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Iron and ROS control of the DownSTream mRNA decay pathway is essential for plant fitness

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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 21 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the description of the iron and ROS sensitive DST decay pathway to be potentially interesting and important, however, they currently provide mixed recommendations with referee #2 and #3 being more positive than referee #1. As a consequence the referees require further experimental analysis to make the study suitable for The EMBO Journal. This includes a further analysis of the link between Fe induced expression and stability of the DST containing transcripts and further discussion of potential models. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, 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.

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Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Continuing their work on an iron-inducible AtFer1 gene the authors measured AtFer1 mRNA stability and demonstrated that it is destabilized by the addition of iron. Interestingly, they identified a DST motif in the 3'UTR of AtFer1 mRNA and showed that AtFer1 mRNA became stabilized in dst1 and dst2 mutants. Other iron-inducible genes containing the putative DST sequences also displayed altered expression pattern. Moreover, the dst1 and dst2 mutants were altered in their oxidative physiology and growth. The authors concluded that the oxDST pathway is an essential mechanism allowing plants to cope with adverse environmental conditions.

mRNA turnover is a subject of considerable current interest and manuscripts describing such results should be of potential interest to EMBO journal. Based on their results the authors suggested that the oxDST pathway is an essential mechanism allowing plants to cope with adverse environmental conditions. If fully substantiated, this finding is novel and would advance our understanding of the DST pathway. However, for reasons detailed below, I feel that that the manuscript is not acceptable in its present form. Additional experiments are needed to solidify the current data set, particularly to address the relationship between Fe induced expression and Fe induced mRNA distabilization. Major comments are listed below.

1. Figure 1. The authors demonstrated that CHX treatment extended the half-life of AtFer1 mRNA from 60min to 5h (page 5). First of all, no error bar was given in the figure although 3 biological replicates were used in the qRT-PCR measurement. Second, it is not clear the exact effect of CHX to the cell. Blocking protein synthesis would be a major one. However, there are many reports that CHX will affect mRNA stability due to possibly arresting mRNAs on polysomes. Considering the latter, the authors would need to include other transcripts as controls for this experiment. E.g. the authors can use AtFer3 to check the specificity of CHX effect.

2. As shown in Figure 2A, the half-life of the AtFer1 mRNAs was dynamically changed overtime. There was no obvious acceleration in mRNA degradation rate at 1h or 1.5h after 300uM Fe-citrate treatment, compared to untreated controls (time 0h). The author should explain why they chose the degradation rate at 3h after Fe treatment as the only time point used for comparison. AtFer3 and AtAPX1 showed steady degradation rate and their abundance was only induced 1-3 fold by Fe. The authors should address the issue whether the higher transcription rate change (25-30 fold) might affect the measurement of AtFer1mRNA stability.

3. In Figure 3B, the half-life of AtFer1 mRNA after 3h of Fe treatment was shown as 42min. This value is quite different from the 90min shown in Fig 2. Please clarify.

4. Figure 4C showed the LUC mRNA abundance was different with different 3'UTRs. However, the author should measure the half-life for each mRNA species to demonstrate the relationship between the degradation rate and the 3'UTRs.

5. Figure 6 was not properly labeled. I could not find Fig. 6B and At2g30390 (page 8) in the figure. There is big difference for mRNA abundance between dst1 and dst2, e.g. at5g63790 was only changed in dst1 but not dst2. Fe response for some transcripts was abolished in dst mutant which would suggest that DSTs are required for Fe response.

6. Minor issues:
fig 8 picture quality is poor;
conclusion and future challenge part is too long;
Data not shown: SAUR-AC1 was not modulated in response to Fe (page 7);

Referee #2:

This is a very interesting manuscript that demonstrates the importance of the DST-dependent mRNA degradation mechanism for regulation of At Fer1 by iron and reactive oxygen species (ROS). The authors present strong evidence that both stimuli can decrease the half-life of AtFer1 mRNA. In the case of regulation by iron, data from reporter genes indicate that a functional DST element is required and regulation of AtFer1 is impaired in dst mutants 1 and 2. These mutants also alter the expression of several other genes that are iron-regulated and contain DST elements. Finally, they show that the fitness of the dst1 and 2 mutants under high Fe conditions is reduced, arguing that the pathway is essential for regulating growth and stress responses.

Overall, this manuscript represents a strong contribution to our understanding of sequence-dependent mRNA degradation and should be of interest to a broad audience. It is clearly written, and the data is convincing for the DST-dependent regulation of AtFer1 in response to Fe treatment and that it goes through ROS as in the model in Fig 8. However, it has yet to be shown that H2O2 regulation is DST-dependent since only iron was used in Fig 4 and H2O2 was not used to test the mutants. In addition, iron treatment can lead to H2O2 production. To better represent that there is less evidence for H2O2 going through DST than iron, a dashed arrow from the Fe to H2O2 and a set of dashed arrows parallel and to the right of the solid arrows from ROS to Protein Synthesis and Protein Synthesis to the DST machinery, should be added to Fig 8.

Several minor, but very important, corrections are necessary as listed below, especially related to the figures and methods:

1. In Fig 1, the last time point is at 48 hours after Fe addition, but the legend indicates 24 hours, so this discrepancy needs to be corrected, and the time of Fe addition should be added to the graph. A key or labels should be added for the gray and black curves that do not go past 12h.

2. A key to the treatments (i.e., black versus gray) should also be indicated in Fig 2. The number of biological replicate half-life experiments that have been done to produce Figs 1 and 2 should be indicated in the legend or methods.

3. The y-axis of Fig 4C needs to be corrected because the abundance of LUC mRNA was quantified rather than AtFer1 mRNA as indicated.

4. It seems important to add the actual SAUR-AC1 DST sequence to Fig 4A, since the text refers to SAUR-AC1 (as at the top of p.8). It differs from the DST of the SAUR-AC1-like DST shown in Fig 4A in that SAUR-AC1 has less space between the motifs and is more typical. Since the AtFer1 DST has the greatest spacing, this contrast would be nice to provide a better point of reference for the search for DSTs that was described in the manuscript with spacing of up to 50 for the each "n".

5. Fig 6B in the text should be changed to read Fig 6. Alternatively, A, B, and C should be added to the image and indicated in the text.

6. Data in Fig 6 does not match the text in two places. In the second graph in the 3rd row, the text indicates At2g30390 whereas At5g26030 is in Fig 6. Also, At2g31890 abolished the Fe response in dst1, rather than dst2 as the text states. These discrepancies need to be corrected.

