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Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity

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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 10 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments to the authors are shown below. As you will see both referees are very positive about the paper and would support its publication here after appropriate revision. We will therefore be happy to consider a revised manuscript once you have addressed the referees' comments in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

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Referee #1 (Remarks to the Author):

This is a very nice study on an important issue of plant pathogen response and of plant cell biology. With the aim of identifying regulators of EFR function the authors have performed a screening for Arabidopsis mutants impaired in the response to the EFR inducer elf18. Using genetics and biochemistry assays they found that a complex formed by BiP, SDF2 and the co-chaperone Erdj3B is required for EFR synthesis. Importantly, the structurally related receptor FLS2 is much less affected by mutations that impair the complex. In general, the data are of high quality and the interpretation of the results is in most cases convincing, but the following questions should be solved:

- Page 8. "Notably, SDF2 was not found in complex with other ER resident proteins (Supplementary Figures 8 and 9), demonstrating that the association of SDF2 with ERdj3B and BiP was specific." The data demonstrate that the association with BiP survives the assay procedures and that association with Calreticulin or SHD are either non-existing or too weak or transient to be detected in the assay. I think that, to be safer, the authors should mention this, because it is well established that ligand associations with calreticulin or endoplasmin are much more difficult to detect than those with BiP, and often require cross-linking for detection.
- The authors failed to stabilize EFR in the sdf2-2 background by MG132 treatment. This experiment needs a positive control for the effect of the proteasome inhibitor on a known ERAD substrate in the assay conditions used by the authors. If the reliability of the assay is confirmed, this deserves more discussion. Kifunensine is an inhibitor of mannosidase and therefore only indirectly affects ERAD degradation in the cytosol. Because it interferes with interactions with ER residents its effect on EFR can be due to enhanced retention of the receptor in the ER, a scenario that is also compatible with degradation of EFR occurring in locations other than the cytosol. It has been recently shown that plant ER quality control degradation can also occur in the vacuole (Foresti et al, Mol. Plant 1: 1067).
- Isn't it surprising that single BiP knockouts show wild type levels of elf18 response? In this respect the authors should comment on the fact that a BiP2 knockout is impaired in SA-induced resistance and hypersensitive to SA analogs (Wang et al 2005, cited in the manuscript).
- Figure S1. There is no amplification of SDF2 in sdf2-4. Is this expected?

Additional points

- Page 3, sixth line from bottom. Change "Misfolded" to "Unfolded". Newly synthesized proteins that eventually will fold properly are unfolded, not misfolded. Misfolded proteins are usually not recoverable and will aggregate or get destroyed.
- Add molecular mass markers in both panels of Figure S4A.
- Figure 7B is never cited.

Referee #2 (Remarks to the Author):

The ms by Nekrasov et al. describes the results of a genetic screen for Arabidopsis mutants impaired in the response towards elf18, the ligand for the EFR protein. The work is complete, up to the point of providing a mechanism for the proteins encoded as elements of 2 ER-QC events. The first of these employs the SDF2-ERdj3B-BiP complex, while the second is a component of the N-glycosylation mechanism. Somewhat surprisingly, the sdf2 and erdj3b mutants do not show any developmental changes and may indeed have a different degree of specificity for the EFR and FLS2 receptors. However, this point is not really addressed in the ms and I think it would full outside the scope of this work and could be addressed elsewhere.
Given the overall very high quality of the experiments I have only a few minor points.

1. It appears to me that the SDF containing complex has a more specific role in folding the EFR and FLS2 receptors rather than acting to remove misfolded proteins as seems to be inferred from yeast and animal cell experiments. This distinction could be made somewhat more specific in the discussion and emphasize this aspect for the plant members of these protein families.
2. In fig. 7 there are numbers above the lanes that are not really explained. Since these figures are essentially non-quantitative WBs, I assume they represent some form of quantification of the bands below but this does not seem to correspond to all of the bands. It would be informative to add here a sentence or 2 about the quantitative effects of the sdf and erdj3B mutant backgrounds on the residual amounts of EFR proteins. This may help further to quantitatively understand the link between reduced receptor protein level and the observed effects on efl18/fls22 SGI and sensitivity towards pathogens.

