SMC5/6 Complex Subunit NSE4A is Involved in DNA Damage Repair and Seed Development in Arabidopsis

Mariana Diaz, Petra Pecinkova, Anna Nowicka, Celia Baroux, Takuya Sakamoto, Priscilla Yuliani Gandha, Hana Jeřábková, Sachihiro Matsunaga, Ueli Grossniklaus, Ales Pecinka

Plant Cell. Advance Publication April 29, 2019; doi: 10.1105/tpc.18.00043

Corresponding author: Ales Pecinka pecinka@ueb.cas.cz

Review timeline:

TPC2018-RA-00043	Submission received:	January 18, 2018
	1st Decision:	March 2, 2018 *revision requested*
TPC2018-RA-00043R1	1st Revision received:	June 6, 2018
	2nd Decision:	June 26, 2018 *revision requested*
TPC2018-RA-00043R2	2nd Revision received:	March 26, 2019
	3rd Decision:	March 31, 2019 *acceptance pending, sent to science editor*
	Final acceptance:	April 26, 2019
	Advance publication:	April 29, 2019

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-RA-00043 1st Editorial decision – *revision requested* **March 2, 2018**

The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

The precise mechanism of how NSE4A is involved in DNA damage repair is desirable but might be out of the scope of the current study. However, a number of other conclusions should be strengthened or clarified, including additional phenotype descriptions (origin of *nse4a* lethality, response to HU, etc.) and characterization of the expression of genes involved in DNA damage repair in the *nse4a* mutant.

--- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-RA-00043R1 1st Revision received **June 6, 2018**

Reviewer comments and **author responses**:

RESPONSE TO EDITOR: Thank you for your positive evaluation of our work. We fully agree that knowing the detailed mechanism of NSE4 (SMC5/6) complex action in DNA damage repair would be desirable. However, this is difficult task, which has not been solved in any model organism to date. All the more, we think that our work represents a significant step towards achieving this goal in plants. We addressed all points and have performed almost all suggested experiments. Most notably, we have:

- **re-structured the figures and improved their quality. Due to additions of new data, the number of the figures increased from six to eight.**
- **performed transmission assays using the *nse4a-1* allele and show that it is normally transmitted through both male and female gametophytes and that the defects occur post-zygotically.**

- **tested sensitivity of all mutants to hydroxyurea, which revealed that *nse4a-2* and *smc6b-1* are only weakly sensitive to this drug.**
- **measured the frequency of homologous recombination using the B11 reporter construct.**
- **performed a genome-wide expression analysis of *nse4a-2* under control and zebularine stress conditions by RNA-sequencing. This provided additional support for the role of NSE4A in DNA damage repair and new data on its potential involvement in plant immune responses.**

These results further strengthened our conclusions. We hope that you and the three reviewers will find our revision satisfactory.

Reviewer #1:

The manuscript by Diaz et al. reports the characterization of the NON-SMC ELEMENT 4 (NSE4) in *Arabidopsis thaliana*. The authors show that NSE4A is essential for DNA damage and reproduction while the role of NSE4B is more elusive. The study starts with the molecular and phenotypical analysis of insertional mutants. Two allelic mutants for each NSE4 homologs are characterized. The analysis is well performed and promotor GUS fusions allow the expression profiling of each of the gene.

Point 1. I strongly suggest to perform a transmission assay for nse4a-1 in order to determine the origin of the lethality.

RESPONSE: Reciprocal crosses between wild-type and mutant plants were performed and showed that aborted seeds occur only if the mutation is transmitted by both parents, indicating a recessive, zygotic embryo lethal mutation. The remaining 3% of aborted ovules (originally included in our counts but not in the revised version) are background infertility also observed in wild-type control plants under our growth conditions.

Point 2. The authors nicely show that G2 is prolonged in *nsa4-2*. I am wondering how single and double mutant plants behave under hydroxy urea (HU) treatment that blocks cell cycle progression.

