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The plant specific CDKB1-CYCB1 complex mediates homologous recombination repair in Arabidopsis

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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 06 November 2015

Thank you for submitting your manuscript on plant HRR regulation by CDKB1-CYCB1 to The EMBO Journal, where it has now been evaluated by three expert referees. I apologize for the fact that it has taken somewhat longer than usual to obtain reviews and get back to you with a decision in this case. In any case, I am pleased to inform you that all three reviewers are overall very positive about the study, and would support publication pending the addressing of a limited number of specific concerns, as detailed in the reports below. Among these, the most significant conceptual issue is the one brought up by referee 3, regarding unclear epistasis between CDKB1 and CYCB1 in phenotypic assays as well as in mechanistic (recombination) assays.

Should you be able to adequately address the various points raised by the reviewers, then we should be happy to consider a revised version further for EMBO Journal publication. However, please bear in mind that our policies allow only a single round of major revision, making it important to carefully answer to all points raised at this stage. When revising the paper, please also make sure to carefully edit and proofread the final manuscript text, including replacing jargon terms (such as 'dephosphomutants') with more formal descriptions to improve readability and accessibility for general readers.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension. Finally, please note that

we now require a completed 'author checklist' to be submitted with all revised manuscripts (see below for more detail).

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any feedback or questions regarding the referee reports or this decision. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

This work aimed at investigating the connection between DNA repair and cell cycle control in plants. The task is difficult because plants have a large panoply of CDKs-only few of which were characterized so far. Moreover, plant cells seem to be more resistant to DNA damage than their mammalian counterparts, therefore a simple road map cannot be followed.

One particular member of the Cyclins family, CYCB1-1 is up-regulated during DNA damage treatment. The authors followed this perplexing lead and addressed the role the B1-type cyclins (CYCB1s) in DNA repair. The lead was perplexing because CYCB1-1 is thought to promote cell cycle, while DNA repair requires usually arrest of the cell cycle. The authors searched for partners of CYB1-1, through a combination of genetic analysis, using single, double and triple mutants, and pharmacogenetics using genotoxic compound in WT and mutant background (such BLM, a DSB-inducing agent, or cisplatin, a DNA crosslinker whose effect can be overcome by homologous recombination).

In addition, they used an impressive battery of assays for functional analysis of the mutants-This included, root growth, FACS analysis, protein localization, immunostaining, CHIP, phosphorylation assays, etc.. They also ran a wide range of controls to determine if effects were direct or indirect, connected to cell death or the changes in ploidy or to cell cycle arrest and whether the type of DSB was more likely to occur via HR or non-homologous end-joining.

This lead to a number of major findings:
B1-type CDKs work together with B1-type cyclins in promoting DNA damage response - probably HR as this was most pronounced when damage was induced by cisplatin.
In vitro analysis showed RAD51 phosphorylation by a CDKB1-CYCB1 complex, supporting the role of the complex in HR.
The promoters of CDKB1-1 and CYCB1-1 are activated upon cisplatin treatment, suggesting a role of SOG1 a major TF in the DNA damage response. It turned out, using CHIP experiments that the promoter of CYCB1-1 but not of CDKB1-1 is bound by SOG1.

Overall both the scope and the quality of the work are impressive and the findings are novel and significant to the field.

I have a few minor comments:
1-I am not sure the data is strong enough regarding statements that the CDKB1-CYCB1 complex affects the decision on the type of repair (HR versus NHEJ). There are multiple NHEJ pathways and also multiple HR pathways-- maybe such statement should be toned down or conversely better argued.
2- Some references are a bit old and more recent ones, specially regarding the RAD51 homologs should be quoted.
3- Few typos: p12, middle,
"So far, a few enzymes involved HR" should be
"So far, a few enzymes involved in HR"

In the same long sentence:
"Wohlbold et al., 2012), and we asked whether RAD51" should be
"Wohlbold et al., 2012). Here, we asked whether RAD51"

Referee #2:

CDK activity is inhibited to block cell cycle progression as part of the DNA damage response. Paradoxically, some CDK activity appears to be needed for some DNA repair pathways, typically homologous recombination (HR). To answer this question the authors of this study take advantage of a fair collection of single and multiple mutants in the CYCB and CDK genes to measure their growth and DNA damage response. They conclude that CYCB1 and the plant-specific CDKB1 are major regulators of HR in Arabidopsis. They also provide evidence that RAD51 is phosphorylated in vitro by these CDK/CYC complexes and that it fails to accumulate in foci in cycb and cdkb1 mutants. Finally, they show the role of the SOG1 transcription factor in regulating the expression of CYCB1 in response to DNA damage. The author's overall conclusion is that there is division of labor between CDKA and CDKB1 in cell proliferation and DNA damage response, respectively.

