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ROS-Mediated Vascular Homeostatic Control of Root-to-Shoot Soil Na Delivery in Arabidopsis

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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 28 June 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.

As you can see, the referees find the analysis interesting and suitable for publication in the EMBO Journal. The referees raise a number of points that shouldn't involve too much additional work to address. Referee #3 does feel that some more molecular insight into how AtrbohF protects against salt stress (point #2) would strengthen the paper. It would be good to try to add some more insight to this point. If this is not straightforward to do then we can discuss this issue further. Given the comments of the referees, I would like to invite you to submit a revised version that addresses the issues raised. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the major issues 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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Authors have conducted a novel forward genetic screen and isolated a soil salinity hypersensitive mutant, leading to a discovery of ROS-regulated root-shoot Na+ transport machinery in plants. They show that a gene encoding a NADPH oxidase, AtrbohF, is expressed specifically in root vascular tissue and controls the local salt-induced ROS production. Lack of AtrbohF function confers a decrease in ROS accumulation and an increase of Na+ level in root stele cells and stem xylem sap. They further isolated a suppressor of this hypersensitive mutant, indicating that local salt-induced ROS can prevent the delivery of excess Na+ from root to shoot via transpiration. The results are well stated and convincing. The findings of this study may have important implication on the improvement of salt tolerance of agricultural crops through genetic engineering.

Minor comments:
1. The pictures of the cross section of the root shown in Figure 3 and Figure 4 are not very clear. Better images should be provided to indicate the location of protoxylem, metaxylem, phloem and pericycle cells.
2. Xylem sap was collected from the shoot, not from the root based on the materials and methods. The statement needs to be clarified.

Referee #2

The authors identified a salt hypersensitive mutant of Arabidopsis (soil salinity sensitive1-1; sss1-1) by a genetic screen. The SSS1 locus was found to encode a NADPH oxidase, AtrbohF. The AtrbohF was demonstrated to be essential for reducing shoot Na+ accumulation via controlling xylem Na+ retrieval, indicating that Reactive oxygen species (ROS) regulate the Na+ level in the xylem vessel thus in shoots under salinity stress. The authors further showed that the sss1-1 mutation was suppressed by the collapse of stem xylem due to the disruption of the AtCesa7 gene encoding the cellulose synthesis catalytic subunit7. These results provide evidence that AtrbohF-mediated ROS signal controls xylem Na+ levels and plant salinity tolerance. Presented results are interesting and valuable information in the field of plant salt tolerance. Comments are listed below.

1. Descriptions related to Figure3B are not clear (page 6, the third paragraph, lines 4-8). The authors explain that 35S-driven AtrbohF partially complemented the sss1-1 mutation. However, on the other hand, they also show in Figure S2A and S2B that many independent lines do not retrieve the salt sensitivity. It looks to me that every line is not sufficient and I wonder which line presented in the FigureS2A corresponds to the line shown in Figure 3B. The results do not appear to support the conclusion that over-expression of AtrbohF complemented the mutation. The complementation part of Figure 3B should be removed or new complementation lines and data set using the native AtrbohF promoter should be presented. Or having two alleles could suffice.

2. The quality of root section data (in Figures 3F, H and 4A) are poor. The results are very interesting. But, the authors should increase the quality.

3. Fluorescence images are shown in Figure 5, F to P. It seems that results from two independent plants appear to be shown. I do not know why the authors presented two plants in the case of mutants. Do they show any difference? If not, the authors leave only the representative results and try to reduce the panel number as the current status of this Figure is quite busy. If they wish, additional data could be added to the supplement. In panel Q, the authors show a quantitative data set confirming the reduction.

4. In the panel D of Figure 7, the second column from the left is labeled as "atrbohF-F3 salt". This may be "atrbohF-F3 control", instead?