1st Revision - Authors' Response 20 July 2009

Referee #1
Page 8. "Notably, SDF2 was not found in complex with other ER resident proteins (Supplementary Figures 8 and 9), demonstrating that the association of SDF2 with ERdj3B and BiP was specific." The data demonstrate that the association with BiP survives the assay procedures and that association with Calreticulin or SHD are either non-existing or too weak or transient to be detected in the assay. I think that, to be safer, the authors should mention this, because it is well established that ligand associations with calreticulin or endoplasmin are much more difficult to detect than those with BiP, and often require cross-linking for detection.
We have modified the sentence to “SDF2 was not found in complex with other ER resident proteins under the conditions tested” to take in account the referee’s comments.

The authors failed to stabilize EFR in the sdf2-2 background by MG132 treatment. This experiment needs a positive control for the effect of the proteasome inhibitor on a known ERAD substrate in the assay conditions used by the authors. If the reliability of the assay is confirmed, this deserves more discussion. Kifunensine is an inhibitor of mannosidase and therefore only indirectly affects ERAD degradation in the cytosol. Because it interferes with interactions with ER residents its effect on EFR can be due to enhanced retention of the receptor in the ER, a scenario that is also compatible with degradation of EFR occurring in locations other than the cytosol. It has been recently shown that plant ER quality control degradation can also occur in the vacuole (Foresti et al, Mol. Plant 1: 1067).
We thank the referee for this constructive comment. We cannot provide a positive control for the effect of the proteasome inhibitor on a known physiological ERAD substrate in our Arabidopsis mutants, as all currently known plant ERAD substrates are either heterologously expressed proteins (*e.g.* RTA, RCA A and mutant forms of MLO) (reviewed in Vitale and Boston, Traffic 2008) or mutant forms of the Arabidopsis BRI1 receptor (Jin et al., Mol Cell 2007; Hong et al., Plant Cell 2008; Jin et al., PNAS 2009). This would therefore require to over-express these proteins in *sdf2*, or to cross *sdf2* with *bri1-9* or *bri1-5*. Although informative, we do not believe that these experiments are physiological and are required for the current manuscript. Here we report that the biogenesis of the endogenous EFR, in physiological conditions, depends on SDF2 and other ER proteins.
ERAD is commonly associated with proteasome-dependent degradation of misfolded proteins in the cytosol (Anelli and Sitia, EMBO J 2008). Our results (Fig. 7F) show that EFR level in *sdf2* is not restored after treatment with the proteasome inhibitor MG132, suggesting that EFR degradation is proteasome-independent. Interestingly, the cytosolic ATPase CDC48 that is required for efficient degradation of plant ERAD substrates is also required for degradation of non-ubiquitinated substrates, suggesting that a proteasome-independent pathway does indeed exist in plants (Marshall et al., JBC 2008).
We however agree with the referee that a positive control for the MG132 treatment in our experiments should be performed. We have tried to use a series of antibodies to detect proteins that are regulated by ubiquitination (Maor et al., Mol Cell Proteomics 2007). Using commercial antibodies against Arabidopsis Hsp70/Hsc70, we found that the signal of non-specific bands corresponding to unknown proteins was increased in the samples treated with MG132 (see figure in response to referees), suggesting that the treatment was effective. However, in the absence of more

convincing evidence, we have decided to remove our results on the MG132 treatment and have changed the text accordingly.
Our remaining results with the kifunensine still show that degradation of EFR in *sdf2* is an active process and requires ER exit. We realised that we have neglected to give more explanation about the possible effect of kifunensine and have changed the text accordingly.