7. More explanation for the model Fig 8 should be added. It would seem that the arrows from circadian to the DST machinery should be black solid lines rather than dashed and CCL should be black since the dst mutant effect has been shown. Since not all circadian genes are affected by dst, probably the Fig should include less than 1500 genes not more; however, more explanation should clarify that.

8. Conditions for the H2O2 treatment need to be provided in the methods.

9. Since the dst mutants affect circadian events, indicating when the lights come on relative to the starting times for the experiments is recommended.

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Referee #3:

This is a very interesting manuscript that describes a ROS-activated mRNA decay pathway that requires DST1, and perhaps DST2, which are loci identified more than a decade ago and implicated in selected mRNA decay. The initial identification of DST1 and DST2 was based on a 3' sequence of an unstable mRNA (SAUR), but these two loci have yet to be characterized molecularly. A subsequent study of circadian clock-regulated mRNAs confirmed a role for DST1, but not DST2, in selected mRNA decay. This study makes a major contribution by confirming a role for DST2 in selective mRNA decay, identifying ROS as a signal that activates decay of a specific mRNA subset, and revealing that decay of some mRNAs requires both DST1 and DST2, while others require either DST1 or DST2.

This study arose from an analysis of AtFER1 mRNA stability, which the authors found to be sensitive to cycloheximide (implying the requirement for an unstable protein). Their analysis indicates that while Fe plays a role in this mRNA destabilization pathway, its effect is through production of ROS. Their study links this destabilization pathway to a DST element in the AtFER1 mRNA, and identifies additional Fe (ROS) transcripts regulated by stability. Interestingly, their work confirmed a role for DST2 in RNA decay, and identified additional mRNAs that decay in an ROS-dependent pathway.

I think this paper is a significant contribution in the mRNA decay field, however, there are a number of problems that need to be addressed before this paper is publishable.

Major concerns:

1. The labeling of figures, and the figure legends are both inadequate. The legends provide the methods for the experiments, but they fail to explain the graphs. Many (most) graphs contain lines of different colors (e.g. see figures 1 and 2), and for the most part the meaning of the colors is not explained. Many graphs have "time" as the label for the X axis, and in different graphs, this might mean time after addition of CHX, or addition of Fe, etc. Better labeling would make this manuscript easier to read, and thereby increase its potential impact.

2. The qRT-PCR methods need to be more robust. Only a single internal control was used, there is no analysis to show that this particular internal control is not affected by their treatments (Fe, ROS, and dst mutants). The method does state that they used biological replicas, but they also need technical replicas. The methods should be explained in more detail.

3. One of the really important contributions of this research is a clarification of the DST pathway. The AtSAUR mRNA is the quintessential DST mRNA, and the manuscript would benefit from a more thorough comparison to SAUR decay. The authors claim that their analysis reveals a new degradation pathway (bottom of page 7), but really, I think they show it reveals an additional way that mRNAs are selected for a particular decay pathway. In support of identifying a new pathway, the authors state that SAUR decay is not regulated by ROS, but with data not shown. These data should be shown.

4. Another of the really interesting findings, at the beginning of this manuscript, is that an unstable protein is required for AtFER1 decay (Fig 1a). This observation is not followed up with the subsequent analysis of other Fe-(ROS)-regulated mRNAs that require DST1 and/or DST2. This story would be more complete if the SAUR and other DST-requiring mRNAs (Fig 6) were also analyzed for a requirement for an unstable protein. This one simple experiment will help the authors flesh out their ideas of a single pathway or multiple pathways. The authors should show these data, and also test whether SAUR requires an unstable protein (implied by a CHX experiment shown in Fig 1A). Also the unstable protein should be included in their discussion.

5. The authors claim to have shown that the AtFER1 3'UTR is necessary and sufficient for the transcript accumulation pattern. In fact, they have shown that the genes affected in the dst1 and dst2 mutants are required, but their data linking this to the 3' UTR (figure 4) is weak.

6. Table 1 is cited on page 8 as showing that 14 genes are regulated in a manner that requires DST1 and DST2, but its only a table giving gene identity.

7. Analysis of the DST sequence (figure 4) needs to be expanded. It should include the mutations analyzed in part C of the figure, as well as the putative DST sequences identified in the transcripts analyzed for Figure 6 (or another figure should show the DST sequences after they have been introduced in figure 6), and perhaps analyze the mRNA sequences identified by Lidder et al. as requiring DST1 for similarity to their DST sequences.

8. The final section of the results focuses on catalase activity (page 9). This is not well linked to the story, and I could not understand its relevance.

9. A model is presented in the discussion (page 11, Figure 8) that suggests that the DST1 and DST2 gene products are parts of a core machinery. I do not agree with this model. It is not clear how the differential requirement for DST1 and/or DST2 in decay of several mRNAs can be explained by a single core containing both proteins. In their model, I do not see how defects in a core (e.g. in dst2 or dst1 mutants) would allow normal decay rates. An alternative model is that DST1 and DST2 represent distinct decay pathways or distinct machinery to select mRNAs for decay. Alternative models need to be considered in the discussion, and critically analyzed. Also the figure legend is problematic - AtFER1 is described as conditionally processed, but this vague phrase will be confusing to readers, and it should be made more specific.

Minor concerns:
1. page 8 - last paragraph - says oxDST mRNA degradation pathway is controlled by Fe and ROS, but I think this is misleading - the authors clearly show that Fe regulation is through ROS.

2. page 5 - its unclear what "green cells" are.

3. Figure 1a,b: axis should be labeled time after Fe addition,
in general, labeling of graphed to indicate time after what (e.g. Fe, CHX Cordycepin) would help.

4. page 8 - awkward - sentence "we screened among transcripts regulated in response to Fe for those containing potential DST-like sequences" seems misplaced, because the next sentence says they looked at all Arabidopsis transcripts first, then screened for Fe response. Its really not clear what they did, figure legend 6 and text imply different things.

5. Figure 2 - I don't know what is meant by the term "evolution", I think they mean degradation? This figure includes dark colored lines and then lighter gray lines, but there is no information about what these different lines mean.

6. page 10 - near bottom - sentence begining "Recently, a large-scale analysis..." doesn't make sense - I do not understand what the authors mean by "that category".