This is an extensive and systematic study using a collection of mutants with affected CYCB and CDK activity. The experiments are nicely done and clearly described in most cases and they follow a logical trend. The long-standing question of the upregulation of CYCB1 expression in response to DNA damage (as well as in several mutant backgrounds that could be also at least mentioned) is addressed here to obtain results supporting the authors' main conclusions. This is a very relevant topic showing how plants deal with cell cycle control under normal conditions and in response to DNA damage using distinct CDK/CYC complexes for each pathway.

A general comment is that I feel insufficient the discussion (and implications) of the apparent specificity of cisplatin to trigger the CYCB1/CDKB1-dependent HR pathway, while several other agents/conditions also end up in HR activation.

I have also a list of more specific comments to be addressed.
- In general the quality of the images that I received is not sufficient to appreciate the details (examples, Fig 1, Fig. 4, Fig 5, Fig 6 GUS assays). Some figures (Fig 1) are not very friendly (too many think bars in the graph to easily follow their changes).
- Fig. 1. The double cycb1;1 cycb1;2 mutant had shorter roots than wt. What's the significance of this? Any speculation?
- Changes in Fig. EV5 are relatively small. Is the stat ok?
- Fig. 2C. I would expect to see signal of H2A.X in wt treated with cisplatin.
- Page 11. I think that expression of B1 cyclins "from late S to M" could be a bit misleading, since levels at the end of S might not be significant or biologically relevant (just a consequence of the synchronization wave).
- Fig. 5A-C. Are the differences biologically relevant?
- 2nd paragraph, section on RAD51 localization... "These foci... instead of These loci... 3rd paragraph, "both process DNA...
- Fig. 5D,E. Some images in panel D do not seem to match the quantification shown in panel E: compare atr mutant and wt, for example.
- Authors mention that they "created reporter lines for all four CYCB1 genes". A detailed description should be provided in Methods. Do the primers correspond to those in Expanded Table 1?
- Page 13, 2 lines from bottom. Authors state: ... depends on ATM and SOG1 (Culligan et al., 2006). I think that SOG1 is not used in this work. Please complete reference.
- Page 14. Panel 6H,I is cited but apparently panel I is missing in Fig. 6.
- Page 17. Line 8 from bottom. Should be resection of the 3' end....

Referee #3:

DNA repair mechanisms operate in cell-cycle dependent manner with HR repair being prevalent in S and G2 when a sister chromatid is available as a template, while NHEJ is mainly active in G1. Studies in yeast and mammals showed that CDK activity is one of the key decision makers in the choice of DNA repair pathways, as it promotes HR, mainly at the resection step. This study reports that instead of using the CDKA;1, the main regulator of cell cycle progression, Arabidopsis regulates its DNA repair activity through specialized CDKB1 kinases and their associated CYCB1s.

In support of this conclusion, the authors collect a number of data linking these cell cycle regulators to DNA repair: they show that 1) mutants deficient in CYCB1s or CDKB1s are sensitive to DNA damage inducing agents, particularly to cisplatin known to induced lesions that require HR, and accumulate gamma H2AX damage foci 2) CYCB1s are required for intrastrand recombination in response to DNA damage, 3) cdkb1 and cycb1 mutant plants fail to form Rad51 foci, and 4) CDKB1;1-CYCB1;1 phosphorylate Rad51 in vitro. Discovery of CDK/cyclins dedicated for DNA repair regulation is an interesting concept that not only underlies complexity and importance of DNA damage response in plants, but it may be of an interest for non-plant scientists as well.

Nevertheless, I still feel that some key conclusions drawn in this study need more experimental back-up. The authors propose that HR regulation is specifically mediated by CDKB1s-CYCB1s complexes. This conclusion is based on a similar behavior of mutants in response to cisplatin (sensitivity, formation of Rad51 foci) and ability of CDKB1;1-CYCB1;1 to phosphorylate Rad51. There seem to be also differences as cdkb1, but not cycb1 mutants, show sensitivity to bleomycin. Although this may be explained by genetic redundancy among CYCB1s, and alternative explanation is that CDKB1 and CYCB1 act in related, but mechanistically distinct pathways. This can be genetically tested by combining cdkb1 and cycb1 mutations and looking at epistatic interactions. Furthermore, the authors should perform the recombination assay with the cdkb1 mutants to demonstrate CDKB1 requirement in HR. Ideally, the authors would include in this assay other CDKs and cyclins (hypomorphic CDKA;1, CYCA2) to demonstrate specific effect on HR. While Rad51 phosphorylation assay provides a mechanistic evidence for concerted action of CYCB1 and CDKB1, I miss specificity controls. Namely control reactions that include other combinations of CDK-CYC complexes.

