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# Dehydration stress activates Arabidopsis MPK6 to signal DCP1 phosphorylation

Jun Xu, Nam-Hai Chua

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**Review timeline:**

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| Submission date: | 15 July 2011 |
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## Transaction Report:

(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 05 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise that it has unexpectedly taken longer than usual to have your manuscript reviewed but have now received final report and I enclose all the referees' comments below. As you will see they are in general positive about the role of dehydration stress and MPK6 in regulated mRNA decapping and expression, however, they raise a number of issues that need to be experimentally addressed before a revised manuscript can be further considered by the EMBO Journal. Many of these are required to strengthen the current data in the manuscript, which are central to the main conclusions, the referees would also like to see a description and comparison of the phenotypes in the complementation experiments, including that of a kinase dead MPK6 line. Given the support from the referees I would like to invite you to submit a revised version of the manuscript.

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.

 

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript proposes that phosphorylation of DCP1 (via MAP Kinase 6) leads to interaction with DCP5 (a homolog of RAP55), and that this interaction regulates drought-response gene expression. Understanding the role of RNA decay in regulation of gene expression is an important topic, and so the topic of this paper is appropriate for publication in the EMBO J. I think the authors might be correct that DCP1 phosphorylation is important for DCP2 activity, and I found this an interesting story.

The authors show clearly that DCP1 is phosphorylated, but this is not new (it was previously reported in animals). The two isoforms the authors use to identify their phosphorylated version were also reported previously in paper published in Plant Cell by the same authors.

Probably the best part of this paper is their demonstration that the ratio of phosphorylated:non-phosphorylated DCP2 changes in response to drought (Figure 1). The authors also developed some very nice tools for investigating the role of DCP1 phosphorylation -- these are transgenic plants carrying a phospho-mimic version and a transgenic carrying a version that cannot be phosphorylated. These constructs appear to have been placed into the mutant dcp1 genetic background, but it was not clearly stated. It is also necessary that the authors add a complete description of the degree of rescue by each construct (supported by actual photographs and complete descriptions), the number of transgenic lines recovered, and the variability of rescue.

The authors examine primary root growth (+/- mannitol, Figure 2b) of various mutants to link DCP1 phosphorylation (and DCP5) mediate stress responses. These data are not convincing. Rather it appears that both normal and 5% mannitol growth were both affected in the mutants, while the root length ratios were similar.

Half life of EXPL1 mRNA was examined in various mutant lines. I was not convinced that there was a significant difference between the two different DCP2 transgenic lines, especially given that one is described as having a 30 minute half life, but the 60 minute time point is just as intense as the 30 minute time point. The authors would be well served by using a more quantitative method.

The authors go on to analyze whether phosphorylation changes the affinity of DCP1 for the RAP55 homology (DCP5). This experiment appears to have used the same FLAG epitope for both DCP2 and DCP5. Because the figure legend is incomplete, and I was left unable to determine whether this core premise was supported or not. These authors are well established scientists, and so I generally think the data are probably correct, but they need to supply the appropriate explanations.

The authors carried out microarray experiments, and found many mRNAs that had altered dehydration responses in dcp5 mutants. One might predict that if decapping were partially defective, then direct targets of the DCP5 regulation would fail to decay, and so would show no abundance change in the treatment. Instead, more mRNAs had diminished levels in dcp5 than accumulated. It is not clear to me what this means, nor is it adequately explained.

To connect DCP2 phosphorylation and DCP5 interaction to RNA decapping, the authors compared the ratio of capped/uncapped CRC mRNA in WT and dcp5-1 (RNAi line). I am convinced that in WT, dehydration led to strong reduction in cap (Table 2, described in results on page 11). However, the authors state that there is not a similar decrease in the dcp5-1 mutant (page 10/11), implying a role for dcp5 in regulation of decapping. BUT if you look at their data, in fact the issue is not the dehydration response, but instead it is their T=0 timepoint. There is almost no capped CRC RNA in

 

dcp5, making a change in ratio in response to stress uninterpretable. This is a serious flaw - both the data and the way the data were presented.

The next experiment carried out entailed comparing RNA on polysomes. The authors appear to be showing that they have DCP1 contamination in their polysomes. What is the purpose of showing this, and what are the numbers (1-12) under the blots shown in 4B? The authors state that their "response ratio" was greater for the polysomal RNA; this could simply be due to the requirement for a cap in order for RNA to be in the polysome fraction. Further, there needs to be an analysis of relative expression of each RNA, not simply the response ratios.

Reference might be incomplete, I only looked at a handful, and found Yoon et al., 2010, cited in the introduction, not included among the references.

Page 6: authors describe their earlier paper as showing that the phosphorylated form of DCP1 accumulates during seed development - I looked at this paper, figure 6 - shows that in fact both forms (larger and smaller) accumulate. So - factually correct the way it is stated is misleading, it gives an incorrect impression that the putative phospho form does not show greater accumulation than the non-phospho form.

page 3 - EVH1 "domain" - domain needs to be added for clarity. Strong editing is required throughout the manuscript.

The methods appear incomplete or wrong, they describe the DCP1 rescue line as a CFP fusion, but I found no other explanation of CFP.