5. A part of references could be improved. In page 3, the last sentence at the bottom (Second, HKT transporters can...before it reaches the shoots), the authors refer Rus et al., 2001 PNAS for xylem Na+ retrieval mechanism. This is incorrect. Rus et al concluded that AtHKT1 mediates Na+ influx

at the root epidermis from the outside of environment, as a soil to root entry mechanism of toxic Na+ influx under salinity stress. Researchers have not confirmed this model presently and have found a different mechanism. Also the paper of Berthomieu et al concluded that AtHKT1 mediates Na+ loading into the phloem in contrast to the text.
As far as I know, there are a few important papers that could be cited in this sentence. Maser et al., 2002 FEBS showed the root-leaf Na+ distribution function of AtHKT1 in reducing Na+ accumulation in leaves, vascular expression and the Na+ accumulation in roots. Ren et al., 2005 Nature Genetics and Sunarpi et al., 2005 Plant J showed xylem parenchyma expression in rice and Arabidopsis and plasma membrane AtHKT1 protein localization in xylem cells in Arabidopsis and the function of these HKT transporters in removing Na+ from the xylem.

This is a very interesting manuscript that will be of significant impact.

Referee #3

This is a very interesting and potentially important piece of work describing a new regulator of salt stress in soil grown plants, RBOHF, and an associated suppressor mutation cesa7. The manuscript is well written and presented and the data appears robust. However, at present, the data is rather short on mechanistic insight. Specific points below.

1. RBOHF would have been an obvious gene to identify in a mutant screen for soil grown plants hypersensitive to salt because this gene has previously been implicated in ABA induced stomatal closure. Therefore, the identification of RBOHF is no great revelation. However, it is very surprising that rbohF mutants do not have increased transpiration rates relative to WT plants exposed to high salt. This observation would therefore suggest that RBOHF function is not physiologically relevant to stomatal aperture control under the conditions employed. Therefore, this work uncovers a potentially exciting new role for RBOHF, which is great! However, the authors observations therefore appear at odds with previous high profile data implicating RBOHF in the control of ABA regulated stomatal apperture. Hence, the authors should discuss this important point in the manuscript.

2. If RBOHF is not controlling the transpiration rate through stomatal apperture then a key question is how does RBOHF provide protection against salt stress at the molecular level? Little attempt is made by the authors to address this key point and thus the manuscript is rather short on mechanistic insight for an EMBO paper. Mechanisms for counteracting root-to-shoot Na+ delivery include Na+/H+ antiporters that can pump Na+ back into soil solution, reducing the net influx of Na+ into inner cell layers of the root and HKT transporters that can retrieve Na+ from the transpiration stream in the xylem before it reaches the shoots. Therefore, are the activities of any of these transport proteins impacted by RBOHF function? This would be one good place to start.

3. It is somewhat surprising and slightly worrying that the 35S::RBOHF transgene does not fully complement the sss1 mutation. The authors suggest that the specific cellular location of AtrbohF expression may be essential to confer full soil-salinity tolerance. Does the 35S promoter fail to drive expression in the root cell types that express RBOHF? It would be easy to check the relevant literature for 35S expression and compare it with that driven by RBOHF::GUS.

4. The provided evidence suggests sss1 and rbohf-f3 phenotypes are quantitatively similar, so it is unlikely one or more additional mutations contribute to the sss1 salt sensitive phenotype. However, it would be helpful to include rbohf-f3 in the data panels 4 and 5 alongside sss1 for comparison.

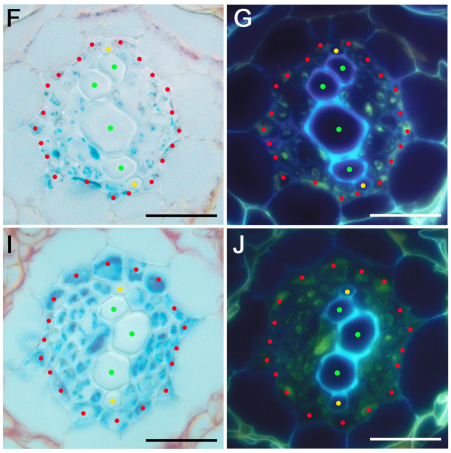
1st Revision - authors' response 23 August 2012

Referee #1

Minor comments:
1. The pictures of the cross section of the root shown in Figure 3 and Figure 4 are not very clear. Better images should be provided to indicate the location of protoxylem, metaxylem, phloem and pericycle cells.