RESPONSE: We analyzed root growth phenotypes of *nse4a-2* and *smc6b-1* in response to 1 mM HU treatment. Both *atr-2* and *wee1-1* controls showed hypersensitivity, confirming the functionality of this assay in our hands. In contrast, *nse4a-2* and *smc6b-1* were only weakly sensitive, suggesting that the SMC5/6 complex is not a key player in the repair of HU-induced DNA damage.

Point 3. Are there any DNA damage/repair relates genes deregulated in *nsa4* mutant plants?

RESPONSE: In order to obtain an unbiased answer to this question, we conducted RNA-sequencing experiments using wild-type and *nse4a-2* plants under control and zebularine stress conditions. Indeed, we found six DNA damage repair genes significantly up-regulated in at least one of the datasets. While there was a relatively mild DNA damage repair response in control-treated *nse4a-2*, the DNA damage repair genes were strongly up-regulated in combination with zebularine treatment. By comparison with the existing microarray data for *sni1-1* (mutation in another SMC5/6 complex subunit), we found that both mutants show up-regulation of many immune response genes. This confirms previous data suggesting that the SMC5/6 complex functions as a suppressor of the immune hyper-response (Yan et al., 2013).

Point 4. I think that this also an important point to clarify that may help to better decipher the repair process in which NSE4 proteins are involved. Genetic interaction could also help.

RESPONSE: We agree that this is an interesting question. We are currently producing many double mutants and running a forward genetic screen to answer it. However, this large and labor-intensive task is beyond the scope of the current study.

Point 5. The YTH and the BiFC assay are convincing and clear although in vivo interactions (Co IP) would have been greatly appreciated as added value to the study.

RESPONSE: We agree that the results of a third independent method may further strengthen our conclusions. However, given the facts that (a) we have already performed two independent methods, which give congruent results, and (b) our results confirm interactions already known from other model organisms, we gave this

experiment a low priority and did not perform it.

Reviewer #2:

I read your manuscript "SMC5/6 Complex Subunit NSE4A is Involved in DNA Damage Repair and Seed Development in Arabidopsis" with interest, and was impressed by the amount of work and diversity of experiments. In summary, your work lead to the conclusion of NSE4A involvement in DNA repair, the distinctive expression and phenotypes due to NSE4A and NSE4B in spite of neatly demonstrated biochemical redundancy regarding seed development, and to similar seed defect phenotypes in NSE4A as in the well-established SMCs condensin and cohesin.

Point 1. Briefly describe "VENUS" since it is not as commonly known yet as GFP.

RESPONSE: We have now included a short description of VENUS and the original reference.

Point 2. In the promoter expression study, add an analysis of shared and unique cis-elements in the NSE4A and NSE4B promoter regions since this might explain the different expression patterns.

RESPONSE: We spent considerable amount of time trying to solve this request, but can provide only a preliminary answer. First, we analyzed the promoters of *NSE4A* and *NSE4B* using several web-based tools, which predicted binding of several hundreds of transcription factors. To simplify this experiment, we conducted phylogenetic shadowing of *NSE4A* and *NSE4B* promoters using sequences of several other Brassicaceae species. This indicated conserved block directly flanking the transcription start site in the *NSE4A* promoter (A1). There was a shorter and generally less conserved block at similar position in the *NSE4B* promoter (B1). We assume that these regions may harbor the key cis-regulatory sequences. However, their analysis using a software predicting binding of transcription factors did not reveal any known candidates associated with DNA damage repair. Therefore, we consider these results as preliminary.

Point 3. Refer to prior work from Watanabe et al 2005 where NSE4A was found expressed but not NSE4B, which lead them to the suggestion that NSE4A is functional in Arabidopsis.

RESPONSE: The paper by Watanabe et al. (2005) deals with the DNA methylation status and spatial organization of artificially introduced repetitive sequences (lacO) in Arabidopsis, but not with SMC5/6 complex (A. Pecinka is co-author on this paper). We think that the reviewer #2 means publication Watanabe et al. (2009), which we cited 3x in the introduction and 2x in the discussion.

Point 4. Provide possible explanations for the seed phenotypes in *nse4a-2* mutants, or examples of other mutants with similar seed phenotype combinations and identified causes if available.