Referee #2 (Remarks to the Author):

Xu and Chua identified a specific, dehydration-responsive phosphorylation site in DCP1, which is modified by MPK6 during seed dehydration and development. DCP1 mutants that do not support phosphorylation at this site are developmentally aberrant, and more sensitive to osmotic stress with enhanced DCP1 transcript stability. These data support a role for DCP1 phosphorylation in development and stress responses, although the DCP1 S237A mutant phenotype discussed is not shown in the manuscript. The data to support these findings are otherwise clear and the figures well-presented, although there are a number of apparent errors in the manuscript (below). An elegant assay for comparing transcript half-lives was employed to illustrate compromised decay in phosphor-mutant DCP1. Xu and Chua propose a mechanism whereby DCP1 phosphorylation enhances RNA binding and mRNP formation, leading to enhanced decapping activity. This is based on enhanced RNA binding by DCP5 in the presence of phosphor-mimic DCP1, and enhanced interaction in vitro and in vivo between phosphor-mimic DCP1 and DCP5 or DCP1. Microarray analysis of wild type vs the dcp5-1 loss-of-function mutant response to dehydration showed significantly altered expression of several hundred genes, specifically several transcription factors. Some, but not all, of these altered transcripts are shown to be targets of DCP5-dependent decapping, as capped transcripts prevail in the dcp5 mutant. Finally, dehydration-induced MPK6-mediated phosphorylation of DCP1 is shown in vitro, with genetic evidence for the role of MPK6.

In summary, the topic is of interest for readers of EMBO Journal, and a connection between MPK6 and the decapping component DCP1 is of general interest. However, some issues outlined below should be resolved.

General points
1-There are many inconsistencies in verb tenses. For example on page 6 -'phospho-DCP1 accumulates, which likely resulted'.
2-There are several spelling and grammatical errors which are too numerous to list. For example on page 3, delete 'that' (line6), 'multi-cell' to multi-cellular' (line 8) and 'a long extended' to 'a long' or 'an extended' (line 14).
3-Xu and Chua demonstrate that dehydration leads to increased phosphorylation of DCP1 (Figure 1A). In addition, they show that the phosphorylation requires MPK6 (Figure 5E). While these observations are interesting, no evidence of their biological relevance is presented. Would it not be biologically informative to compare the phenotypes of dcp1-1 complemented DCP1 S237A to that

 

of dcp5-1 and dcp1-1 complemented DCP1 S237D under different abiotic stresses including dehydration. More importantly, it might be very informative to include mpk6 mutants expressing kinase inactive versions of MPK6 (Bush & Krysan 2007, J Exp Bot. 58, 2181). While the plant community generally ignores this type of experiment when working with MAP kinases, researchers working with other organisms discovered a long time ago that such tools are revealing (Madhani et al., 1997. Cell 91, 673). For example, MPK3 and 6 are often considered redundant, but mpk6 mutants exhibit a clear stomatal phenotype when expressing dead versions of MPK6. Such experiments could strengthen the authors findings in a biologically relevant context.

Specific points
1-Page 7 - It is stated that the fact that DCP1 S237A and dcp5-1 are sensitive to osmotic stress suggests that their association is important for these responses. Does the mutant DCP1 compete out DCP5 interactions due to the increased association with other DCP1 moieties and DCP2?
2-Figure 2A - This provides evidence for enhanced association between DCP1 phosphor-mimic mutant and DCP5, but the description given is insufficient. It would be nice to have a better description of precisely what was done.
3-As per 2 above, does this apply to all abiotic stresses, or is it specific to osmotic and dehydration stress?
4-Figure 2D - It would be informative to have DCP1 wild-type as a control reference for the wild-type level of interaction between DCP1, DCP2, DCP5 and VCS.
5-Page 6, last paragraph - The authors claim that dcp1-1 mutants complemented with DCP1-S237A grow poorly, similarly to dcp5-1 mutants. It would be very informative to see representative images of these plants, perhaps in a supplementary Figure.
6-DCP1 phosphorylation enhances mRNA decapping and contributes to some of the decapping activities in vivo. What else could contribute to this?
7-Fig 2E lane 9 vs lane 7 - Why is there less RNA binding by DCP5 in the presence of DCP1?
8-Why were the genes CRA1 and DREB selected? A small explanation of their function would be helpful to the non-specialist reader. Also, more discussion of the other genes with altered profiles would be enlightening.
9-There is no reference to Figure 4B in the body text of the manuscript.
10-Is there any proposed mechanism to explain why phosphorylation of S237 may alter the properties of DCP1, given its context in an apparently unstructured region surrounded by a stretch of Prolines with a 'stretch of Serines' downstream?

Referee #3 (Remarks to the Author):

This manuscript nicely showed that the dehydration response in Arabidopsis involves induction of DCP1 phosphorylation through MPK6. The data indicate that phosphorylation promotes DCP1 association with DCP5 and stimulates decapping. The location of the phosphorylation site in DCP1 was also mapped. The manuscript uses a range of complementary approaches to provide novel insights about decapping and stress in plants and is also relevant to stress-related decapping mechanisms in other multicellular eukaryotes.

Several revisions are recommended before publication.
1. Figure 1 shows that phosphorylated DCP1 is increases during dehydration. However, the total amount of DCP1 protein also appears to be affected during dehydration and rehydration in Figure 1A and 1B. The authors should comment on this and whether the control of DCP1 is solely by phosphorylation or by both induction and phosphorylation. The rationale for using chloroplast rbcL for the internal control for dehydration should be discussed. The unlabled control in Figure 5B-D (rbcL?) should be indicated in the figure or legend.
2. On page 7, the statement, that the osmotic stress sensitivity phenotype of DCP1-S237A and dcp5 mutants suggests "DCP5 association with phospho-DCP1" is important for stress responses, is unclear. Figure 2B showed that WT and all the mutants showed similar sensitive phenotypes to osmotic stress if the dehydration response ratio is considered as in Figure 3. Also, the osmotic stress experiment should be described in methods in addition to that for dehydration. The method for rehydration also needs to be added.
3. Potential targets of the DCP5-phosphoDCP1 association during dehydration were identified by microarray experiments using the dcp5-1 mutant. Even though results from the dcp5-1 mutant might be expected to be similar to that of unphosphorylated DCP1 (or DCP1-S237A), comparing the data

 

between them will be more informative. Moreover, DCP1-S237D could provide a better control for this experiment. It is assumed that all DCP1-S237A and DCP1-S237D lines referred to in the manuscript are complementation lines in a dcp1-1 background. However, this should be stated. Is it necessary to use different notation for these lines in Table 1? If so, the new abbreviations should be included in the legend.
4. CRC and CRA1 were selected for further analysis of the effect of decapping during dehydration stress. In Table 2, rather than just the ratios, normalized transcript levels of capped and decapped transcripts like Table1 should be provided to judge the potential roles of these genes in dehydration responses. Also, the effect of rehydration on these genes might be more supportive. Finally additional discussion about how decapping of CRC and CRA could contribute to dehydration or drought responses should be included, such as whether the genes have ever reported to be in an abiotic stress pathway.