Minor comments:
Fig EV5: The authors tested whether cisPt increases endoreplication in roots. The experimental design is not clearly described. Were plants grown for five days after cisPt treatment and then measured, or were they grown for five days, then treated with cisPt for 8 hrs and measured for ploidy? If latter is the case, I am just wondering whether 8 hrs treatment is sufficient for producing detectable increase in endoreplication. Is it know how long does endoreplication cycle last in Arabidopsis?

Fig 2: It seems that induction of breaks in wt samples was much less efficient than in cycB mutant samples. How do authors interpret this difference? Such difference in the initial level of breaks will affect repair during recovery period. To meaningfully interpret comet data, the authors should perform a time course experiment to the time point when all breaks are repaired, in order to determine kinetics of DSB repair in wt and mutant plants.

Page 4: ATR is predominantly involved in replication stress response by sensing single stranded DNA, not in single strand break response.

Page 6, 2nd sentence: this sentence implies that HR is active only in S phase, but in fact, HR is preferable DSB repair pathway in G2 as well.

Page 6, last sentence of the 1st paragraph: To which organism this sentence refers to?

Fig. 1C: correct to: The rightmost plant is wee1 mutnat that shows high sensitivity to HU.

Fig. 1E: correct to: The rightmost plant is ku70 mutnat that shows high sensitivity to Bleocin treatment.

Page 17, last paragraph: replace resurrection with resection or formation.

Page 18, 1st paragraph: Failure to recruite Rad51 to DNA damage foci may also be caused by insufficient resection of DSBs. This alternative should be mentioned in discussion.

1st Revision - authors' response 09 June 2016

We would like to thank the editor and the reviewers for their positive and constructive comments. We have addressed the raised questions below and performed additional experiments to show the specificity of the CYCB1-CDKB1 complex in homologous recombination repair in Arabidopsis. Furthermore, we generated the triple mutant cycb1;1 cdkb1;1 cdkb1;2 and tested its response to various DNA damage treatments. Due to additional experiments and text changes requested we made a number of changes to the figures and the text as described in detail in our point-by-point response below.
The changes are highlighted in yellow in our revised manuscript.
We believe these changes have significantly improved our manuscript and we hope that the reviewers will now find the revised manuscript suitable for publication in EMBO Journal.

Referee #1

This work aimed at investigating the connection between DNA repair and cell cycle control in plants. The task is difficult because plants have a large panoply of CDKs- only a few of which were characterized so far. Moreover, plant cells seem to be more resistant to DNA damage than their mammalian counterparts, therefore a simple road map cannot be followed.

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In addition, they used an impressive battery of assays for functional analysis of the mutants-This included, root growth, FACS analysis, protein localization, immunostaining, CHIP, phosphorylation assays, etc.. They also ran a wide range of controls to determine if effects were direct or indirect, connected to cell death or the changes in ploidy or to cell cycle arrest and whether the type of DSB was more likely to occur via HR or non-homologous end-joining.

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Overall both the scope and the quality of the work are impressive and the findings are novel and significant to the field.

We would like to thank reviewer 1 for his/her positive feedback on our work.

I have a few minor comments:
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We agree with reviewer 1 that we cannot say anything about the role of CDKB1-CYCB1 complexes for the decision whether a cell executes NHEJ versus HR. In the discussion of the previous version, we have used several times the term “promote HR” which is indeed misleading as it suggests

inhibition of NHEJ in favor of HR. We have carefully double-checked the text and replaced this statement with more descriptive terms, such as “required for HR”, “control HR”, etc. In addition, we explicitly state that it is currently unclear whether there is an antagonistic relationship between NHEJ and HR in plants. Finally, we have adjusted the model and removed all aspects that addressed the question of which repair pathway is chosen.

2- Some references are a bit old and more recent ones, specially regarding the RAD51 homologs should be quoted.

We thank the reviewer for pointing this out have updated the text with more recent literature. In addition, we added some information about RAD51 homologs in the introduction.

3- Few typos: p12, middle,
"So far, a few enzymes involved HR" should be
“So far, a few enzymes involved in HR”

We apologize for these mistakes and have now double-checked the text for typos.

In the same long sentence:
"Wohlbold et al., 2012), and we asked whether RAD51" should be
"Wohlbold et al., 2012). Here, we asked whether RAD51"

We have rewritten this paragraph and hope the readability has improved now.