1st Revision - Authors' Response 15 June 2011

<u>*Referee #1:*</u>

Continuing their work on an iron-inducible AtFer1 gene the authors measured AtFer1 mRNA stability and demonstrated that it is destabilized by the addition of iron. Interestingly, they identified a DST motif in the 3'UTR of AtFer1 mRNA and showed that AtFer1 mRNA became stabilized in dst1 and dst2 mutants. Other iron-inducible genes containing the putative DST sequences also displayed altered expression pattern. Moreover, the dst1 and dst2 mutants were altered in their oxidative physiology and growth. The authors concluded that the oxDST pathway is an essential mechanism allowing plants to cope with adverse environmental conditions.
mRNA turnover is a subject of considerable current interest and manuscripts describing such results should be of potential interest to EMBO journal. Based on their results the authors suggested that the oxDST pathway is an essential mechanism allowing plants to cope with adverse environmental conditions. If fully substantiated, this finding is novel and would advance our understanding of the DST pathway. However, for reasons detailed below, I feel that that the manuscript is not acceptable in its present form. Additional experiments are needed to solidify the current data set, particularly to address the relationship between Fe induced expression and Fe induced mRNA distabilization. Major comments are listed below.

1. Figure 1. The authors demonstrated that CHX treatment extended the half-life of AtFer1 mRNA from 60min to 5h (page 5). First of all, no error bar was given in the figure although 3 biological replicates were used in the qRT-PCR measurement. Second, it is not clear the exact effect of CHX to the cell. Blocking protein synthesis would be a major one. However, there are many reports that CHX will affect mRNA stability due to possibly arresting mRNAs on polysomes. Considering the latter, the authors would need to include other transcripts as controls for this experiment. E.g. the authors can use AtFer3 to check the specificity of CHX effect.

Error bars are now included in the Figure 1 A and B.
We do agree that such pharmacological experiments needs to be carefully controlled and similar experiments as described in Figure 1A were performed on *AtFer3*, *AtAPX1*, *AtFer4* and *AtSAUR-AC1* transcripts (see below, Additional Figure 1). Since several decades, mRNA over-accumulation after a CHX treatment (usually referred as "superinduction") has been shown to reveal both increase of mRNA transcription (see for example Lau & Nathans 1987 PNAS 84: 1182; Linial et al., 1985 Science 230: 1126) and decrease of mRNA stability (see for example Shaw & Kamen 1986 Cell 46: 659; Hershko et al., 2004 J Cell Biochem 91: 951). Indeed protein synthesis inhibition has pleiotropic effects that alter the accumulation of most of the transcripts analyzed (all except *AtFer4*). These indirect effects lead to the over-accumulation of the transcripts after 12 to 24 hours of CHX treatment. These effects are observed in cells treated with Fe but also (in a less extent) in control cells. As pointed out by the reviewer, CHX may have several direct and indirect effects on mRNA stability like mRNA aggregation on the polysomes. It is also likely that 24 hours of absence of *de novo* protein synthesis may affect the overall ability of the cells to degrade mRNA molecules. However, *AtFer1* mRNA exhibits a particular accumulation pattern where it over-accumulates as soon as 3 hours after CHX addition. This comparative analysis led us to consider this *AtFer1*-specific behaviour as an indication of a potential destabilising mechanism. Then, we validated that hypothesis by monitoring *AtFer1*, *AtFer3* and *AtAPX1* mRNA half-life after 3 hours of Fe treatment (Figure 2). Furthermore, our overall set of data converges towards a mechanism occurring between 3 and 6 hours, which correlates with this early *AtFer1* accumulation in response to CHX.

<u>Additional Figure 1:</u> Effect of CHX on AtFer3, AtFer4, AtAPX1 and AtSAUR-AC1 accumulation in response to Fe. Arabidopsis cells were pre-treated with cycloheximide (+CHX) or not (-CHX) for 1 hour prior to Fe addition (300 μM). CHX effect was also tested on cells untreated with Fe (+$CHX_{control}$). AtFer3, AtFer4, AtAPX1 and AtSAUR-AC1 mRNA accumulation was measured using qRT-PCR using Sand transcript (At2g28390) as a reference. Values and standard errors were obtained from 3 independent experiments.

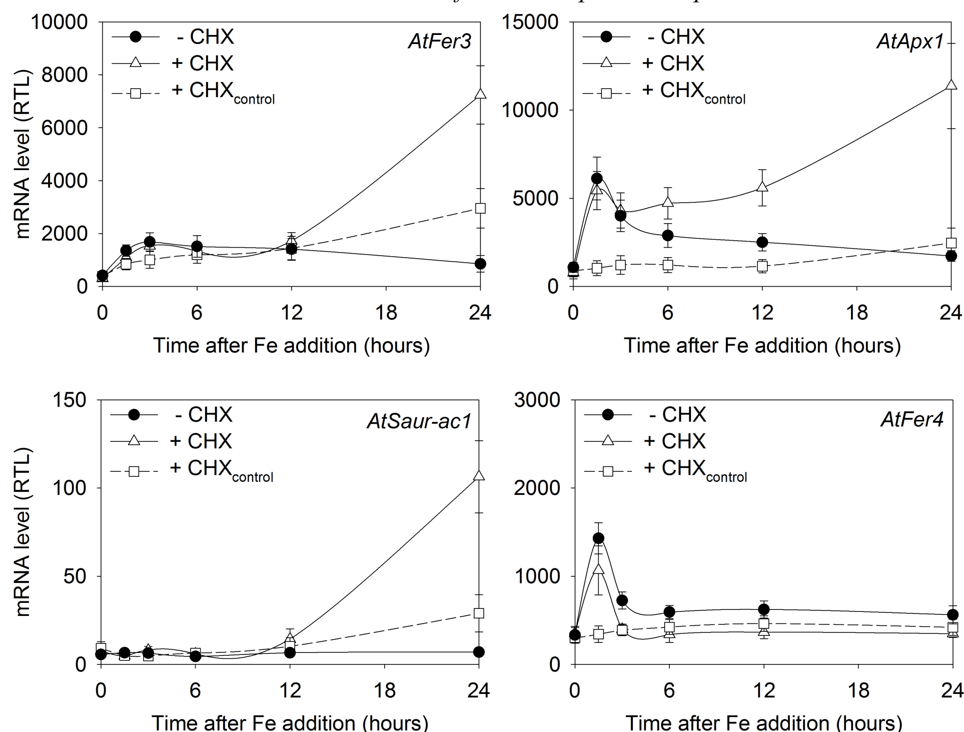
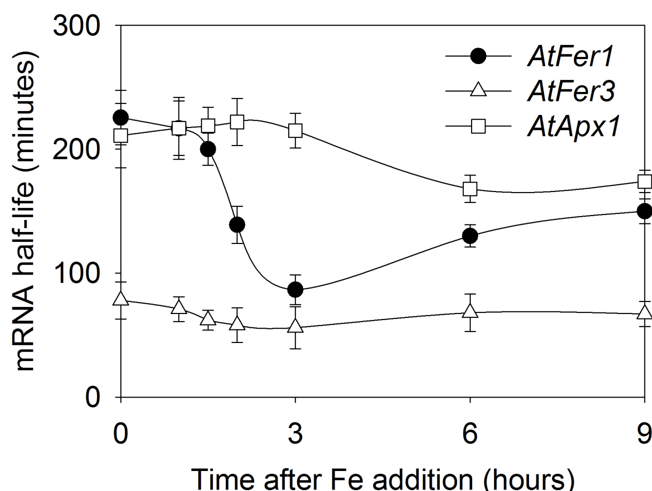