RESPONSE: In the discussion (page 13-15), we suggest that NSE4A is required to avoid the propagation of genetic lesions and/or accumulation of toxic DNA replication intermediates. Endosperm of *nse4a* mutants may thus not develop properly; since it has a role in embryo nurturing, altered endosperm development results in the delay of embryo development. The embryo can form more or less properly, but progresses very slowly or arrests before organogenesis, which is typical for endosperm malfunctions. Hence this phenotype is typical for many functionally diverse endosperm developmental mutants. To elucidate the developmental origin of aberrant seed formation in *nse4a* would require in-depth analyses of tissue-specific markers and their expression with temporal resolution, genetic dissection of the embryo and endosperm contribution to the phenotype, which are all beyond the scope of this study.

Reviewer #3:

The authors characterized two NSE4 paralogs in Arabidopsis thaliana. They found that nse4a exhibits hypersensitive to DNA damage and defects in seed development, suggesting that NSE4a functions in DNA repair and reproductive development. This manuscript is a first repot to describe the detail function of NSE in plants. This information will be useful to plant researchers in plant DNA repair and sexual reproduction.

Point 1. Introduction: Please add the information of organisms, for example, human, yeast and plant. Readers do not know what organism's subunit function is described.

RESPONSE: We added this information.

Point 2. The authors write "there was no *ProNSE4A* activity in root meristem". However, the signal of NSE4A -Venus was detected in the stele of RAM. Please explain this.

RESPONSE: Indeed, we did not observe *ProNSE4A* activity in the stele of the root apical meristems under the non-stress conditions, while there was a weak protein accumulation. We hypothesize that this could be due to *NSE4A* transcript mobility. *NSE4A* promoter is active in the differentiated zone of Arabidopsis roots under ambient conditions and the mRNA could be transporter to the apical meristem tissues from here. We tried validating this hypothesis by exploring the most comprehensive genome-wide datasets containing information on >2000 Arabidopsis root - shoot mobile mRNAs (Thieme et al., Nature Plants, 2015). Unfortunately, *NSE4A* CDS sequence is identical between the used accessions, which hinders assessing transcript mobility for this gene. Nevertheless, Thieme et al. identified mRNA of *ASAP1* and *SNI1* being cell-to cell mobile, indicating that at least some SMC5/6 complex subunits are subject to this activity.

Point 3. Figure 2; There is an inconsistency between GUS staining pattern and Venus signals at stages 13 and 14. Please explain it.

RESPONSE: We re-analyzed our images and confirmed that they are representative. In addition, this phenotype should not be caused by positional effects because we confirmed these phenotypes over multiple independent transformants. The ProNSE4A::GUS reporter lines produces GUS mRNA, which is translated into very stable GUS protein. The second construct uses the same promoter, but produces NSE4A:VENUS fusion mRNA and protein. We hypothesize that either the GUS mRNA and/or protein are much more stable than the NSE4A-VENUS protein, leading to the apparent discrepancy in distribution patterns.

Point 4. Figure 4D; The *smc6b-1* is sensitive to Zeb and MMC but *nse4a-2* did not exhibit the sensitivity to MMC. Please discuss this result although both subunits are members in the same complex. There is a difference in the phenotype between *nse4b-1* and *nse4b-2*. The evidence of loss of function of two alleles should be shown using qRT-PCR.

RESPONSE: Differential sensitivity of *smc6b-1* and *nse4a-2* to DNA damaging agents is probably result of: (a) differential expression patterns and (b) different degree of functional redundancy of *SMC6* and *NSE4* paralogs in Arabidopsis. Both *SMC6A* and *SMC6B* are widely transcribed and existing data suggest that *SMC6A* can fully substitute for *SMC6B* in non-damaging situations. However, their double mutants are lethal (see Watanabe et al., 2009; Yan et al., 2013). In contrast, strong *NSE4A* mutations are lethal due to limited transcription from *NSE4B*. Furthermore, *smc6b-1* is most likely null mutant, while the *nse4a-2* is very likely only a partial loss of function allele. This may explain remaining *nse4a-2* capacity for the repair of MMC-induced damage. We included a shorter version of this explanation on page 9.