Referee #2

CDK activity is inhibited to block cell cycle progression as part of the DNA damage response. Paradoxically, some CDK activity appears to be needed for some DNA repair pathways, typically homologous recombination (HR). To answer this question the authors of this study take advantage of a fair collection of single and multiple mutants in the CYCB and CDK genes to measure their growth and DNA damage response. They conclude that CYCB1 and the plant-specific CDKB1 are major regulators of HR in Arabidopsis. They also provide evidence that RAD51 is phosphorylated in vitro by these CDK/CYC complexes and that it fails to accumulate in foci in cycb and cdkb1 mutants. Finally, they show the role of the SOG1 transcription factor in regulating the expression of CYCB1 in response to DNA damage. The author's overall conclusion is that there is division of labor between CDKA and CDKB1 in cell proliferation and DNA damage response, respectively.

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We would like to thank reviewer 2 for his/her positive feedback and suggestions on how to improve the paper. Indeed, *CYCB1;1* becomes upregulated in several mutants that affect DNA repair and we provide now two examples in which this is the case: Mutants in *JING HE SHENG 1 (JHS1)*, in which the Arabidopsis homolog of the human and yeast DNA Replication Helicase/Nuclease2 is not functional and in mutants of the Arabidopsis *DNA REPLICATION FACTOR C1 (RFC1)* gene.

A general comment is that I feel insufficient the discussion (and implications) of the apparent specificity of cisplatin to trigger the CYCB1/CDKB1-dependent HR pathway, while several other agents/conditions also end up in HR activation.

Indeed, other genotoxic agents besides cisplatin can also lead to homologous recombination repair, for instance bleomycin, and our data even suggests that at least some of the bleomycin induced damaged can be repaired by HR, visible in *ku70 mutants*, which were depleted of CYCB1 activity.

I have also a list of more specific comments to be addressed.

- In general the quality of the images that I received is not sufficient to appreciate the details (examples, Fig 1, Fig. 4, Fig 5, Fig 6 GUS assays). Some figures (Fig 1) are not very friendly (too many think bars in the graph to easily follow their changes).

We have carefully addressed this comment and changed the figures to improve readability. In Figure 1, we decreased the number of data points and only show root length 10 days after germination (before: day 8-10 that led to the same conclusion). Likewise we have streamlined Figure 4. We hope that this improves the quality of the figures while it did not take away any information necessary. The quality of the Figure 3 (recombination assays) was also checked. Possibly, the downsampling of the figures might have caused some visible quality loss and provide now images with higher resolution.

- Fig. 1. The double cycb1;1 cycb1;2 mutant had shorter roots than wt. What's the significance of this? Any speculation?

Mutants of *cycb1;1 cycb1;2* have shorter roots on control medium without drugs compared to WT (Fig 1). In order to properly analyze the sensitivity to drug treatment, we calculated the root growth ratio (which is the % of root growth on drug medium compared to control medium). The results are displayed in Expanded View Figure EV3. The reason for the defect of *cycb1;1 cycb1;2* lies in compromised cell division activity due to their function as mitotic regulators. As far as we can tell at the moment, the mutants have a compromised mitotic spindle – this defect is neither found in the single mutants nor any other double mutant combination. A detailed description of this mutant aspect requires additional experimentation and is currently in progress.

- Changes in Fig. EV5 are relatively small. Is the stat ok?

We performed the Mann-Whitney-Wilcoxon statistical to test for significant changes in ploidy levels with and without treatment. This statistical test calculates significances of the overall distribution of changes of ploidy levels. Although the ploidy levels are indeed statistically significant, we agree that they appear very small raising doubts about their biological relevance.

- Fig. 2C. I would expect to see signal of H2A.X in wt treated with cisplatin.

We quantified the gamma-H2AX foci (Figure 2D) in WT and more than 60% of nuclei did not show foci. Around 20% showed 1-2 gamma-H2AX foci. For Figure 2C we chose a representative picture showing two gamma-H2AX foci for WT. The plants were treated for 24 h with 50 µM Cisplatin.

- Page 11. I think that expression of B1 cyclins "from late S to M" could be a bit misleading, since levels at the end of S might not be significant or biologically relevant (just a consequence of the synchronization wave).

This is true and we adapted the text to: “Putative candidates are the plant-specific CDKB1s since the expression pattern of B1-type cyclins in G2 phase overlaps with the expression pattern of CDKB1s during the cell cycle (Menges et al., 2005).”

- Fig. 5A-C. Are the differences biologically relevant?

The difference was at least obvious and appeared in all repetitions to be significantly shorter on 15 µM cisplatin (one asterisk, p value <0.05, calculated by student’s t-test).

- 2nd paragraph, section on RAD51 localization... "These foci... instead of These loci... 3rd paragraph, "both process DNA...

We apologize for this typo and corrected it.

- Fig. 5D,E. Some images in panel D do not seem to match the quantification shown in panel E: compare atr mutant and wt, for example.