Figure 1 in the manuscript corresponds to the introductive part of our work. The results presented in the following figures confirmed the hypothesis we raised. Thus, in order to avoid introducing confusion, and since the results we provided on Figure 2 are robust, we think that the results shown above in the Additional Figure 1 should not be incorporated into the revised version of the manuscript.

2. As shown in Figure 2A, the half-life of the AtFer1 mRNAs was dynamically changed overtime. There was no obvious acceleration in mRNA degradation rate at 1h or 1.5h after 300uM Fe-citrate treatment, compared to untreated controls (time 0h). The author should explain why they chose the degradation rate at 3h after Fe treatment as the only time point used for comparison. AtFer3 and AtAPX1 showed steady degradation rate and their abundance was only induced 1-3 fold by Fe. The authors should address the issue whether the higher transcription rate change (25-30 fold) might affect the measurement of AtFer1mRNA stability.

During the course of these experiments, we raised the same point than the referee.

Additional Figure 2: *Effect of Fe on AtFer1, AtFer3 and AtAPX1 mRNA half-life. Arabidopsis cells were treated with Fe (300 µM). AtFer1, AtFer3,and AtAPX1 mRNA accumulation was measured using qRT-PCR using Sand transcript (At2g28390) as a reference as described in Figure 2 A, B and C. Half-life values were calculated according to Gallie et al, 1991 and shown are averages from 3 biological replicates ± SD.*



Based on the results presented in Figure 2 in the manuscript, we analysed the modulation of *AtFer1*, *AtFer3* and *AtAPX1* mRNA half-life during 9 hours after Fe addition. As expected based on the mRNA degradation patterns observed in Figure 2A, B and C, *AtFer3* and *AtAPX1* decay were not affected by Fe. However, *AtFer1* mRNA half-life was quickly and strongly decreased between 2 and 6 hours. We therefore selected that point “3 hours” for all subsequent experiments, since it represents the most contrasted condition (when compared to the half-life obtained in control condition). This additional figure 2 is used as supporting information in the present letter, but we did not consider useful to incorporate it into the revised version.

Concerning the half-life determination, we used a logarithmic regression curve according to Gallie (1991). To ensure the absence of relationship between the amount of accumulated *AtFer1* mRNA (related to the transcription rate change mentioned by the referee) and the relative half-life of the transcript, we determined both *AtFer1* accumulation and relative half-life at different iron concentration ranging from 0 to 1 mM. These results are now included in the revised version (Figure 2D). We first check that whatever the iron concentration used, the maximal amount of mRNA accumulation was obtained around 3 hours (these data are not included in the paper, but are available upon request). When the maximal amount of *AtFer1* mRNA accumulation was plotted as a function of iron concentration used, a plateau was raised around 1 mM. Increasing the concentration after this point did not increase further *AtFer1* mRNA accumulation. By contrast, *AtFer1* mRNA relative half-life was not modulated in response to concentrations ranging from 50 µM to 1 mM, and between these two iron concentrations, *AtFer1* accumulation was about doubled.

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Moreover, during the selection of transgenic plants used for experiments presented in Figure 4, we isolated lines with variable level of *LUC* mRNA accumulation. And for all the lines, the relative half-lives measured were almost the same (see new Figure 4 panel D). Thus it appeared from these experiments, that there was no relationship between mRNA accumulation and mRNA half-life. This point was also raised and discussed in the work by Newman et al. (Newman et al., 1993 Plant Cell 5: 701).
Figure 2D and the corresponding section in the "Results" part were changed according to the answer provided.

3. In Figure 3B, the half-life of AtFer1 mRNA after 3h of Fe treatment was shown as 42min. This value is quite different from the 90min shown in Fig 2. Please clarify.

Two systems were used in this study to determine *AtFer1* mRNA half-life: *Arabidopsis* cell cultures and liquid grown seedlings. These systems are quite different (de-differentiated cells, seedlings, different media and growth conditions etc) and could be considered as independent systems. Since metal homeostasis and response to environmental factors are probably very different in these two systems, we are not surprised to find different relative mRNA half-lives. It has to be noted that in both systems, the decrease of *AtFer1* mRNA half-life was almost the same. In the different studies she performed, Pr Pamela Green showed also differences in *SAUR* transcripts half-lives depending on the system used (see for example Newman et al., 1993; Gil & Green 1996 EMBO J 15: 1678; Sullivan & Green 1996 RNA 2: 308). To our opinion, such differences clearly illustrate the complexity of the pathways involved, and the physiological differences between the systems we used.

4. Figure 4C showed the LUC mRNA abundance was different with different 3'UTRs. However, the author should measure the half-life for each mRNA species to demonstrate the relationship between the degradation rate and the 3'UTRs.

Indeed, *Luciferase* mRNA abundance was determined and not *AtFer1* as previously mentioned on the Y-axis of the Figure 4C. We changed the figure accordingly.
We did replicate the experiment presented in Figure 4C and analyzed the half-life of the *LUC* transcripts, both in control condition (without Fe addition) and after 3h of Fe treatment. The values have been incorporated as a new panel in Figure 4 and the results are now introduced and discussed in the manuscript. We agree that this data reinforces the link between the mRNA decay, the DST motif and the 3'-UTR of *AtFer1*.

5. Figure 6 was not properly labeled. I could not find Fig. 6B and At2g30390 (page 8) in the figure. There is big difference for mRNA abundance between dst1 and dst2, e.g. at5g63790 was only changed in dst1 but not dst2. Fe response for some transcripts was abolished in dst mutant which would suggest that DSTs are required for Fe response.