Both *nse4b* mutants were positively tested for the homozygous constitution of T-DNA by PCR. Estimating gene functionality based on qRT-PCR is popular test in Arabidopsis. Although this method may work in some cases, we are not convinced that it is reliable method to estimate loss of gene function. First, some of the used T-DNAs (e.g. SALK and GABI-KAT) contain outward directed strong viral 35S CaMV promoter. This may even increase the amount of transcript at specific position of the gene, but this transcript will not encode for a functional protein. Second, this method is problematic when T-DNA is close to gene 3′ end. We can demonstrate this using the *nse4a-2* allele. Although, we provide a clear evidence that the T-DNA insertion has mutagenic effect (sensitivity to zebularine and complementation with genomic construct), we detected *NSE4A* among the up-regulated genes in *nse4a-2* mutant by RNA-sequencing. This is because T-DNA is inserted at the very 3′ end of the gene and the NSE4A′s DNA damage inducible promoter is not affected by it. Furthermore, also in this case the transcript does not lead to a functional protein. Third, *NSE4B* gene is very lowly transcribed in limited number of tissues and use of RT-qPCR based method would be is unreliable in such case. We believe that our genetic and molecular experiments provide multiple levels (promoter reporter lines, mutants, ProNSE4A::NSE4B swap line) complementary evidence that *NSE4B* is expressed in only small number of tissues and not involved in somatic DNA damage repair.

Point 5. The authors report dead cells in RAM. Is it possible that *NSE4a* has a function in RAM without the induction of DNA damage?

RESPONSE: Data from other organisms suggest that the SMC5/6 complex plays multiple roles in repair of

spontaneously occurring replication errors and is also important for organization of DNA strands during DNA replication and sister chromatids during chromatid unwinding. This indeed suggests NSE4 functions also outside of DDR process.

Point 6. DNA damage repair: Overall, the present manuscript includes a weak evidence that NSE4 directly functions in DNA repair. Data of comet assay and assay of homologous recombination assay will reinforce their hypothesis of NSE4 in DNA damage repair.

RESPONSE: We have discussed possibility of doing comet assays extensively (the method is established in our lab). However, we have previously shown that zebularine does not cause detectable amount of ssDNA or dsDNA breaks in comet assays (see Liu et al., 2015). Therefore, we hesitate to perform the suggested experiment.

We have done and included homologous recombination experiment using B11 single strand annealing HR type reporter. This showed that neither *nse4a-2* nor *nse4b-2* mutations alter somatic HR frequency. This differs from previously published results for *SMC6A*, *SMC6B* and *NSE2/HPY2/MMS21* mutants. On the one hand this may suggest that NSE4 proteins are not controlling the single-strand annealing type of HR in Arabidopsis. But on the other hand, this result should be interpreted with caution because (a) *nse4a-2* is not a null allele and (b) *nse4b* mutants are not sensitive to DNA damaging treatments.

TPC2018-RA-00043R1 2nd Editorial decision – *revision requested* June 26, 2018

The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision.

While all three reviewers agreed that the manuscript was improved, several concerns were raised regarding the hypersensitivity of the nse4a mutant, RNA-seq analysis, interaction assays and data interpretation/discussions. These concerns should be addressed in the revision.

TPC2018-RA-00043R2 2nd Revision received March 26, 2019

Reviewer #1:

Point 1. The manuscript was greatly improved.

RESPONSE: Thank you.

Point 2. It is appreciable to have included expression profile experiments. I would suggest to calculate the "representation factor" for each overlap in Figure 6C. It will help to test whether the overlap is significant or not and to support the conclusion that the SMC complex modulates expression of immune genes.

RESPONSE: We have re-analyzed the data using DESeq software (as suggested by the reviewer 3), which should provide statistically more robust conclusions. After the re-analysis, there were minor changes, which did not affect our general conclusions. In Figure 6D we show frequencies of genes associated with biotic stress, defense responses or their combinations, which serve as a proxy for immune responses, and compare them to the frequency among genes up-regulated in *nse4a-2* and *sni1-2*. This shows significant enrichment in the mutants versus genome background. We hope this is a satisfactory answer. In case not, please specify what is "representation factor".

