical analysis for almost all the data. But I think there are still a few points must be
clarified.

Please see my comments in details.

Major points:

- Both reviewers pointed out the partially non-overlapping expression domain of XAL2 compared with PIN1 and PIN4 in the previous version of the manuscript. One of the most striking phenotypes of xal2 is the two layers of columella initials just below QC, however, from the in situ PCR data, there is almost no expression of XAL2 at the columella initials. Moreover, PIN1 is not expressed in that region either (Figure 4C), only PIN4 is strongly expressed there. All in all, current data are not solid enough to prove the overlapping expression domain of XAL2 with PIN1 and PIN4 in meristem. I would like to ask the authors to clarify this aspect by either provide better in situ data
ANSWER: The *xal2-2* loss of function allele has several phenotypes and some of them could be explained by the loss of function of *PIN1* and *PIN4*, which mRNA patterns are regulated by XAL2, among other factors. Indeed, we suggested that the phenotype of the loss of function of

the *xal2-2* allele related to the extra file of root cap or columella initials, that is present in approximately 25% of the mutant roots, could be due to the lack of *PIN4,* which loss of function allele also has an extra file of columella initials (Friml et al., 2002). In principle, one would expect that the expression patterns of these two genes overlapped, particularly so in the columella initials. However, there are other more important phenotypes of *xal2-2* loss of function roots that suggest its role in regulating *PIN1* and *PIN4* in other tissues where they clearly overlap with *XAL2*. Moreover, the columella initials phenotype is not completely penetrant in the *xal2-2* mutant as we mention above and in the ms. Nonetheless, and as indicated by the reviewer, this was not clear in the ms; and we have now clarified this point. In any case, while *PIN4* has been shown to be expressed in the columella initial cells (see Friml et al., 2002; Vieten et al., 2005), we have demonstrated in this study, that *PIN4* is expressed in the central cylinder of the root (see our Figure 4E) where both PIN genes mRNA expression patterns clearly overlap. Indeed, our *in situ* data show that *XAL2* is also expressed in other tissues, such as the lateral root-cap, epidermis, endodermis, and columella including the initials, although we agree that this is not very clear from the pictures shown, but this is not a fundamental issue for the main conclusions of the ms under consideration. Furthermore, it is quite hard to obtain good *Arabidopsis* root sections for *in situs* and we have tried such experiment several times and have not been able to recover better sections or images. So we used the *in situ* PCR or whole mounts, but these do not reveal the expression of this gene in the QC and the initials probably because of its low expression levels and issues related to the penetration of the probe into internal cells. Published microarray data, however, does show *XAL2* expression in the QC (see Figure S1). In conclusion, we consider that with the data at hand, we can substantiate that both genes overalp in their expression patterns in several cell types. Moreover, several transcription factors, including some MADS-box members have non-autonomous functions (Kim et al., 2003; Urbanus et al., 2010; Lu et al., 2012). Therefore, if the expression patterns of the mRNAs of *XAL2* and the *PIN genes* that this MADS regulates, are not 100% coincidental, the XAL2 protein could still move to cells where it regulates these *PIN* genes and others. The same is true for *PIN1* (Grunewald and Friml, 2010) and the data presented in our paper.

OR construct longer promoter version perhaps using fluorescent reporter rather than GUS. On the other hand, they can also try to complement the mutant phenotypes with the existed construct p1Kb::XAL2::gXAL2-GUS to prove if the 1kb promoter is sufficient.
ANSWER: We tried for a long time to clone longer upstream sequences of the *XAL2* promoter with no success maybe because of peculiarities of the genomic DNA region where it is found. So we decided to work with the 1kb construct with GUS that is useful to test its induction with auxin and corroborates part of the spatio-temporal expression pattern of *XAL2* that we have also documented with the *in situ* data. Indeed, the GUS line expression (unless left for several days) does not reproduce the complete "*in situ*" pattern probably because it only has 1Kb of the promoter, and hence it probably lacks some enhancer motifs upstream of the promoter fragment used.

On the other hand, I suggest the authors move some GUS/GFP data presented in Figure S7 to the main text.
ANSWER: Yes, this is a good idea, and we have moved all of Figure S7 to Figure 4 (E) and corrected the numbers and citations to figures in the main text.

Also I think Figure 1G and H are redundant, one of them can be removed.
ANSWER: Thank you, we now have corrected this.