We tried to chose representative pictures for every genotype tested (see also above comment). Indeed, the nucleus shown for atr had more foci than nuclei from the wildtype that was misleading since atr has fewer foci according to our quantitative analyses. We have now replaced the panel showing at atr nucleus with a more representative picture.

- Authors mention that they "created reporter lines for all four CYCB1 genes". A detailed description should be provided in Methods. Do the primers correspond to those in Expanded Table 1?

We added a Material & Method section about the cloning strategy we used to created the CYCB1 marker lines. The corresponding primers are added to Expanded View Table 1.

- Page 13, 2 lines from bottom. Authors state: ... depends on ATM and SOG1 (Culligan et al., 2006). I think that SOG1 is not used in this work. Please complete reference.

We indeed forgot to mention one reference here and apologize for this mistake. We added the paper in which the authors show that CYCB1;1 upregulation depends on SOG1: Yoshiyama et al. (2009) Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. PNAS.

- Page 14. Panel 6H,I is cited but apparently panel I is missing in Fig. 6.

We thank the reviewer for pointing out this mistake. The sentence referred to Figure G and H instead of H and I. We corrected the text accordingly.

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Referee #3

DNA repair mechanisms operate in cell-cycle dependent manner with HR repair being prevalent in S and G2 when a sister chromatid is available as a template, while NHEJ is mainly active in G1. Studies in yeast and mammals showed that CDK activity is one of the key decision makers in the choice of DNA repair pathways, as it promotes HR, mainly at the resection step. This study reports that instead of using the CDKA;1, the main regulator of cell cycle progression, Arabidopsis regulates its DNA repair activity through specialized CDKB1 kinases and their associated CYCB1s. In support of this conclusion, the authors collect a number of data linking these cell cycle regulators to DNA repair: they show that 1) mutants deficient in CYCB1s or CDKB1s are sensitive to DNA damage inducing agents, particularly to cisplatin known to induced lesions that require HR, and accumulate gamma H2AX damage foci 2) CYCB1s are required for intrastrand recombination in response to DNA damage, 3) cdkb1 and cycb1 mutant plants fail to form Rad51 foci, and 4) CDKB1;1-CYCB1;1 phosphorylate Rad51 in vitro. Discovery of CDK/cyclins dedicated for DNA repair regulation is an interesting concept that not only underlies complexity and importance of DNA damage response in plants, but it may be of an interest for non-plant scientists as well.

We would like to thank reviewer 3 for his/her positive feedback and the helpful comments on the manuscript.

Nevertheless, I still feel that some key conclusions drawn in this study need more experimental back-up. The authors propose that HR regulation is specifically mediated by CDKB1s-CYCB1s complexes. This conclusion is based on a similar behavior of mutants in response to cisplatin (sensitivity, formation of Rad51 foci) and ability of CDKB1;1-CYCB1;1 to phosphorylate Rad51. There seem to be also differences as cdkb1, but not cycb1 mutants, show sensitivity to bleomycin. Although this may be explained by genetic redundancy among CYCB1s, and alternative explanation is that CDKB1 and CYCB1 act in related, but mechanistically distinct pathways. This can be genetically tested by combining cdkb1 and cycb1 mutations and looking at epistatic interactions.

The reviewer has raised an important point. Although time consuming and exceeding the usual time frame for a revision, we could generate the triple mutant *cycb1;1 cdkb1;1 cdkb1;2* and tested the root growth on cisplatin, bleomycin and hydroxy urea. We show now that the triple mutant *cycb1;1 cdkb1;1 cdkb1;2* has indeed a similar root growth behavior on control plates and plates supplemented with drugs as the *cdkb1;1 cdkb1;2* double mutant indicating that there is no additive effect of when both *CYCB1* and *CDKB1* are lost. The genetic evidence corroborates our cytological and morphological analyses indicating that indeed CDKB1;1/CDKB1;2 and CYCB1 act in the same pathway. We added these data as a new supplementary figure (Expanded View Figure EV7).

Furthermore, the authors should perform the recombination assay with the cdkb1 mutants to demonstrate CDKB1 requirement in HR. Ideally, the authors would include in this assay other CDKs and cyclins (hypomorphic CDKA;1, CYCA2) to demonstrate specific effect on HR.