Point 3. The interaction experiments would be better supported by CoIP experiments using either transient expression in *N. benthamiana* or stable lines in *A. thaliana*.

Response: We have performed requested Co-IP experiments using transient expression system in *N. benthamiana*. The results are summarized in Figure 7 and Supplemental Figure 9. This confirmed all interactions predicted by Y2H and BiFC, except for SMC5 – NSE4 interactions, which we could not show, as we did not detect SMC5 tagged protein in western blots. That is most likely due to biochemical properties of the full length SMC5 protein, which renders it difficult to detect. In order to submit a satisfactory answer to this question, we did extensive optimization,

where we tagged SMC5 with YFP, RFP, and FLAG in both N- and C- terminal orientations and also performed microscopic analysis of the signals. In the microscope, we observed that EYFP and -tagRFP N- and C-terminus tagged SMC5 protein strongly localized to nucleus, which differed to the localization of the negative control (35S promoter driven GFP) into the membranes and nuclei. Then, we detected changes in NSE4 localization when co-expressed with SMC5. We observed formation of speckles inside of nuclei, which we did not observe when NSE4 was infiltrated alone or co-infiltrated with a negative control (Figure 7C). This remained true for all SMC5 – NSE4s combinations and orientations where we could test NSE4 localization by confocal microscopy (SMC5-tagRFP and NSE4A, NSE4B-EYFP; SMC5-EYFP and NSE4A, NSE4B-tagRFP and even in SMC5-Flag and NSE4s EYFP, tagRFP tagged), strongly suggesting that this interaction indeed occurs. Hence, most of the proposed interactions were now validated with three independent methods.

Reviewer #2:

Point 1. Addition of HR-GUS assays showed that none of the *nse4* mutants is defective in somatic HR repair in the chosen reporter lines which is a bit surprising. I did not see the authors' explanation for the zebularine hypersensitivity of *nse4a-2* contrasting with the HR GUS result. What could be the reason for this?

In line with this inconsistency and currently missing explanation, I would highly appreciate the addition of qRT-PCR data for all *nse4a* and *nse4b* mutants used, as previously suggested by reviewer #3 (line 300 and 312 indicate this data is already available). Optimally include primers spanning the insertions as well as primers for the intact 3', mid and 5' end of the respective gene to distinguish knockout/knock-down with dominant-negative effects.

RESPONSE: The wild type-like levels of HR in *nse4a-2* could be caused by the fact that this is not a null mutant (null mutants such as *nse4a-1* are homozygous embryo lethal). Speculatively, the recombination could take longer, but may be completed at some point. We did not find any indication for that NSE4B is involved in somatic DNA damage repair based on multiple independent experiments.

Concerning effectiveness of the used mutations. *nse4a-1* allele is lethal. For *nse4a-2* we performed 3′ RACE, which showed that the transcript progresses into T-DNA and based on *in silico* translation predict that the mutated protein should be longer than in WT (Supplemental Figure 2). The last 67 amino acids of NSE4A in *nse4a-2* are exchanged for a nonsense sequence based on the T-DNA. Given viability of homozygous *nse4a-2* and lethality of *nse4a-1* plants, we believe that the *nse4a-2* is only partial loss of function mutant as mentioned at multiple positions in the manuscript.