1st Revision - authors' response 07 October 2011

Response to referees:

Referee #1(Remarks to the Author):
*This manuscript proposes that phosphorylation of DCP1 (via MAP Kinase 6) leads to interaction with DCP5 (a homolog of RAP55), and that this interaction regulates drought-response gene expression. Understanding the role of RNA decay in regulation of gene expression is an important topic, and so the topic of this paper is appropriate for publication in the EMBO J. I think the authors might be correct that DCP1 phosphorylation is important for DCP2 activity, and I found this an interesting story.*

*1. The authors show clearly that DCP1 is phosphorylated, but this is not new (it was previously reported in animals). The two isoforms the authors use to identify their phosphorylated version were also reported previously in paper published in Plant Cell by the same authors.*

We have previously reported the detection of a DCP1-related doublet by western blot (Xu et al., 2009). However, the biochemical nature of this doublet was unknown. Here, we identified the upper band of the doublet as phosphorylated DCP1. In addition, we showed that S237 is the only phosphorylated site on DCP1.

Although phosphorylation of human DCP1a has been reported the kinase mediating this modification has not yet been described and its connection to stress responses is unknown.

*2. Probably the best part of this paper is their demonstration that the ratio of phosphorylated:non-phosphorylated DCP2 changes in response to drought (Figure 1). The authors also developed some very nice tools for investigating the role of*
*DCP1 phosphorylation -- these are transgenic plants carrying a phospho-mimic version and a transgenic carrying a version that cannot be phosphorylated. These constructs appear to have been placed into the mutant dcp1 genetic background, but it was not clearly stated. It is also necessary that the authors add a complete description of the degree of rescue by each construct (supported by actual photographs and complete descriptions), the number of transgenic lines recovered, and the variability of rescue.*

We assume there is a typographical error in the reviewer's comment above. We described the phosphorylation of DCP1, not DCP2.
In response to the above, we have added to the manuscript Fig 1S as well as more detailed descriptions of the complemented lines in the text (p.6-7).
"The T-DNA insertion in the *DCP1* locus of the *dcp1-1* mutant conferred resistance to sulfadiazin whereas the T-DNAs we subsequently introduced into *dcp1-1* conferred resistance to glufosinate. We recovered more than 100 T1 plants resistant to both antibiotics. Since the transgene carried a FLAG tag it was possible to compare the expression levels of native DCP1 and FLAG tagged DCP1 using appropriate antibodies. Among 15 T1 plants of each complemented line, we identified 4

 

putative homozygous *dcp1-1* complemented with FLAG-DCP1S237A and 4 complemented with FLAG-DCP1S237D. Growth on double selection medium along with protein detection (a typical western blot was shown in Fig. 1D) were used to select plants of the T2 and subsequent generations. Finally, we characterized 3 individual T4 lines homozygous with respect to both T-DNAs."

*3. The authors examine primary root growth (+/- mannitol, Figure 2b) of various mutants to link DCP1 phosphorylation (and DCP5) mediate stress responses. These data are not convincing. Rather it appears that both normal and 5% mannitol growth were both affected in the mutants, while the root length ratios were similar.*

We have added Fig. S1 to show growth of 12-day old seedlings of different genotypes on different media. On p7 of the text, we have added the following statement: "The hypersensitivity was readily visible after 12 days of growth on treatment medium, such that the two lines were unable to produce lateral roots, which was a typical response in WT (Fig. S1)."

*4 .Half life of EXPL1 mRNA was examined in various mutant lines. I was not convinced that there was a significant difference between the two different DCP2 transgenic lines, especially given that one is described as having a 30 minute half life, but the 60 minute time point is just as intense as the 30 minute time point. The authors would be well served by using a more quantitative method.*

We consider Northern blot analysis to be the best method available for determining transcript half-life. Quantitative method such as qRT-PCR suffers from the lack of size information of the target mRNA.

*5. The authors go on to analyze whether phosphorylation changes the affinity of DCP1 for the RAP55 homology (DCP5). This experiment appears to have used the same FLAG epitope for both DCP2 and DCP5. Because the figure legend is incomplete, and I was left unable to determine whether this core premise was supported or not.*
*These authors are well established scientists, and so I generally think the data are probably correct, but they need to supply the appropriate explanations.*

For greater clarity we have added to legend of Fig. 2A: "ANTI-FLAG (mouse monoclonal) M2 affinity gel was used for immunoprecipitation. Rabbit polyclonal antibodies, anti-DCP5 and anti-DCP2, were used for detection."

*6. The authors carried out microarray experiments, and found many mRNAs that had altered dehydration responses in dcp5 mutants. One might predict that if decapping were partially defective, then direct targets of the DCP5 regulation would fail to decay, and so would show no abundance change in the treatment. Instead, more mRNAs had diminished levels in dcp5 than accumulated. It is not clear to me what this means, nor is it adequately explained.*

If a direct target of DCP5 regulation should fail to decay, the transcript response ratio of this target in the *dcp5* mutant is expected to be higher than the one in WT. This is why we resorted to measuring the transcript response ratio but not the absolute transcript levels. Indeed, more mRNAs showed a down regulation of transcript response ratio of more than 2-fold in *dcp5* compared to WT suggesting an impaired response.