While this is certainly a valuable experiment, it is a more demanding procedure than it may appear. The recombination system needs to be tested for functionality (i.e. lack of recombination in the meristem) and the genetic base has to be unambiguously known (i.e. hemizygosity versus homozygosity). The assays with *cycb1* mutants that we show in the paper took already more than one year. To repeat this now with *cyca2* triple mutants will by far exceed this time frame. Similarly, the work with the completely sterile hypomorphic *cdka;1* mutants (such as DE) is also challenging. We have followed the appreciated comment of this reviewer and generated the triple mutant *cycb1;1 cdkb1;1 cdkb1;2* in the last six months (see above) and show that its growth is not significantly different from the double *cdkb1* mutants. Hence, we conclude that CYCB1s and CDKB1s act in the same pathway. We hope that the reviewer agrees that this finding together with the observation that *cdkb1;1 cdkb1;2* double mutants are hypersensitive to cisplatin, the finding that RAD51 foci are missing in *cdkb1;1 and cdkb1;2* and the updated kinase assays (see below) builds a sufficiently strong case to support our conclusions.

While Rad51 phosphorylation assay provides a mechanistic evidence for concerted action of CYCB1 and CDKB1, I miss specificity controls. Namely control reactions that include other combinations of CDK-CYC complexes.

In order to address the specificity of the CYCB1-CDKB1 complex in phosphorylating RAD51 we performed further kinase assays with different CYC-CDK complexes; CDKA;1-CYCA2;3, CDKB1;1-CYCA2;3, CDKB1;1-CYCD2;1, CDKA;1-CYCB1;1, CDKB1;1-CYCB1;1 and without kinase as a control. The new kinase assays are displayed in Figure 5F. These assays show that CYCB1;1-containing complexes, i.e. CDKA;1-CYCB1;1 and CDKB1;1-CYCB1;1 have the highest activity among the tested combinations against RAD51 in vitro. This is consistent with data from yeast and animals that show that the cyclin partner is a key determinant of substrate specificity (Loog and Morgan, Nature, 434, 2005) Why CDKA;1 does apparently not contribute to the DNA response pathway *in planta* remains to analyzed. One possibility is that CDKA activity is blocked by CDK inhibitors. In any case the scope of this paper was to show that CDKB1s have a previously not recognized key role in DNA damage response and the updated kinase assays support this conclusion.

Minor comments:
Fig EV5: The authors tested whether cisPt increases endoreplication in roots. The experimental design is not clearly described. Were plants grown for five days after cisPt treatment and then measured, or were they grown for five days, then treated with cisPt for 8 hrs and measured for ploidy? If latter is the case, I am just wondering whether 8 hrs treatment is sufficient for producing detectable increase in endoreplication. Is it know how long does endoreplication cycle last in Arabidopsis?

This comments addresses a similar point as reviewer 2. Aim of the experiment was to see if WT and the tested mutants show an increase in endoreplication upon expose to cisplatin. Plants geminated on control plates and were transferred five days after germination to plates supplemented with 50 μM cisplatin for 8 hours or on control plates for 8 hours. Plants were then harvested and measured for their ploidy. We calculated statistical differences by performing a Mann-Whitney-Wilcoxon test. According to this reviewer and reviewer 2, we have now repeated the ploidy analyses during which we have treated plants for 24 hours essentially leading to the same result as before with our 8-hour

treatment. However, based on the comments from both reviewers we only show now these new results.
Our major conclusion is that endoreplication is actually not a major response to cisplatin, neither in the wildtype nor in the mutants.

Fig 2: It seems that induction of breaks in wt samples was much less efficient than in cycB mutant samples. How do authors interpret this difference? Such difference in the initial level of breaks will affect repair during recovery period. To meaningfully interpret comet data, the authors should perform a time course experiment to the time point when all breaks are repaired, in order to determine kinetics of DSB repair in wt and mutant plants.

All probes, i.e. the wildtype and mutants were handled the same way. In addition, we have repeated the experiment several times with always the same outcome. The analysis of DSBs by gamma H2AX detection also indicated much higher levels of damage in the mutants versus the wildtype. Our interpretation of this finding is that indeed repair pathways are compromised in the mutants and, while wildtype cells already repair the damage (during the treatment), the mutants accumulate damage or repair only very slowly.

At least under our assay conditions, the experimental read out of a comet assay is variable as seen by the rather large error bars. This makes a kinetics analysis a very difficult and demanding procedure since dozens of experiments have to be performed to reach statistically significant results.

None-the-less, we have performed as a test recovery experiments for a longer time with *cdkb1;1 cdkb1;2* mutants grown on cisplatin and found that it takes many days for the mutant to resume growth (as expected by the severe damage seen). The mutants often even died after a few days again adding variability and interfering with a detailed kinetics.

The major point of this experiment is to cytologically backup the root growth analysis and demonstrate that the mutants accumulate DNA damage. This is our major point here and we hope the reviewer agrees with the qualitative statement.

Page 4: ATR is predominantly involved in replication stress response by sensing single stranded DNA, not in single strand break response.

We corrected the sentence accordingly.

Page 6, 2nd sentence: this sentence implies that HR is active only in S phase, but in fact, HR is preferable DSB repair pathway in G2 as well.