- The authors claimed that only PIN1 and PIN4 (not PIN2 and PIN7) expression are affected in xal2, however, from the data presented in Figure S8, it's quite obvious to me that the expression domain of PIN7 is expanded to xylem axis.

ANSWER: In response to the referee´s worries concerning the possible differences between wild type and *xal2-2* backgrounds in the expression levels and patterns of *PIN2* and *PIN7*, we have analyzed a larger number of roots. For *PIN2* we show data for wild type and *xal2-2* plants with the reporter GFP (see Figure S8) and qRT-PCR (see Figure 4D). Both clearly show that the levels or spatio-temporal patterns of mRNA of this gene are not altered in the *xal2-2*

mutant in comparison to wild type. The data for this *PIN* gene then suggest that XAL2 does not regulate all the *PINs*.
Concerning *PIN7* expression, we have analyzed now 40 plants and did not find clear differences in the expansion of expression to the xylem axis as it could be suggested by the pair of images that we had provided, and the referee noted. We found, and summarize these data in the ms (see Figure S8), that 62% of the wild type plants and 54% of *xal2-2* showed this pattern; therefore, it does not seem to be part of a pattern peculiar to the *xal2-2* phenotype. These results suggest that while *XAL2* regulates *PIN1* and *PIN4* mRNA levels, it does not seem to regulate *PIN2* and *PIN7* mRNA expression levels.

The authors also respond to the reviewer about the radial pattern of PIN1 is not changed in the mutant but there is no data to support the statement.
ANSWER: Eventhough we did a carefull confocal analysis of both longitudinal and radial sections in order to corroborate our conclusions according to the comments of the reviewers, we only reported the longitudinal sections because these are clearer then the radial ones (see Figure 4C). However, we are now including some of the radial sections as well in a supplemental figure to further support our claims as suggested by the reviewer (See Figure S7).

Therefore, I would like to ask the authors to provide optical cross sections for PIN1 and PIN7 to confirm the phenotypes.
ANSWER: As we state above, we have now included the cross-sections for *PIN1* (See Figure S7).
Given the new data that we are now providing for *PIN7* that clearly show that the mRNA patterns of this gene are not altered in the *xal2-2* mutant with respect to wild type, we consider that this is not necessary anymore.

- I suggest the authors put the information about the variation of the expression patterns of some marker genes such as J2341 in the text as they responded to the reviewer. On the other hand, in the figure legend of Figure 3, it is written "specific enhancer trap J2341 expression (25%; n=30)", I wonder what is the data for other 75% plants? Please clarify this.
ASWER: Yes, this is a good suggestion, and we have now included this detailed description of the data on the main text of the ms. In the case of J2341 we found that 25% of the plants have two tiers of columella initials and the 75% remaining have only one tier. We clarify this now.

- Concerning about the qRT-PCR data in Fig. 4, I appreciate that the authors add Col+IAA data, I think it will make the statement stronger if they can also add Col (without IAA) data.
ANSWER: We are presenting the data for qRT-PCR as it is generally shown. Hence, we DO NOT show absolute expression levels for each gene in Col-0. As is generally the case, we really cannot make a comparison on some absolute scale from the qRT-PCR data. For this, we provide the microarray values for seedlings. The conditions for growing the seedlings would be the same as in Schmid et al., 2005 (Nature genetics). We used delta Ct for the measurements and are comparing expression of each gene in Col-0 to the change in genotype/treatment. As such, the Col-0 values are all = 1. However, to know the Standard Deviations it is correct to multiply the Sds for Col-0 by 2 since we use division to get the result. These procedures are standard when using qRT-PCR data analyses.

Also, please indicate if the increase of ABCB19 is significant or not.
ANSWER: Thank you; it was not clear. As for the significance of change in ABCB19, the change with IAA (Col-0 and xal2) is significant at P<0.001 by Student's t test followed by Neuman Keuls post hoc ANOVA. We have now explicitly included this in the main text and figure legend of the ms as well.

Minor point:

- I would like the authors to modify the description of mutant phenotypes regarding to radial patterning in xal2 in the text on page 8. It is not informative to say "the radial cellular structure of xal2-2 roots was altered with rounded cells in 20% of 30 plants analyzed (Figure S4F)". At least, I can see the pericycle cell division is altered.

ANSWER: Yes, this is also an important point and we have clarified this in the ms.

- There is no data for PLT1 but there is data for COL148 in Figure S5. Please make the description consistent in the text and figure.
ANSWER: Yes, thank you for pointing this issue to us as well. We have also clarified this.