*7. To connect DCP2 phosphorylation and DCP5 interaction to RNA decapping, the authors compared the ratio of capped/uncapped CRC mRNA in WT and dcp5-1 (RNAi line). I am convinced that in WT, dehydration led to strong reduction in cap (Table 2, described in results on page 11). However, the authors state that there is not a similar decrease in the dcp5-1 mutant (page 10/11), implying a role for dcp5 in regulation of decapping. BUT if you look at their data, in fact the issue is not the dehydration response, but instead it is their T=0 timepoint. There is almost no capped CRC RNA in dcp5, making a change in ratio in response to stress uninterpretable. This is a serious flaw - both the data and the way the data were presented.*

Again, we assume that there was a typographical error. The reviewer meant DCP1 phosphorylation, not DCP2 phosphorylation.
*dcp5-1* is a T-DNA insertion mutant, a knock-down mutant of DCP5, but it is not a null allele. The capped/uncapped ratio of *CRC* transcripts in *dcp5* was decreased from 0.08 to 0.01, far less than the

change from 11.65 to 0.01 in WT. However, the ratio of 0.08 does not indicate that the absolute level of *CRC* mRNA is low; it just reflects the presence of relatively more uncapped mRNA.

*8. The next experiment carried out entailed comparing RNA on polysomes. The authors appear to be showing that they have DCP1 contamination in their polysomes. What is the purpose of showing this, and what are the numbers (1-12) under the blots shown in 4B? The authors state that their "response ratio" was greater for the polysomal RNA; this could simply be due to the requirement for a cap in order for RNA to be in the polysome fraction. Further, there needs to be an analysis of relative expression of each RNA, not simply the response ratios.*

The transcript response we observed with total RNAs could result from either transcriptional or post-transcriptional changes. The purpose for measuring mRNA levels on polysomal RNAs was to reconfirm the changes observed with total RNAs. The similarity in results indicates that these changes are largely post-transcriptional.
We have already provided the relative expression level of each mRNA in Fig. S2 for both the total RNA and polysomal RNA fraction. We have expanded the description in the figure legend and indicated the 1-12 fractions.

*9. Reference might be incomplete, I only looked at a handful, and found Yoon et al., 2010, cited in the introduction, not included among the references.*

This comment is incorrect. Yoon et al., 2010 was included in the list of references. Actually it was the last one on the list and this might have escaped the attention of the reviewer.

*10. Page 6: authors describe their earlier paper as showing that the phosphorylated form of DCP1 accumulates during seed development - I looked at this paper, figure*
*6 - shows that in fact both forms (larger and smaller) accumulate. So - factually correct the way it is stated is misleading, it gives an incorrect impression that the putative phospho form does not show greater accumulation than the non-phospho form.*

Fig. 6 (Xu and Chua, 2009) shows both DCP1 forms accumulated during seed maturation. However, in dry seed the larger form of DCP1 accumulated much more than the smaller form. This is consistent with what we have observed with seedlings in dehydration response.

*11. page 3 - EVH1 "domain" - domain needs to be added for clarity. Strong editing is required throughout the manuscript.*

*The methods appear incomplete or wrong, they describe the DCP1 rescue line as a CFP fusion, but I found no other explanation of CFP.*

Thank you for pointing out these errors, which we have corrected in this revised version. Indeed, there were grammatical errors and stylistic issues elsewhere in the text and these have been rectified as well.

Referee #2 (Remarks to the Author):

*Xu and Chua identified a specific, dehydration-responsive phosphorylation site in DCP1, which is modified by MPK6 during seed dehydration and development. DCP1 mutants that do not support phosphorylation at this site are developmentally aberrant, and more sensitive to osmotic stress with enhanced DCP1 transcript stability. These data support a role for DCP1 phosphorylation in development and stress responses, although the DCP1 S237A mutant phenotype discussed is not shown in the manuscript. The data to support these findings are otherwise clear and the figures well-presented, although there are a number of apparent errors in the manuscript (below). An elegant assay for comparing transcript half-lives was employed to illustrate compromised decay in phosphor-mutant DCP1. Xu and Chua propose a mechanism whereby DCP1 phosphorylation enhances RNA binding and mRNP formation, leading to enhanced decapping activity. This is based*

 

*on enhanced RNA binding by DCP5 in the presence of phosphor-mimic DCP1, and enhanced interaction in vitro and in vivo between phosphor-mimic DCP1 and DCP5 or DCP1. Microarray analysis of wild type vs the dcp5-1 loss-of-function mutant response to dehydration showed significantly altered expression of several hundred genes, specifically several transcription factors. Some, but not all, of these altered transcripts are shown to be targets of DCP5-dependent decapping, as capped transcripts prevail in the dcp5 mutant. Finally, dehydration-induced MPK6-mediated phosphorylation of DCP1 is shown in vitro, with genetic evidence for the role of MPK6.*

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We have corrected spelling and grammatical errors.

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Please see response to Referee 1, #2,3 for phenotypic description of *dcp5* mutant and complementation lines. We did not attempt to compare mutant phenotypes between *mpk6* and these lines for two reasons: (1) mRNA decapping is only a cytoplasmic function of MPK6 in stress response. As this kinase also regulates a large number of nuclear functions additional mutant phenotypes are expected that are not related to cytoplasmic mRNA decapping. (2) in vitro phosphorylation assay already showed that MPK3 and MPK6 are redundant for DCP1 phosphorylation.

*Specific points*
*1-Page 7 - It is stated that the fact that DCP1 S237A and dcp5-1 are sensitive to osmotic stress suggests that their association is important for these responses.*
*Does the mutant DCP1 compete out DCP5 interactions due to the increased association with other DCP1 moieties and DCP2?*

This is unlikely to be true because we found that DCP1S237A displayed reduced affinity to DCP1 and DCP2 as compared with DCP1S237D (Fig 2D).