We agree that this sentence was misleading and corrected it to: “HR requires a homologous template to repair the damaged DNA and can therefore only occur after DNA replication in late S and G2 phase of the cell cycle when sister chromatids are available.”

Page 6, last sentence of the 1st paragraph: To which organism this sentence refers to?

This sentence referred to RAD51 in Arabidopsis thaliana. We changed the sentence for more clarity to “Mutants of rad51 and other members of this group in Arabidopsis show fewer homologous recombination events after DNA damage confirming an important role for these proteins during plant HR (Abe et al, 2005), which is in line with studies in other eukaryotes (Hosoya and Miyagawa, 2014).”

Fig. 1C: correct to: The rightmost plant is wee1 mutnat that shows high sensitivity to HU.

We added this sentences to the figure legend.

Fig. 1E: correct to: The rightmost plant is ku70 mutnat that shows high sensitivity to Bleocin treatment.

We added this sentences to the figure legend.

Page 17, last paragraph: replace resurrection with resection or formation.

We corrected this typo.

Page 18, 1st paragraph: Failure to recruite Rad51 to DNA damage foci may also be caused by insufficient resection of DSBs. This alternative should be mentioned in discussion.

The reviewer raised a good point here. We have included this alternative possibility in the discussion.

2nd Editorial Decision 11 July 2016

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by all three original referees, whose comments are copied below. I am happy to say that the reviewers are satisfied with the revisions and now consider the study suitable for publication in The EMBO Journal!

Referees 2 and 3 still mention a few specific points, which should be changed/incorporated in the text (and if necessary in some figures) before formal acceptance of the paper.

REFEREE COMMENTS

Referee #1:

My comments were properly addressed.

Referee #2:

Authors have submitted a significantly modified version of their original manuscript. They have addressed most issues that I raised in my report. In most cases I have no further concerns and I am convinced with their arguments and/or the new data included. However, there a few cases where I think there are still points not sufficiently clear. They are listed below.

1. page 5. New comment on mutants where upregulation of CYCB1;1 occurs. The two examples provided are fine. References to mutations in the FAS1 gene should be included.
2. Page 6. Discussion on availability of sister chromatids for recombination. It seems that HR occurs in G2. However, formally speaking, replication of DNA early in S-phase immediately leads to sister chromatids (even early in S-phase). Therefore, the sentence should be rephrased.
3. Fig. 1F, H-N. It would be helpful to separate the color codes for the H-J and L-N panels respectively, as not all of them are used in both groups.
4. Fig EV5. I appreciate that the data after 24h treatment could be more enlightening than those after 8h. Still not sufficient to discard long term effect. A comment is needed to make explicit such possibility.

Referee #3:

I am to a large extend satisfied with the corrections and amendments in the new manuscript. I particularly appreciate new data on characterization of cycb1,1 cdkb1,1 cdkb1,2 triple mutants and in vitro phosphorylation of Rad51. I have only a few minor comments:

1.While the authors provide a compelling evidence on importance of CDKB1 kinases in DNA repair, they suggest on several occasions that there is a division of labor between CDKB1 and CDKA1 with CDKA playing no role in DNA repair (e.g. abstract; discussion - page 18). However, this is an overinterpretation of provided data. First, the data on DNA repair phenotypes were obtained with a hypomorphic allele of CDKA1 that is still presumably active. Second, CDKA1

CYCB1,1 complex is obviously quite efficient in phosphorylating Rad51. The authors should tone down these statements as they cannot refute the possibility that CDKA1 is involved in DNA repair.

2.Source of the gamma H2AX antibody should be provided.

3.Figure 5F, the CDK phosphorylation assay - the figure is not well described. It is not explained why there are multiple bands of different sizes in the Rad51 samples and which ones represent specific phosphorylation events. What is the meaning of asterisk?

4.Description of the sensitivity of ku70 cycb1,1 cycb1,2 mutants is deceiving (page 16), they should not be compared to wild type but to not treated triple mutants that also show reduced root length.

2nd Revision - authors' response 12 July 2016

We are happy that all three reviewers find our revision has sufficiently addressed their previous questions. Once again, we like to thank them for the last round of constructive comments that we have now fully addressed, as outlined below.

Referee #1:

My comments were properly addressed.

Referee #2:

Authors have submitted a significantly modified version of their original manuscript. They have addressed most issues that I raised in my report. In most cases I have no further concerns and I am convinced with their arguments and/or the new data included. However, there a few cases where I think there are still points not sufficiently clear. They are listed below.

1. page 5. New comment on mutants where upregulation of CYCB1;1 occurs. The two examples provided are fine. References to mutations in the FAS1 gene should be included.