***nse4b-1* allele carries T-DNA in the second intron and *nse4b-2* allele in the fifth exon. We performed multiple RT-qPCR based quantifications of NSE4B transcripts with pairs of primers placed before and after the T-DNAs. However, this gave conflicting results with more transcript than in wild type, where there should be none (not shown). The most likely reason is (antisense?) transcription from T-DNAs. This phenomenon has been reported previously. Ülker et al. (2008) showed that the 1´2´ bidirectional promoter (contained in T-DNAs of GABI-Kat and SAIL lines) is capable of activating expression of flanking genomic sequences. This suggests that the results from RT-qPCR performed in T-DNA insertion lines should be interpreted with caution. Therefore, for the revision we adapted another strategy, where each primer is placed on one side of the T-DNA (see Supplemental Figure 3) and therefore does not amplify spurious transcripts originating from T-DNA. This revealed no or little amplification in *nse4b-1* and *nse4b-2*, respectively (Supplemental Figure 3), indicating that both used mutations indeed destroy *NSE4B* ORF. Based on multiple other experiments using *PromoterNSE4B::GUS* fusion reporter line, we suggest that NSE4B acts only in specific tissues and defects in its mutants (if any) may be masked by NSE4A (the double mutant *nse4a-2 nse4b-2* will show residual NSE4A activity).**

Point 2. The RNA-seq data does not add that much, and could be condensed to the main key message in the text (e.g. "2-3 times more genes differentially regulated in *nse4a* than in WT after zebularine treatment"; then the specific genes).

RESPONSE: We have reduced this part and made the text more compact.

Point 3. Please include newly added results in the discussion to put them into context.

RESPONSE: We discussed the newly added results in the manuscript. In most these cases we felt that explaining the problem directly in the results section will be more appropriate.

Reviewer #3:

Several experimental data and additional discussion have successfully reinforced their conclusion about NSE4 functions. The revised manuscript by Diaz et al. is more convincing. The following comments were raised up from this revised version.

RESPONSE: Thank you for positive evaluation.

Point 1. Thank you to make kind responses to my comments on the inconsistency between GUS and Venus pattern and different sensitivity to DNA-damaging agents. Do you have the idea to explain the reason why nse4a mutant did not exhibit significant sensitivity to bleocin as shown in Fig. 5? You showed that the signals of NSE4a-GUS were induced by the addition of bleocin as shown in Fig. 4. Your explanation of the relationship between GUS induction and the response to DNA-damaging agents will be helpful for Plant Cell audience.

RESPONSE: Speculatively, we believe that multiple DNA repair pathways are activated upon DNA damage (in particular when variable types of damage are induced – bleocin does DBS, SSBs and potentially also smaller amount of other DNA damages). Subsequently, the pathways (or their parts) may be competing for repair at individual DNA damage sites. In case of bleocin treatment it is possible that NHEJ is given priority (or is simply sufficient). In addition, nse4a-2 is only partial loss of function mutant, so we cannot fully exclude involvement of NSE4A in repair of bleocin-induced DNA damage. We added short comment on this directly in the results section.

Point 2. I have some technical questions about your added RNA-seq analyses. My main concern is your experimental replication. Can you add the SD bars to the graph in Figs 6B and 6E although you performed RNA-seq experiments TWICE? More than three replicates enable to calculate SD. You used cuffdiff software when you selected significantly changeable genes. However, the cuffdiff software can work well based on t-test as a significance test with more than three replicates. If you have RNA-seq data with two replicates, you should analyze with edgeR or DEseq based on fisher's exact test with two replicates. If you can analyze two replicated data with cuffdiff, the modified points including parameter changes should be added to the method section.

RESPONSE: Thank you for this important comment. We have reanalyzed our RNA-seq data using DEseq and modified corresponding parts of the manuscript accordingly. This re-analysis let to some modifications, but generally confirmed our previous results.

Point 3. The significant upregulation of DNA repair-related genes was found in nse4a mutant under the normal condition. This suggests the possibility that NSE4a is involved in suppression of DNA damage under the condition without DNA-damaging agents. Taken together with my previous comment No. 5, please add the discussion on this point.

RESPONSE: Indeed, SMC5/6 complex certainly resolves endogenously (spontaneously) occurring DNA damage and data from non-plant models also suggest its involvement in non-DNA damage situations. We added explanation on this at the end of the section dealing with the transcriptomic data.

TPC2018-RA-00043R2 3rd Editorial decision – *acceptance pending* March 31, 2019

We are pleased to inform you that your paper entitled "SMC5/6 Complex Subunit NSE4A is Involved in DNA Damage Repair and Seed Development in Arabidopsis" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor April 26, 2019