*2-Figure 2A - This provides evidence for enhanced association between DCP1 phosphor-mimic mutant and DCP5, but the description given is insufficient. It would be nice to have a better description of precisely what was done.*

The following has been added to legend of Fig. 2A: “ANTI-FLAG (mouse monoclonal) M2 affinity gel was used for immunoprecipitation. Rabbit polyclonal antibodies, anti-DCP5 and anti-DCP2, were used for detection.”

 

*3-As per 2 above, does this apply to all abiotic stresses, or is it specific to osmotic and dehydration stress?*

Figure 2A showed all plants without stress treatment. Because DCP5 protein also accumulated during dehydration stress it is difficult to measure the increased association between DCP1 and DCP5.

*4-Figure 2D - It would be informative to have DCP1 wild-type as a control reference for the wild-type level of interaction between DCP1, DCP2, DCP5 and VCS.*

As the wild-type DCP1 produced in E. coli migrates similarly as DCP1S237A we did not include it in Fig.2D.

*5-Page 6, last paragraph - The authors claim that dcp1-1 mutants complemented with DCP1-S237A grow poorly, similarly to dcp5-1 mutants. It would be very informative to see representative images of these plants, perhaps in a supplementary Figure.*

Please see responses to Referee 1, comment #3.

*6-DCP1 phosphorylation enhances mRNA decapping and contributes to some of the decapping activities in vivo. What else could contribute to this?*

Based on our observation, we think DCP1 is necessary but not sufficient for mRNA decapping in vivo. Unmodified DCP1, phosphorylated DCP1, DCP2, DCP5, and VCS could all contribute to the decapping activities.

*7-Fig 2E lane 9 vs lane 7 - Why is there less RNA binding by DCP5 in the presence of DCP1?*

We think this is likely due to the addition of GST-DCP1 in the MBP-DCP5/RNA mix since the control lane, lane 12, shows a similar effect by GST only.

*8-Why were the genes CRA1 and DREB selected? A small explanation of their function would be helpful to the non-specialist reader. Also, more discussion of the other genes with altered profiles would be enlightening.*

We have added the following to the text (p.10): "*DREB1b* and *DREB2a,* which are typical dehydration stress responsive genes, encode AP2 domain-containing transcription factors (Oono et al, 2003) whereas *CRA1* and *CRC* encode 12S globulins which are typical seed storage proteins"

*9-There is no reference to Figure 4B in the body text of the manuscript.*

We have added this to the text (p.10): "Since DCP5 protein was not detected in the polysomal fractions this protein could not be responsible for the difference in polysomal mRNAs between WT and *dcp5-1*(Fig. 4B)"

*10-Is there any proposed mechanism to explain why phosphorylation of S237 may alter the properties of DCP1, given its context in an apparently unstructured region surrounded by a stretch of Prolines with a 'stretch of Serines' downstream?*

We think phosphorylation of S237 in the unstructured region may affect trimerization of DCP1 as well as the formation of the decapping complex through interactions between DCP1 and DCP5, DCP2, and other components.

Referee #3 (Remarks to the Author):

*This manuscript nicely showed that the dehydration response in Arabidopsis involves induction of DCP1 phosphorylation through MPK6. The data indicate that phosphorylation promotes DCP1 association with DCP5 and stimulates decapping. The location of the phosphorylation site in DCP1 was also mapped. The manuscript uses a range of complementary approaches to provide novel*

 

*insights about decapping and stress in plants and is also relevant to stress-related decapping mechanisms in other multicellular eukaryotes.*

*Several revisions are recommended before publication.*
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The quantification of proteins by western blot is a difficult issue. We have focused on measuring the change of ratio between the upper band and the lower band at the same time point. This made us believe that phosphorylation of DCP1 increased and native DCP1 decreased during dehydration stress. The large subunit of RUBISCO, rbcL, is generally used by the community as a protein loading control.

In our original submission, we have clearly stated that the unlabelled band in Fig 5B-D is the truncated DCP1 substrate stained with Coomassie Brilliant Blue. The reviewer missed reading this description and assumed the band was rbcL. This truncated DCP1 substrate band serves as a convenient loading control.

*2. On page 7, the statement, that the osmotic stress sensitivity phenotype of DCP1-S237A and dcp5 mutants suggests "DCP5 association with phospho-DCP1" is important for stress responses, is unclear. Figure 2B showed that WT and all the mutants showed similar sensitive phenotypes to osmotic stress if the dehydration response ratio is considered as in Figure 3. Also, the osmotic stress experiment should be described in methods in addition to that for dehydration. The method for rehydration also needs to be added.*

Please see response to Referee 1, comment #2.
We have provided additional descriptions to the Materials and Methods section.

*3. Potential targets of the DCP5-phosphoDCP1 association during dehydration were identified by microarray experiments using the dcp5-1 mutant. Even though results from the dcp5-1 mutant might be expected to be similar to that of unphosphorylated DCP1 (or DCP1-S237A), comparing the data between them will be more informative. Moreover, DCP1-S237D could provide a better control for this experiment. It is assumed that all DCP1-S237A and DCP1-S237D lines referred to in the manuscript are complementation lines in a dcp1-1 background. However, this should be stated. Is it necessary to use different notation for these lines in Table 1? If so, the new abbreviations should be included in the legend.*

Thank you for the suggestions.
We have corrected the annotations in Table 1.