Isn't it surprising that single BiP knockouts show wild type levels of elf18 response? In this respect the authors should comment on the fact that a BiP2 knockout is impaired in SA-induced resistance and hypersensitive to SA analogs (Wang et al 2005, cited in the manuscript).
No. We are aware of the results from Wang et al. (2005); this is why we performed the experiments presented in Fig. S4. Our results clearly show that *sdf2* is not impaired in SA-induced resistance, in contrast to what was found for *bip2* previously. This is further evidence that the phenotypes reported in our manuscript for *sdf2* and *erdj3b* are not due to a general defect in the secretory pathway, but specifically affect EFR and potentially other PRRs. Although PAMPs can induce SA-dependent resistance (Mishina and Zeier, Plant J 2007), mutants affected in the establishment of SAR are generally not affected in early PAMP responses.
Given the high homology between BiP1 and BiP2, we even considered a potential functional redundancy between the two genes and generated *bip1 bip2* double-mutants. They were also wild-type for their response to elf18 (Fig. S12). We have now mentioned the SAR phenotype of *bip2* in the text.

Figure S1. There is no amplification of SDF2 in sdf2-4. Is this expected?
This is not necessarily expected, but it is common that point mutations can lead to transcript destabilization.

Additional points
Page 3, sixth line from bottom. Change "Misfolded" to "Unfolded". Newly synthesized proteins that eventually will fold properly are unfolded, not misfolded. Misfolded proteins are usually not recoverable and will aggregate or get destroyed.
Change made.
Add molecular mass markers in both panels of Figure S4A.
Change made.
Figure 7B is never cited.
Citation added.

Referee #2
Given the overall very high quality of the experiments I have only a few minor points.
1. It appears to me that the SDF containing complex has a more specific role in folding the EFR and FLS2 receptors rather than acting to remove misfolded proteins as seems to be inferred from yeast and animal cell experiments. This distinction could be made somewhat more specific in the discussion and emphasize this aspect for the plant members of these protein families.
We have now further emphasized the specific roles of SDF2 and ERdj3B in plant innate immunity in the text.

2. In fig. 7 there are numbers above the lanes that are not really explained. Since these figures are essentially non-quantitative WBs, I assume they represent some form of quantification of the bands below but this does not seem to correspond to all of the bands. It would be informative to add here a sentence or 2 about the quantitative effects of the sdf and erdj3B mutant backgrounds on the residual amounts of EFR proteins. This may help further to quantitatively understand the link between reduced receptor protein level and the observed effects on efl18/fls22 SGI and sensitivity towards pathogens.
We apologise for this confusion. The numbers above the lanes correspond to the reference of the transgenic lines used in the experiments; they do not correspond to a quantification of the EFR signal. Therefore we have now replaced the numbers with “1” and “2” in all cases to avoid confusion.

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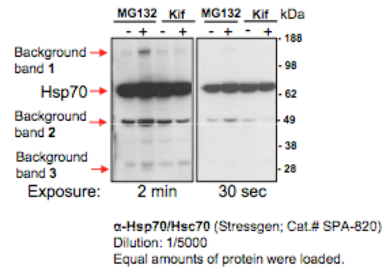

Figure in response to referees

2nd Editorial Decision 04 August 2009

Thank you for sending us your revised manuscript. Our original referee 2 has now seen it again, and you will be pleased to learn that in his/her view you have addressed their criticisms in a satisfactory manner, and we feel that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider following the minor suggestion by referee 1 regarding the MG132 experiments (see below). Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORT

Referee #2 (Remarks to the Author):

My quite minor concerns have been adequately addressed and I have no further suggestions for improvement. In my opinion it is a pity that the MG132 experiments have been removed from the ms. I propose to include these data in one of the supplementary files because data such as this need to be accessible for other researchers.

2nd Revision - Authors' Response 08 August 2009

I am delighted to hear about your decision.
I have submitted the new files that include the MG132 results as a supplementary figure.

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