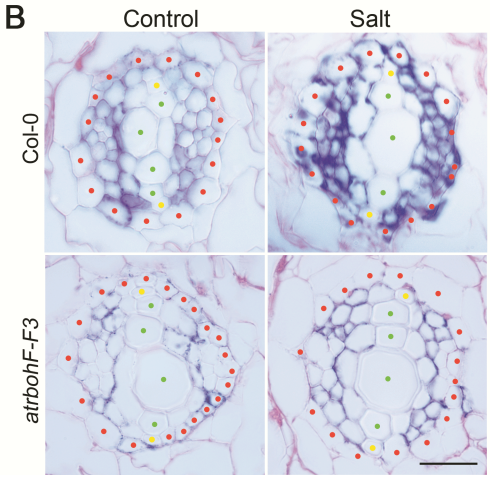
We agree with this comment and have replaced the original cross-section display items with better images, as follows:

1) New images to replace cross section images originally submitted as Figure 3F and 3H:



These images provide better resolution regarding the *AtrbohF* expression pattern, with UV fluorescence images in panels G and J clearly indicating the location of xylem cells. The locations of specific cell types are highlighted with red (pericycle), yellow (protoxylem) and green (metaxylem) dots.

2) New images to replace the cross-section images originally submitted for Figure 4:



These new images show with improved cellular resolution the patterns of NBT staining. The locations of specific cell types are highlighted with red (pericycle), yellow (protoxylem) and green (metaxylem) dots.

2. Xylem sap was collected from the shoot, not from the root based on the materials and methods. The statement needs to be clarified.
This comment reflected a slight lack of clarity in our original description of the method used for xylem-sap collection. Xylem sap was in fact collected, after excision of the shoot, from the top of the de-topped root system, not from the bottom of the excised shoot. To clarify this, we have changed a few words in the relevant section of Materials and Methods and believe the improved description of the methodology will ensure there is no ambiguity.

Referee #2

1. Descriptions related to Figure3B are not clear (page 6, the third paragraph, lines 4-8). The authors explain that 35S-driven AtrbohF partially complemented the sss1-1 mutation. However, on the other hand, they also show in Figure S2A and S2B that many independent lines do not retrieve the salt sensitivity. It looks to me that every line is not sufficient and I wonder which line presented in the FigureS2A corresponds to the line shown in Figure 3B. The results do not appear to support the conclusion that over-expression of AtrbohF complemented the mutation. The complementation part of Figure 3B should be removed or new complementation lines and data set using the native AtrbohF promoter should be presented. Or having two alleles could suffice.
The purpose of Figure 3B in the original submission was actually not primarily to determine if over-expression of *AtrbohF* could complement the phenotype of the *atrbohF-F3* mutant (and thus to validate the conclusion that the *atrbohF-F3* mutation confers that phenotype). In fact, the results shown in Figure 2C (*sss1-1* and *atrbohF-F3* homozygotes and *sss1-1*/*atrbohF-F3* heterozygotes all display soil-salinity hypersensitivity) had already proved that *sss1-1* is a novel mutant *AtrbohF* allele (as the referee says, 'two alleles will suffice').
However, as suggested by this referee, and in light of the comment from referee *#3* (comment 3), we have removed the data in the original Figure 3B (and the text discussing these data) from the revised manuscript.