Labels in figure 6 and the AGI number page 8 are corrected in the revised version.

6. Minor issues:
fig 8 picture quality is poor; conclusion and future challenge part is too long;
Data not shown: SAUR-AC1 was not modulated in response to Fe (page 7);

The quality of Figure 8 has been improved and modified according to the other reviewers' suggestions.

The data concerning *SAUR-AC1* mRNA accumulation and relative half-life in response to iron are now included in the revised version as a supplemental Figure 1.

<u>Referee #2:</u>
This is a very interesting manuscript that demonstrates the importance of the DST-dependent mRNA degradation mechanism for regulation of AtFer1 by iron and reactive oxygen species (ROS). The authors present strong evidence that both stimuli can decrease the half-life of AtFer1 mRNA. In the case of regulation by iron, data from reporter genes indicate that a functional DST element is required and regulation of AtFer1 is impaired in dst mutants 1 and 2. These mutants also alter the

expression of several other genes that are iron-regulated and contain DST elements. Finally, they show that the fitness of the dst1 and 2 mutants under high Fe conditions is reduced, arguing that the pathway is essential for regulating growth and stress responses.
Overall, this manuscript represents a strong contribution to our understanding of sequence-dependent mRNA degradation and should be of interest to a broad audience. It is clearly written, and the data is convincing for the DST-dependent regulation of AtFer1 in response to Fe treatment and that it goes through ROS as in the model in Fig 8. However, it has yet to be shown that H2O2 regulation is DST-dependent since only iron was used in Fig 4 and H2O2 was not used to test the mutants. In addition, iron treatment can lead to H2O2 production. To better represent that there is less evidence for H2O2 going through DST than iron, a dashed arrow from the Fe to H2O2 and a set of dashed arrows parallel and to the right of the solid arrows from ROS to Protein Synthesis and Protein Synthesis to the DST machinery, should be added to Fig 8.

We do agree with this comment, and we modified the model accordingly. The fact that there is actually less evidence for H_2O_2 is also highlighted in the legend to the model.

Several minor, but very important, corrections are necessary as listed below, especially related to the figures and methods:
1. In Fig 1, the last time point is at 48 hours after Fe addition, but the legend indicates 24 hours, so this discrepancy needs to be corrected, and the time of Fe addition should be added to the graph. A key or labels should be added for the gray and black curves that do not go past 12h.

A new version of the Figure 1 is provided in the revised version of the manuscript. The related text sections are modified accordingly.

2. A key to the treatments (i.e., black versus gray) should also be indicated in Fig 2. The number of biological replicate half-life experiments that have been done to produce Figs 1 and 2 should be indicated in the legend or methods.

Indications referring to the number of biological replicates are now specified in the legends of all the figures, as well as in the methods section.

3. The y-axis of Fig 4C needs to be corrected because the abundance of LUC mRNA was quantified rather than AtFer1 mRNA as indicated.

Indeed, *Luciferase* mRNA abundance was determined and not *AtFer1* as mentioned previously on the Y-axis. We changed the Figure 4C accordingly.

4. It seems important to add the actual SAUR-AC1 DST sequence to Fig 4A, since the text refers to SAUR-AC1 (as at the top of p.8). It differs from the DST of the SAUR-AC1-like DST shown in Fig 4A in that SAUR-AC1 has less space between the motifs and is more typical. Since the AtFer1 DST has the greatest spacing, this contrast would be nice to provide a better point of reference for the search for DSTs that was described in the manuscript with spacing of up to 50 for the each "n".

The DST sequence alignment in Figure 4A presents (from the top to the bottom) the consensus established in Sullivan et al (1996), the one in the actual *Arabidopsis SAUR-AC1* (At4g38850), and the one *AtFer1*. We previously described in the legend to Figure 4A the second sequence as SAUR-AC1-like, we apologize for this misleading information.
However, the spacing between the different motifs (referred as n) is larger in our alignment since the conserved motifs we described are smaller than in the Sullivan *et al* report. In other words, our n=14 between the ATGATC and the CGTAT motifs in *SAUR-AC1* mRNA corresponds to the n=8 between the CATAGATCG and the CAATGCGTAT motifs described in the same transcript in the previous report.
Concerning the n=50 used in our screen for DST-containing transcripts, we agree that this number is larger than the spacing observed in *SAUR-AC1* and *AtFer1*. How the spacing affect the DST functionality has not yet been studied. Based on the DST sequences found in CCL and SIN1, spacing up to 50 may be relevant (see answer to comment 7 of the reviewer 3 and the additional figure 4). Here, we aimed to analyze the regulatory effect of Fe on their expression. Therefore we tested a large enough number of DST containing genes.

5. Fig 6B in the text should be changed to read Fig 6. Alternatively, A, B, and C should be added to the image and indicated in the text.

Figure 6B is now replaced by Figure 6.

6. Data in Fig 6 does not match the text in two places. In the second graph in the 3rd row, the text indicates At2g30390 whereas At5g26030 is in Fig 6. Also, At2g31890 abolished the Fe response in dst1, rather than dst2 as the text states. These discrepancies need to be corrected.

These discrepancies are now corrected.

7. More explanation for the model Fig 8 should be added. It would seem that the arrows from circadian to the DST machinery should be black solid lines rather than dashed and CCL should be black since the dst mutant effect has been shown. Since not all circadian genes are affected by dst, probably the Fig should include less than 1500 genes not more; however, more explanation should clarify that.

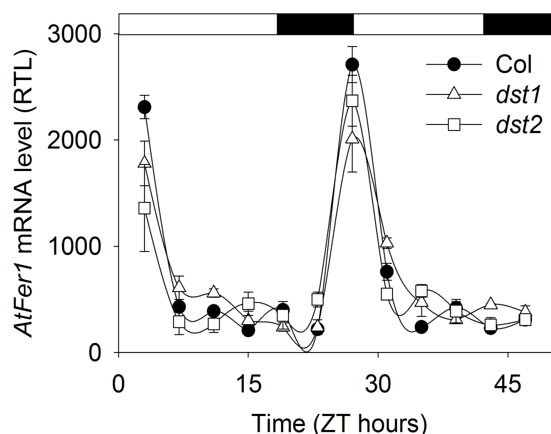
We modified the model and the corresponding legend. We did take in account these comments in the revised version

8. Conditions for the H2O2 treatment need to be provided in the methods.

H_2O_2 treatment conditions were added in the Methods section and in the legend to Figure 3.