*4. CRC and CRA1 were selected for further analysis of the effect of decapping during dehydration stress. In Table 2, rather than just the ratios, normalized transcript levels of capped and decapped transcripts like Table1 should be provided to judge the potential roles of these genes in dehydration responses. Also, the effect of rehydration on these genes might be more supportive. Finally additional discussion about how decapping of CRC and CRA could contribute to dehydration or drought responses should be included, such as whether the genes have ever reported to be in an abiotic stress pathway.*

We have now included detailed qRT-PCR results as Table S4.
Thank you for the nice suggestion. We have added the following to the DISCUSSION section:"The role of this subset of genes, including genes encoding seed storage proteins, in the abiotic stress pathway has not yet been reported. Here, we provided evidence that decapping of their mRNAs could contribute to dehydration responses in plants."

 

2nd Editorial Decision 24 November 2011

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. I apologise that it has taken so long to have your manuscript reviewed but this was in part because one of the referees was unavailable for a significant amount of time. It has now been seen by two of the original referees and as you will see from their comments referee #1 finds that you have not satisfactorily addressed all his/her initial concerns and finds that the current manuscript is not suitable for publication in its current form. It is the policy of The EMBO Journal to only allow a single round of revision, however, given the positive support of two referees and the willingness of referee #1 to look at the manuscript once more means that we are willing to allow a second round of review in this case. However, it is important that the remaining issues raised by referee #1 are addressed prior to publication. At this stage we would like to invite you to submit a second revised version of the manuscript.

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.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is my second time reviewing this manuscript. As stated with the earlier review, there is some excellent, interesting, and exciting data in this paper. Unfortunately it is bogged down with data that continues to not be explained, or is simply flawed. The response by the author's to my earlier comments largely commented on typographic mistakes, and did not address the concerns that I raised.

My concerns about the co-immunoprecipitation have not been answered. The figure legend states that the Co-IP used extracts from seedlings expressing FLAG-DCP1 ... and DCP5-FLAG. Extracts were run on affinity resin that binds to the FLAG tag. There is no way for the authors to distinguish between the affinity resin capturing both proteins based on their FLAG tags, as opposed to pulling down the proposed DCP1-DCP5 heterodimer. The data do not tell me that DCP5 interacts with only one the phosphorylated form. These data are in Fig 2A, and results presented on page 6.

A second concern raised with the previous version of this manuscript is interpretation of root growth sensitivity to mannitol. The authors have included a new supplemental figure, but my concerns still stand. In each genotype, mannitol causes root growth to be diminished. There is still no evidence that dcp5 or DCP1-S237A is more sensitive to mannitol.

The authors and I disagree on whether Northern blots or more quantitative approaches are better for RNA decay analyses. I have stated my position already, the authors and I simply disagree. The decision on whether Northern blots suffice should sit with the editor.

 

The authors tested interaction between DCP1 and DCP2 for their two DCP1 versions. A minor problem is that they refer to the wrong figure (text page 8), but I could not determine how they did the experiment nor how to interpret the data they present. I think a part of the problem is that reader is supposed to know what the 75 kda protein is, and make an interpretation based on that. The text (page 8) also refers to only part of the data, further making interpretation confusing.

The authors address the question of whether a phosphorylated DCP1 (phosphomimic) promotes DCP5 association with RNA, data presented in Fig 2E. I appreciate the organization of this panel, it is quite readily understood. Unfortunately I do not think their data support the phospho-DCP1 promoting DCP5-RNA interaction. The readers are asked to compare the lanes where DCP5 (a level of "4") is mixed with 3 forms of DCP1, and the amount of RNP formed is analyzed (lanes 9-12). However, lane 7 shows DCP5 (the "4" level) alone promoting maximum formation of RNP, an amount equivalent to DCP5 plus DCP1-S237D. These data are simply too variable to be interpreted quantitatively.

The reason for analyzing polysomes is not well explained. Fig 4B, the western blots, still suggest some DCP1 contamination of the polysomes preparations. Is the point that CRC and CRA1 mRNAs are more abundant in the polysome fraction in dcp5 mutants, then in total RNA? I think these comparisons can be made only if the RNA preparation used matching tissue, and at the same time. Also, the conclusion of the polysome section in the results states that altered response ratios from microarrays were determined by polysome engagement. However I think that is only for RNAs that were up-regulated. The DREB response ratios were the same in total RNA and polysomes.

The authors go on to analyze roles for DCP5 in dehydration-induced decapping. I agree with the authors that CRC RNA is strongly decapped in response to dehydration in the wild type. However, their data do not support a role for DCP5 in this process. Again, as stated in my prior review, you must look at the data (see table S4), not the response ratio. If you look at table S4, there is essentially no capped CRC RNA in the dcp5 mutant prior to the dehydration stress. If there is no capped RNA, then they cannot see a change in capped RNA. Why is there no capped RNA in the dcp5 mutant? In my opinion, it tells us that CRC4 expression requires DCP5, but probably indirectly. The author's rebuttal again cited only the ratios.

Referee #2 (Remarks to the Author):

The revised manuscript of Xu et al., as well as the author's comments to the previous review, meets the requested changes/additions.

2nd Revision - authors' response 05 December 2011

Responses to referees:

Referee #1 (Remarks to the Author):

*This is my second time reviewing this manuscript. As stated with the earlier review, there is some excellent, interesting, and exciting data in this paper. Unfortunately it is bogged down with data that continues to not be explained, or is simply flawed. The response by the author's to my earlier comments largely commented on typographic mistakes, and did not address the concerns that I raised.*

*My concerns about the co-immunoprecipitation have not been answered. The figure legend states that the Co-IP used extracts from seedlings expressing FLAG-DCP1 ... and DCP5-FLAG. Extracts were run on affinity resin that binds to the FLAG tag. There is no way for the authors to distinguish between the affinity resin capturing both proteins based on their FLAG tags, as opposed to pulling down the proposed DCP1-DCP5 heterodimer. The data do not tell me that DCP5 interacts with only one the phosphorylated form. These data are in Fig 2A, and results presented on page 6.*

 

The transgenic seedlings carried only a transgene expressing FLAG-DCP1 or FLAG-DCP1-S237A or FLAG-DCP1-S237D or DCP5-FLAG (Fig 2A). Seedling extracts from these individual transgenic lines were treated with FLAG antibody to retrieve the relevant tagged protein (FLAG-DCP1, FLAG-DCP1-S237A, FLAG-DCP1-S237D or DCP5-FLAG). The immunoprecipitates were then probed for the presence of DCP2 or DCP5 using rabbit polyclonal antibodies to these proteins. The results showed that DCP5 interacted with all DCP1 forms but with a higher binding efficiency to FLAG-DCP1-S237D. By contrast, no such differences in binding were detected for DCP2 which served as a negative control.