We have now also include the example of fas1 mutants in which CYCB1;1 is strongly upregulated (paper from Seiichi Toki's lab).

2. Page 6. Discussion on availability of sister chromatids for recombination. It seems that HR occurs in G2. However, formally speaking, replication of DNA early in S-phase immediately leads to sister chromatids (even early in S-phase). Therefore, the sentence should be rephrased.

The reviewer is right and we have specified our statement now in the following way:
"…HR requires a homologous template to repair the damaged DNA and can therefore only occur after DNA replication in S phase and the subsequent G2 phase of the cell cycle when sister chromatids are available…"

In addition, we have updated our model and how now also the potential promotion of HR in S phase.

3. Fig. 1F, H-N. It would be helpful to separate the color codes for the H-J and L-N panels respectively, as not all of them are used in both groups.

We are unfortunately not sure how to modify this figure. We use the same color always for the same genotype. Indeed, not all genotypes are present in all panels but the labeling is consistent and always in the same order. We are afraid that more label/colors will be rather confusing but are of course happy for any suggestions to improve the readability.

4. Fig EV5. I appreciate that the data after 24h treatment could be more enlightening than those after 8h. Still not sufficient to discard long term effect. A comment is needed to make explicit such possibility.

We have followed the advice of the reviewer and write now:
...Although we cannot exclude long-term effects of cisplatin to promote endoreplication, we did not see a major increase in endoreplication levels in comparison to control plants when we analyzed cells of the root tips of five day old wild-type plants grown for 24 hours on media with 50 µM cisplatin. Likewise, a strong increase in endoreplication was not observed in *cycb1;1 cycb1;3* double mutants when treated with cisplatin for 24 hours (Fig EV5). ...

Referee #3:

I am to a large extend satisfied with the corrections and amendments in the new manuscript. I particularly appreciate new data on characterization of cycb1,1 cdkb1,1 cdkb1,2 triple mutants and in vitro phosphorylation of Rad51. I have only a few minor comments:

1.While the authors provide a compelling evidence on importance of CDKB1 kinases in DNA repair, they suggest on several occasions that there is a division of labor between CDKB1 and CDKA1 with CDKA playing no role in DNA repair (e.g. abstract; discussion - page 18). However, this is an overinterpretation of provided data. First, the data on DNA repair phenotypes were obtained with a hypomorphic allele of CDKA1 that is still presumably active. Second, CDKA1 CYCB1,1 complex is obviously quite efficient in phosphorylating Rad51. The authors should tone down these statements as they cannot refute the possibility that CDKA1 is involved in DNA repair.

We have now incorporated the warning of this reviewer and write:
"...Although we cannot fully exclude a role of CDKA;1 in DNA repair, our combined data suggest that this kinase is largely not required for a proper DNA damage response in Arabidopsis. Instead we found that especially CDKB1 complexes control HR. ..."

We hope that this sufficiently addresses the point of the reviewer. The fact that the triple mutant cdkb11 cdkb12 cycb11 does not show an enhanced mutant phenotype further supports that the major kinase is CDKB1 with no or very little contribution by CDKA;1 (in complex with CYCB1;1) since we would have otherwise expected an at least partially additive mutant phenotype.

2.Source of the gamma H2AX antibody should be provided.

The antibody was kindly provided by Dr. Charles White, CNRS, Clermont-Ferrand, France. We had stated this in the acknowledgments. The source is now also provided in the M&M part with a reference to Charbonnel et al. 2010.

3.Figure 5F, the CDK phosphorylation assay - the figure is not well described. It is not explained why there are multiple bands of different sizes in the Rad51 samples and which ones represent specific phosphorylation events. What is the meaning of asterisk?

The asterisks represent the positions of the cyclins. The amount of the cyclins can vary from reaction to reaction since the complexes were purified using a His-tag that is fused to the cyclin. Thus we can obtain monomeric cyclins and cyclins bound to CDKs. However, we normalized the reaction in such a way that equal amounts of the CDKs (and hence functional CDK-cyclin complexes) are present in the reaction mixture (as shown by the Western blot in the lower panel). We have explained the procedure now in more detail in the M&M part as well as in the figure legend.

4.Description of the sensitivity of ku70 cycb1,1 cycb1,2 mutants is deceiving (page 16), they should not be compared to wild type but to not treated triple mutants that also show reduced root length.

To facilitate any cross-comparison, we have added now a new panel to figure 7 that shows all ratios between the wildtype and mutants grown on media with and without Bleomycin. From these ratios it

become very clear that ku70 cycb1;1 cycb1;2 is much more compromised on media with than without Bleomycin.

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Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2015-93083R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	all samples were treated the same way, each genotype was analyzed at least with three independent biological replicates
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
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18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	not applicable
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