9. Since the dst mutants affect circadian events, indicating when the lights come on relative to the starting times for the experiments is recommended.

All the pharmacological experiments were performed using *Arabidopsis* cells and seedlings grown in a growth chamber under continuous light. These specifications were omitted in the Methods section concerning the liquid grown seedlings culture and have now been incorporated. Under continuous light, circadian oscillation does not occur. However, since all the physiological experiments have been performed in long day conditions (8h light/ 16h dark), we did test the effect of *dst1* and *dst2* mutations on *AtFer1* circadian rhythm.



Additional Figure 3: The plants were grown on soil under 16h light/8h dark cycles for two weeks. Samples were harvested from ZT3 every 4 h for 48 hours. RTLs were assayed by qRT-PCR. Shown are the means of 3 biological replicates ± S.D. ZT, Zeitgeber time.

However, *AtFer1* mRNA accumulation was unchanged in *dst1* and *dst2* mutants. This result was important to obtain since we have previously reported that the *AtFer1* gene is transcriptionnaly regulated by the clock (Duc et al, 2009, J Biol Chem 284 : 36271). It supports the idea of multiple

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DST pathways since *AFer1* decay is modulated by Fe in a DST-dependent manner, but *AtFer1* circadian oscillation is not affected in the *dst1* and *dst2* mutants. We did not add that result to the revised manuscript, but it could be incorporated upon request.

Referee #3:

This is a very interesting manuscript that describes a ROS-activated mRNA decay pathway that requires DST1, and perhaps DST2, which are loci identified more than a decade ago and implicated in selected mRNA decay. The initial identification of DST1 and DST2 was based on a 3' sequence of an unstable mRNA (SAUR), but these two loci have yet to be characterized molecularly. A subsequent study of circadian clock-regulated mRNAs confirmed a role for DST1, but not DST2, in selected mRNA decay. This study makes a major contribution by confirming a role for DST2 in selective mRNA decay, identifying ROS as a signal that activates decay of a specific mRNA subset, and revealing that decay of some mRNAs requires both DST1 and DST2, while others require either DST1 or DST2.

This study arose from an analysis of AtFER1 mRNA stability, which the authors found to be sensitive to cycloheximide (implying the requirement for an unstable protein). Their analysis indicates that while Fe plays a role in this mRNA destabilization pathway, its effect is through production of ROS. Their study links this destabilization pathway to a DST element in the AtFER1 mRNA, and identifies additional Fe (ROS) transcripts regulated by stability. Interestingly, their work confirmed a role for DST2 in RNA decay, and identified additional mRNAs that decay in an ROS-dependent pathway.

I think this paper is a significant contribution in the mRNA decay field, however, there are a number of problems that need to be addressed before this paper is publishable.

Major concerns:

1. The labeling of figures, and the figure legends are both inadequate. The legends provide the methods for the experiments, but they fail to explain the graphs. Many (most) graphs contain lines of different colors (e.g. see figures 1 and 2), and for the most part the meaning of the colors is not explained. Many graphs have "time" as the label for the X axis, and in different graphs, this might mean time after addition of CHX, or addition of Fe, etc. Better labeling would make this manuscript easier to read, and thereby increase its potential impact.

All the figures have been edited accordingly and legend quality has been improved.

2. The qRT-PCR methods need to be more robust. Only a single internal control was used, there is no analysis to show that this particular internal control is not affected by their treatments (Fe, ROS, and dst mutants). The method does state that they used biological replicas, but they also need technical replicas. The methods should be explained in more detail.

For all qRT-PCR experiments, data analyses were performed in technical duplicates and biological triplicates. We thank the reviewer for pointing out this omission. The corresponding section in the manuscript is now revised.

We indeed did all experiments with at least two housekeeping genes that are not affected by the treatment or the genotype (i.e. similar results were obtained using a normalization by either control genes). The SAND (At2g28390) and Ef1α (At1g07930) genes were efficiently used in most of the experiments in the two biological systems we used (cell suspension and seedlings) except the ones involving CHX treatment (Ef1α transcripts were found to accumulate in response to CHX). For this treatment, we efficiently tested the PP2 gene (At1g13320) as a secondary control gene. Therefore, all mRNA level (RTL) values presented in the figures are obtained relative to the SAND gene expression. Upon request, these specifications could be added to the 'Methods section' of the manuscript, and the sequences of the corresponding qRT-PCR primers could be incorporated into the supplemental Table 1.

3. One of the really important contributions of this research is a clarification of the DST pathway. The AtSAUR mRNA is the quintessential DST mRNA, and the manuscript would benefit from a more thorough comparison to SAUR decay. The authors claim that their analysis reveals a new degradation pathway (bottom of page 7), but really, I think they show it reveals an additional way that mRNAs are selected for a particular decay pathway. In support of identifying a new pathway, the authors state that SAUR decay is not regulated by ROS, but with data not shown. These data should be shown.

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We add data concerning *AtSAUR-AC1* mRNA accumulation and half-life in response to Fe treatment. These data are presented as a supplemental Figure 1. We further discussed these data in the manuscript.

4. Another of the really interesting findings, at the beginning of this manuscript, is that an unstable protein is required for AtFER1 decay (Fig 1a). This observation is not followed up with the subsequent analysis of other Fe-(ROS)-regulated mRNAs that require DST1 and/or DST2. This story would be more complete if the SAUR and other DST-requiring mRNAs (Fig 6) were also analyzed for a requirement for an unstable protein. This one simple experiment will help the authors flesh out their ideas of a single pathway or multiple pathways. The authors should show these data, and also test whether SAUR requires an unstable protein (implied by a CHX experiment shown in Fig 1A). Also the unstable protein should be included in their discussion.

As mentioned above (Reviewer 1, point 1), we analyzed *AtSAUR-AC1* mRNA accumulation in response to CHX and Fe (+CHX), as well as in response to CHX only (+$CHX_{control}$). The *SAUR-AC1* mRNA accumulated after 12 to 24 hours (see additional Figure 1), suggesting that *AtSAUR-AC1* mRNA behaves as *AtFer3* and *AtAPX1*.
This result evidences that the early CHX effect observed on *AtFer1* does not affect *AtSAUR-AC1*, thus suggesting that different pathways are involved.
We did not add the CHX results in the supplemental Figure 1 because we think that the late overaccumulation of the transcript after 24 hours may be misleading.