In the interest of space we tried to be brief in the description of these results. However, upon further consideration and to avoid confusion on the part of readers we have expanded description of these results in the figure legend as follows.
"Fig 2 (A) .....
Co-immunoprecipitation was performed with extracts from 12-day old seedlings expressing either FLAG-DCP1 or FLAG-DCP1 mutants or DCP5-FLAG. Anti-FLAG (mouse monoclonal) M2 affinity gel was used for immunoprecipitation and rabbit anti-DCP5 and anti-DCP2 antibodies were used to detect the related proteins in the immunoprecipitates. Input sample...."
and in the text (p6)
"Using transgenic plants expressing a transgene encoding either FLAG-DCP1-S237A or FLAG-DCP1-S237D, we found that DCP5 preferentially associated with FLAG-DCP1-S237D compared to FLAG-DCP1-S237A (Fig. 2A)."

*A second concern raised with the previous version of this manuscript is interpretation of root growth sensitivity to mannitol. The authors have included a new supplemental figure, but my concerns still stand. In each genotype, mannitol causes root growth to be diminished. There is still no evidence that dcp5 or DCP1-S237A is more sensitive to mannitol.*
Supplemental figure S1 shows that in WT mannitol reduced growth of primary root but promoted lateral root growth. However, in dcp5 or DCP1-S237A , growth of both primary and lateral roots was largely arrested by mannitol.

We have stated in the revised version (p. 7) "The hypersensitivity was readily visible after 12 days of growth on treatment medium such that these two lines were unable to produce lateral roots, in contrast to the typical response in WT (Fig. S1)."

*The authors and I disagree on whether Northern blots or more quantitative approaches are better for RNA decay analyses. I have stated my position already, the authors and I simply disagree. The decision on whether Northern blots suffice should sit with the editor.*
The reviewer argued that more quantitative approaches are better for RNA decay analyses but did not describe what these approaches may be. We believe our Northern blots are sufficient to demonstrate that the decay of EXPL1 transcripts is compromised in dcp5-1 and DCP1-S237A, as compared to WT and DCP1-S237D. Note that in these experiments we measured mRNA levels at different time points and mRNA half life was estimated based on mRNA levels relative to that of time zero. The absolute mRNA levels were not that important provided there was no big difference in mRNA levels at the start of the experiment (time 0) between WT and mutants. We were comparing the relative rate of mRNA decay after cordecypin treatment according to the method published by Dr Pam Green, which is generally accepted by the plant community.

*The authors tested interaction between DCP1 and DCP2 for their two DCP1 versions. A minor problem is that they refer to the wrong figure (text page 8), but I could not determine how they did the experiment nor how to interpret the data they present. I think a part of the problem is that reader is supposed to know what the 75 kda protein is, and make an interpretation based on that. The text (page 8) also refers to only part of the data, further making interpretation confusing.*

The reviewer was correct that we should have referred to "Figure 2D" rather than Fig 2C. This error has been rectified (text page 8). The 75 kDa protein is GST-DCP1-S237A or GST-DCP1-S237D. Note that GST-DCP1-S237D migrated slightly slower than GST-DCP1-S237A due to the one amino acid change.
To describe these results more fully, we added the following to the text: "In the in vitro assays with different MBP-tagged proteins as baits we used amylose resins to retrieve the individual MBP-

 

tagged protein. Antibody to GST was then used to detect the presence of GST-DCP1-S237A or GST-DCP1-S237D associated with the MBP-tagged protein."
We also added the following to the legend to figure 2D.
"(D) …… D53, the C-terminus of DCP5 (Xu & Chua, 2009);VCSc, the C-terminus of VCS (Xu et al, 2006). The last two proteins served as negative controls. The 75 kDa band is either GST-DCP1-S237A or GST-DCP1-S237D. Note that the former mutant protein migrated slightly faster than the latter mutant protein because of the amino acid change. "

*The authors address the question of whether a phosphorylated DCP1 (phosphomimic) promotes DCP5 association with RNA, data presented in Fig 2E. I appreciate the organization of this panel, it is quite readily understood. Unfortunately I do not think their data support the phospho-DCP1 promoting DCP5-RNA interaction. The readers are asked to compare the lanes where DCP5 (a level of "4") is mixed with 3 forms of DCP1, and the amount of RNP formed is analyzed (lanes 9-12). However, lane 7 shows DCP5 (the "4" level) alone promoting maximum formation of RNP, an amount equivalent to DCP5 plus DCP1-S237D. These data are simply too variable to be interpreted quantitatively.*

Lane 7 shows that DCP5 (the "4" level) alone promoted maximum formation of RNP but this binding to RNA was reduced by the addition of GST (lane 12). As the DCP1 proteins were tagged with GST, we consider lane 12 rather than lane 7 to be the appropriate control. Comparison of lanes 9-12 shows that addition of GST-DCP1 (lane 9) or GST-DCP1-S237A (lane 10) had no effect on RNP formation over the control (lane 12); by contrast, addition of GST-DCP1-S237D enhanced RNP formation (cf lane 11 & 12). On p9 of the text, we have expanded on the description of these results for greater clarity.