2. The quality of root section data (in Figures 3F, H and 4A) are poor. The results are very interesting. But, the authors should increase the quality.
This is essentially the same comment as made by Referee #1 (comment 1), please see our responses above.

3. Fluorescence images are shown in Figure 5, F to P. It seems that results from two independent plants appear to be shown. I do not know why the authors presented two plants in the case of mutants. Do they show any difference? If not, the authors leave only the representative results and try to reduce the panel number as the current status of this Figure is quite busy. If they wish, additional data could be added to the supplement. In panel Q, the authors show a quantitative data set confirming the reduction.
We agree with this comment. We now present one fluorescence image for each sample in the revised version of Figure 5.

4. In the panel D of Figure 7, the second column from the left is labeled as "atrbohF-F3 salt". This may be "atrbohF-F3 control", instead?
This has been changed.

5. A part of references could be improved. In page 3, the last sentence at the bottom (Second, HKT transporters can...before it reaches the shoots), the authors refer Rus et al., 2001 PNAS for xylem Na+ retrieval mechanism. This is incorrect. Rus et al concluded that AtHKT1 mediates Na+ influx at the root epidermis from the outside of environment, as a soil to root entry mechanism of toxic Na+ influx under salinity stress. Researchers have not confirmed this model presently and have found a different mechanism. Also the paper of Berthomieu et al concluded that AtHKT1 mediates Na+ loading into the phloem in contrast to the text.
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We agree with the referee. We have replaced the Rus et al., 2001 PNAS reference with the references suggested by the referee (Mäser et al., 2002 FEBS; Ren et al., 2005 Nature Genetics; Sunarpi et al., 2005 Plant J).

Referee #3

This is a very interesting and potentially important piece of work describing a new regulator of salt stress in soil grown plants, RBOHF, and an associated suppressor mutation cesa7. The manuscript

is well written and presented and the data appears robust. However, at present, the data is rather short on mechanistic insight. Specific points below.

1. RBOHF would have been an obvious gene to identify in a mutant screen for soil grown plants hypersensitive to salt because this gene has previously been implicated in ABA induced stomatal closure. Therefore, the identification of RBOHF is no great revelation. However, it is very surprising that rbohF mutants do not have increased transpiration rates relative to WT plants exposed to high salt. This observation would therefore suggest that RBOHF function is not physiologically relevant to stomatal aperture control under the conditions employed. Therefore, this work uncovers a potentially exciting new role for RBOHF, which is great! However, the authors observations therefore appear at odds with previous high profile data implicating RBOHF in the control of ABA regulated stomatal apperture. Hence, the authors should discuss this important point in the manuscript.

We agree with these comments and had indeed initially imagined that the altered soil-salinity responses resulting from lack of *AtrbohF* function could be explicable in terms of the previously described effects on regulation of stomatal aperture. We were therefore ourselves surprised by the observation that loss of *AtrbohF* function did not alter transpiration rates in our experimental conditions. However, this is a robust result that we have observed consistently in replicate experiments. To help draw attention to the importance of this observation, we have accordingly added a sentence to the text of the revised manuscript: 'Given that *AtrbohF* mediates ABA-regulation of stomatal aperture (Kwak et al., 2003), the absence of observed effect on transpiration rate in our conditions is intriguing.'