5. The authors claim to have shown that the AtFER1 3'UTR is necessary and sufficient for the transcript accumulation pattern. In fact, they have shown that the genes affected in the dst1 and dst2 mutants are required, but their data linking this to the 3' UTR (figure 4) is weak.

Indeed, a genetic proof (transforming each *dst* mutants with the constructs used in Figure 4) would reinforce the direct link between DST factors and the 3'-UTR of *AtFer1*. However, the production of these transgenic lines requires more time than that allowed for the revision of the present manuscript.
In addition, as already mentioned above (Reviewer 1, point 4), we performed new analyses concerning the effect of Fe on the half-life of the LUC mRNAs containing the 3'-UTR of *AtFer1* or the corresponding DST-mutated version. Data are added to Figure 4 (Figure 4D). We believe that these data reinforce strongly the involvement of the DST sequence present in the 3'-UTR of *AtFer1* in the Fe-mediated degradation of the transcript, which itself requires DST1 and DST2 factors.

6. Table 1 is cited on page 8 as showing that 14 genes are regulated in a manner that requires DST1 and DST2, but its only a table giving gene identity.

This table should present the 13 additional genes (i) regulated by Fe, (ii) containing the DST sequence in their 3'-UTR and (iii) therefore tested in our study. However, only 9 of them were expressed in our experimental conditions and are presented in Figure 6. The corresponding part of the text is now modified.

7. Analysis of the DST sequence (figure 4) needs to be expanded. It should include the mutations analyzed in part C of the figure, as well as the putative DST sequences identified in the transcripts analyzed for Figure 6 (or another figure should show the DST sequences after they have been introduced in figure 6), and perhaps analyze the mRNA sequences identified by Lidder et al. as requiring DST1 for similarity to their DST sequences

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Name	Locus	Sequence
SAUR-AC1	AT4G38850	5'-GGAA- 10 -ATAGATC- 14 -CGTAT-3'
AtFer1	AT5G01600	5'-CGAA- 15 -TTAGATT- 23 -TGTAG-3'
Zn Finger	AT1G26800	5'-TGAA- 26 -TTAGATT- 35 -TGTAG-3'
MATE	AT1G61890	5'-CGAA- 8 -ATAGATG- 8 -TGTAA-3'
UCP5	AT2G22500	5'-TGAA- 4 -TTAGATT- 22 -TGTAA-3'
Rap	AT2G31890	5'-AGAA- 13 -ATAGATA- 46 -TGTAT-3'
FES1A	AT3G09350	5'-AGAA- 7 -ATAGATA- 2 -TGTAT-3'
SAP12	AT3G28210	5'-GGAA- 14 -TTAGATT- 39 -AGTAA-3'
Bme3	AT3G54810	5'-TGAA- 4 -TTAGATA- 7 -AGTAG-3'
		5'-GGAA- 28 -TTAGATG- 15 -TGTAT-3'
OPT3	AT4G16370	5'-AGAA- 4 -TTAGATT- 45 -TGTAC-3'
Hydrolase	AT4G24380	5'-CGAA- 7 -TTGGATA- 16 -TGTAA-3'
WRKY18	AT4G31800	5'-CGAA- 46 -GTAGATG- 7 -CGTAT-3'
		5'-CGAA- 13 -TTAGATG- 12 -TGTAT-3'
FC1	AT5G26030	5'-GGAA- 2 -TTAGATG- 9 -TGTAA-3'
Zat12	AT5G59820	5'-TGAA- 14 -CTAGATT- 37 -TGTAA-3'
Anac102	AT5G63790	5'-CGAA- 0 -GTAGATA- 9 -TGTAA-3'
CCL	AT3G26740	5'-AGAA- 75 -ATAGATT- 9 -TGTAC-3'
SIN1	AT4G35770	5'-TGAA- 43 -GTAGATG- 11 -AGTAT-3'

Additional Figure 4: Sequence alignment of the DST motifs present in the 3'-UTR of validated or predicted DST-containing genes. AtSAUR-AC1 and AtFer1 are presented similarly to the alignment in the Figure 4A. In addition the DST found in the 13 candidate genes are presented as well as the one found in CCL and SIN1 (Lidder et al, 2005). Black symbols indicate nucleotide conserved in all sequences and grey symbols indicate the nucleotides conserve only in AtSAUR-AC1 (white letter) or in AtFer1 (black letter). Two DST motifs were identified in the 3'-UTR of Bme3 and WRKY18 mRNAs. For those, the two alignments are presented underneath

We did perform such analysis (see above Additional Figure 4). However, to search for DST-containing genes, we designed the algorhythm based on the alignment of *Arabidopsis SAUR-AC1* and *AtFer1* (Figure 4A). Indeed, the alignment of all the DST present in the 3'-UTR of the candidate genes confirmed the consensus we established. In addition, we also aligned *CCL* and *SIN1* mRNAs, two transcripts exhibiting a DST sequence in their 3'UTR and shown to be altered in *dst1* background (Lidder *et al*, 2005). Interestingly, these two DST sequences support the alignment we obtained, thus validating the algorhythm we used to identify other potential candidate genes.
However, we do not think that this alignment should be incorporated into the manuscript. In our opinion, it is crucial to base such sequence consensus on DST elements proven to be functional. So far, only the ones in *SAUR-AC1* and the *AtFer1* mRNA are relevant templates, because they represent the only two shown as functional (based on mutagenesis experiments of the DST *cis* element). In this report, we demonstrated that several DST-containing mRNAs do not accumulate in response to Fe in *dst1* and/or *dst2* mutants. A lower accumulation of a mRNA can not be explained by a direct impairment of its destabilization, thus it is rather due to indirect effects of the *dst* mutations. Thus the link between over-accumulation of a transcript and *dst* mutations has to be carefully investigated by analyzing mRNA half-life in these genetic backgrounds. We really believe that determining potential spacing limits or potential additional conserved nucleotide, based on putative DST elements that may appear to be irrelevant in the future, may be misleading. However, if you think that such Figure could be potentially helpful for the audience, we will incorporate it after the Figure 6, or as a supplemental Figure.

8. The final section of the results focuses on catalase activity (page 9). This is not well linked to the story, and I could not understand its relevance.

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The link between the photosynthetic defects in the *dst* mutants observed at high light intensities and the altered activation of the ROS-detoxication system (catalase) at high light has been strengthened by re-writing this part in the results section in the revised manuscript.
One of the major effect of high light treatment on plants is to promote a strong oxidative stress. Consequently, plants activate various enzymatic and non-enzymatic anti-oxidant responses. Among them, catalase activity is known to be strongly induced, and is therefore a good marker of the plant responses to high light exposure. As expected, high light treatment led to a transient increase of catalase activity in control plants, and interestingly, the catalase activity was not increased in the two *dst* mutants under high light.