This question is the same as Q7 from referee #2, and has been addressed in the previous response.

*The reason for analyzing polysomes is not well explained. Fig 4B, the western blots, still suggest some DCP1 contamination of the polysomes preparations. Is the point that CRC and CRA1 mRNAs are more abundant in the polysome fraction in dcp5 mutants, then in total RNA? I think these comparisons can be made only if the RNA preparation used matching tissue, and at the same time. Also, the conclusion of the polysome section in the results states that altered response ratios from microarrays were determined by polysome engagement. However I think that is only for RNAs that were up-regulated. The DREB response ratios were the same in total RNA and polysomes.*

Indeed, total RNAs and polysomal RNAs were prepared from matching tissues and at the same time. The purpose of this experiment was to see if changes in transcript levels would correlate with their polysomal occupancy. We agree there might be some DCP1 contamination of the polysome preparations. However, DCP5 was not detected in the polysomal fractions suggesting this protein could not be responsible for the difference in polysomal mRNAs between WT and dcp5-1 (Fig. 4B). We have modified the original text: "Response patterns of all 4 transcripts tested by using total RNAs were reproduced with polysome-associated RNAs except that the difference between WT and *dcp5-1* was amplified."
to:
"Response patterns of all 4 transcripts tested by using total RNAs were reproduced with polysome-associated RNAs, and for CRC/CRA1 the difference between WT and *dcp5-1* was even amplified."

*The authors go on to analyze roles for DCP5 in dehydration-induced decapping. I agree with the authors that CRC RNA is strongly decapped in response to dehydration in the wild type. However, their data do not support a role for DCP5 in this process. Again, as stated in my prior review, you must look at the data (see table S4), not the response ratio. If you look at table S4, there is essentially no capped CRC RNA in the dcp5 mutant prior to the dehydration stress. If there is no capped RNA, then they cannot see a change in capped RNA. Why is there no capped RNA in the dcp5 mutant? In my opinion, it tells us that CRC4 expression requires DCP5, but probably indirectly. The author's rebuttal again cited only the ratios.*

We agree that CRC transcript levels were relatively low in dcp5-1 total RNAs. We assumed that CRC transcripts/capped RNA did not significantly accumulate in dcp5-1 because capped CRC RNAs may be degraded by other mRNA decay pathways. However, the ratio change of CRC RNAs

 

was detectable and there was a clear difference between WT and dcp5-1. From these data, we believe that DCP5 played a role in the dehydration-related process.

Referee #2 (Remarks to the Author):

*The revised manuscript of Xu et al., as well as the author's comments to the previous review, meets the requested changes/additions.*

3rd Editorial Decision 23 January 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I am sorry for the slight delay in getting back to you with a decision, but I have now received the comments back from the referee.

As referee #1 wasn't available to review the revised version, I asked referee #3 to review the revised version and to comment on the remaining concerns raised by referee #1 and your response to those. As you can see below, referee #3 appreciates the introduced changes and support publication here pending a few minor text changes. Once these last issues have been resolved we will proceed with the acceptance of your paper for publication here.
Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #3:

In my opinion, the authors have adequately addressed reviewer 1's comments except for the last point, which requires minor revisions. Reviewer 1 argued that the capped levels of CRC RNA were too low in dcp5-1 based on table S4 to consider the difference in ratio meaningful, and suggests that CRC may require DCP5 for expression - probably indirectly. The authors concede that the levels are low in the second rebuttal. Based on table S4, this would appear to contribute more to the ratio difference than the relatively more uncapped mRNA they attributed the ratio difference to in the first rebuttal. The authors argue that the ratio change is detectable and believe that the capped CRC RNAs may be degraded by other RNA decay pathways. However, they should mention in the text that capped RNA levels are very low in dcp5, citing table S4, so they cannot rule out that this and reviewer 1's explanation do not contribute significantly to the lower capped/uncapped ratio in dcp5. They should also tone down the discussion sentence at the top of page 15 "This was confirmed by the results from the 5' termini characterization... " I would suggest to change "was confirmed" to "was consistent with" in that sentence.

3rd Revision - authors' response 10 February 2012

Response to Referee:

Referee #3:

*In my opinion, the authors have adequately addressed reviewer 1's comments except for the last point, which requires minor revisions. Reviewer 1 argued that the capped levels of CRC RNA were*

*too low in dcp5-1 based on table S4 to consider the difference in ratio meaningful and suggests that CRC may require DCP5 for expression - probably indirectly. The authors concede that the levels are low in the second rebuttal. Based on table S4, this would appear to contribute more to the ratio difference than the relatively more uncapped mRNA they attributed the ratio difference to in the first rebuttal. The authors argue that the ratio change is detectable and believe that the capped CRC RNAs may be degraded by other RNA decay pathways. However, they should mention in the text that capped RNA levels are very low in dcp5, citing table S4, so they cannot rule out that this and reviewer 1's explanation do not contribute significantly to the lower capped/uncapped ratio in dcp5. They should also tone down the discussion sentence at the top of page 15 "This was confirmed by the results from the 5' termini characterization... " I would suggest to change "was confirmed" to "was consistent with" in that sentence.*

We have inserted the change in the text of page 12, as shown underlined here: "For *CRC* transcripts this ratio was decreased from 11.65 to 0.01 upon dehydration in WT whereas in *dcp5-1* (for unknown reason, the *CRC* transcript level in *dcp5-1* was very low), this decrease was much reduced (Table 2, for details see Table S4)."

We have changed the text of page 15 to the following:
"This was consistent with the results from 5'-termini characterization assay and the finding that phosphorylation of DCP1 signalled by MPK6 promoted association with DCP5 and enhanced mRNA decapping."

 