2. If RBOHF is not controlling the transpiration rate through stomatal aperture then a key question is how does RBOHF provide protection against salt stress at the molecular level? Little attempt is made by the authors to address this key point and thus the manuscript is rather short on mechanistic insight for an EMBO paper. Mechanisms for counteracting root-to-shoot Na+ delivery include Na+/H+ antiporters that can pump Na+ back into soil solution, reducing the net influx of Na+ into inner cell layers of the root and HKT transporters that can retrieve Na+ from the transpiration stream in the xylem before it reaches the shoots. Therefore, are the activities of any of these transport proteins impacted by RBOHF function? This would be one good place to start.
We understand why the referee is raising this point, and indeed it is a major focus of our current research. However, this is not at present a straightforward point to answer. To put it into context, an increasing number of plant signalling pathways involve regulation by ROS. What ROS actually does is relatively unclear for all of these pathways, other than that there is an overall sense that ROS may act through modulation of cytosolic calcium signalling. In none of these pathways have the specifics been worked out. We are currently attempting, via a genetic approach, to determine what ROS targets to regulate Na level in xylem sap, but these experiments are so-far inconclusive. We have also directly investigated the possibility of ROS regulation of the activity of some of the candidate Na ion transporters suggested above by the referee, but these experiments are technically difficult and are currently inconclusive. Thus we can at present only speculate on what ROS might actually be doing at the molecular level to cause the effects that it does. This speculation is briefly summarised at the conclusion of the Discussion section, where we mention the possible involvement of cytosolic free Ca^{2+} in ROS signalling and of modulation of ion transporters such as HKT, but we do not believe a much more extended discussion of these possibilities would be warranted at this stage.
3. It is somewhat surprising and slightly worrying that the 35S::RBOHF transgene does not fully complement the sss1 mutation. The authors suggest that the specific cellular location of AtrbohF expression may be essential to confer full soil-salinity tolerance. Does the 35S promoter fail to drive expression in the root cell types that express RBOHF? It would be easy to check the relevant literature for 35S expression and compare it with that driven by RBOHF::GUS.
See response to referee #2 (comment 1).

4. The provided evidence suggests sss1 and rbohf-f3 phenotypes are quantitatively similar, so it is unlikely one or more additional mutations contribute to the sss1 salt sensitive phenotype. However, it would be helpful to include rbohf-f3 in the data panels 4 and 5 alongside sss1 for comparison.
We are not sure what the referee means by '*panels 4 and 5'*. However, we suggest that this point is covered by the fact that we show *rbohf-f3* alongside *sss1-1* in Figure 2C and Supplementary Figure

S1E.

Additional Correspondence 11 September 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by referee #2. As you can see below, the referee appreciates the introduced changes. I am therefore very pleased to proceed with the acceptance of the paper for publication here. Before doing so referee #2 has a minor text changes suggestion that I would like you to take a look at. If in agreement with the referee then you can send us the amended word file by email.

Once we get this last issue resolved we will proceed with the acceptance of the paper for publication here.

Thank you for submitting your interesting study to the EMBO Journal!

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #2

The authors identify a new salt hypersensitive mutant of Arabidopsis (soil salinity sensitive1-1; sss1-1) by a genetic screen. The SSS1 locus was found to encode a NADPH oxidase, AtrbohF. The AtrbohF mechanism mediating salt tolerance was analyzed by the authors and was demonstrated to be essential for reducing shoot Na+ accumulation via controlling xylem Na+ retrieval, indicating that Reactive oxygen species (ROS) regulate the Na+ level in the xylem vessel under salinity stress. The authors further showed that the sss1-1 mutation was suppressed by the collapse of stem xylem due to the disruption of the AtCesa7 gene encoding the cellulose synthesis catalytic subunit7. These results provide evidence that AtrbohF- mediated ROS production controls xylem sap Na+ levels and plant salinity tolerance. The presented results are interesting and very valuable information and will be of great interest in the field of plant salt tolerance.

The authors have nicely revised the manuscript according to the comments of the reviewers.

Only a minor comment was found. The comparison to stomata effects of RbohF as proposed by one reviewer don't seem to fit the present literature. The single RbohF loss of function mutant has little if any effect on ABA response in stomata, and looking up the cited publications the single RbohF mutant does not increase stomatal apertures with some data showing a small decrease in controls, not lending support to the reviewer comment. Page 9, the last sentence in the paragraph could be deleted to correct this. As the authors correctly state RbohF has many functions in plants. The paper of M. Torres et al. Nature Genetics (2005) could be cited to clarify this relevant point.