9. A model is presented in the discussion (page 11, Figure 8) that suggests that the DST1 and DST2 gene products are parts of a core machinery. I do not agree with this model. It is not clear how the differential requirement for DST1 and/or DST2 in decay of several mRNAs can be explained by a single core containing both proteins. In their model, I do not see how defects in a core (e.g. in dst2 or dst1 mutants) would allow normal decay rates. An alternative model is that DST1 and DST2 represent distinct decay pathways or distinct machinery to select mRNAs for decay. Alternative models need to be considered in the discussion, and critically analyzed. Also the figure legend is problematic - AtFER1 is described as conditionally processed, but this vague phrase will be confusing to readers, and it should be made more specific.

We fully agree with these comments concerning the model we proposed. We modified it accordingly and provided more consideration for alternative models in the legend.

Minor concerns:
1. page 8 - last paragraph - says oxDST mRNA degradation pathway is controlled by Fe and ROS, but I think this is misleading - the authors clearly show that Fe regulation is through ROS.

The corresponding text section has been modified accordingly.

2. page 5 - its unclear what "green cells" are.

We replaced “green cells” by “*Arabidopsis* cell suspension culture”.

3. Figure 1a,b: axis should be labelled time after Fe addition, in general, labelling of graphed to indicate time after what (e.g. Fe, CHX Cordycepin) would help.

All Figures have been modified accordingly.

4. page 8 - awkward - sentence "we screened among transcripts regulated in response to Fe for those containing potential DST-like sequences" seems misplaced, because the next sentence says they looked at all Arabidopsis transcripts first, then screened for Fe response. Its really not clear what they did, figure legend 6 and text imply different things.

This point is now clarified in the text.

5. Figure 2 - I don't know what is meant by the term "evolution", I think they mean degradation? This figure includes dark colored lines and then lighter gray lines, but there is no information about what these different lines mean.

This figure and the corresponding legend are now revised accordingly.

6. page 10 - near bottom - sentence beginning "Recently, a large-scale analysis..." doesn't make sense - I do not understand what the authors mean by "that category".

This sentence is now modified.

2nd Editorial Decision 20 July 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. I apologise for the length of time it has taken to have the manuscript reviewed but this due to the fact that the referee was unavailable for a significant period. One of the referees has reevaluated the study and finds that it is significantly improved and recommends publication one the single issue regarding the RNA decay data is addressed and an additional control is added.

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 REPORT:

Referee #3:

This is a much improved version of the first submission. The authors have made a sincere effort to make figures and text much more understandable. This work is an important contribution to our understanding of mRNA decay pathways, and I expect that it will be of interest to a wide audience - not only those interested in RNA decay, but also people with interests in stress responses and Fe regulation.

Now that I understand the figure legends, I do have one concern about the RNA decay data. In Figure 2, the authors show mRNA decay rates following cordecepin addition at various time points after the Fe addition. It is clear that AtFER1 shows a strong increase in mRNA decay at 3 hours after Fe addition. However, I'm not convinced that AtFER3 does not respond. That is, the time points zero, 6, and 9 all look like they have the same decay kinetics, but time points 1, 2, 3, and 4 look similar to each other - yet a higher rate than the other three time points.

The editor might also want to ask for one additional control. Figure 3 shows their test for whether ROS were required for the Fe response. They showed that preincubation with NAC prevented the cells from responding to H2O2 or Fe (by increasing RNA decay rates), but the did not show whether the NAC pretreatment alone affected RNA decay.

Smaller points:

The Arabidopsis gene nomenclature convention is not followed. Generally gene names are shown in all caps (italics), or all lower case (italics) if mutant. The RNA is also the same - italics all caps. .

Another line states: "As expected, SAUR-AC1 stability was affected in dst1 and dst2." But I cannot figure out if this is from the literature or shown somewhere.

2nd Revision - Authors' Response 10 August 2011

Answers to Referee #3 :

Now that I understand the figure legends, I do have one concern about the RNA decay data. In Figure 2, the authors show mRNA decay rates following cordycepin addition at various time points after the Fe addition. It is clear that AtFER1 shows a strong increase in mRNA decay at 3 hours

after Fe addition. However, I'm not convinced that AtFER3 does not respond. That is, the time points zero, 6, and 9 all look like they have the same decay kinetics, but time points 1, 2, 3, and 4 look similar to each other - yet a higher rate than the other three time points.

In order to clarify this point, we had an additional figure : Figure 2D. Based on the results presented in Figure 2A, B and C, we analysed the modulation of *AtFer1*, *AtFer3* and *AtAPX1* mRNA half-life during 9 hours after Fe addition.
AtFer3 and *AtAPX1* decay were only slightly affected by Fe. By contrast, *AtFer1* mRNA half-life was quickly and strongly decreased between 2 and 6 hours. We therefore selected that point "3 hours" for all subsequent experiments, since it represents the most contrasted condition (when compared to the half-life obtained in control condition).
The text in the "Results" section was changed accordingly.

The editor might also want to ask for one additional control. Figure 3 shows their test for whether ROS were required for the Fe response. They showed that preincubation with NAC prevented the cells from responding to H2O2 or Fe (by increasing RNA decay rates), but the did not show whether the NAC pretreatment alone affected RNA decay.

We do agree that this control is important to show. It was added in the new version of Figure 3.

The Arabidopsis gene nomenclature convention is not followed. Generally gene names are shown in all caps (italics), or all lower case (italics) if mutant. The RNA is also the same - italics all caps.

Nomenclature of ferritin genes was modified by replacing AtFer by AtFER. Caps and italics were used for genes and transcripts, and lower case italic for mutants.

Another line states: "As expected, SAUR-AC1 stability was affected in dst1 and dst2." But I cannot figure out if this is from the literature or shown somewhere.

Three *trans*-acting components of the DST pathway, named DST1-3, were isolated by a forward genetic approach (Johnson *et al*, 2000; Lidder *et al*, 2004; Pérez-Amador *et al*, 2001) based on the deregulation of *AtSAUR-AC1* transcript accumulation. Over-accumulation of *AtSAUR-AC1* in *dst1* and *dst2* backgrounds was presented in Johnson et al. (2000), PNAS 97: 13991-13995.

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