Short Communication

Histone H2AX and the small RNA pathway modulate both non-homologous end-joining and homologous recombination in plants

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ABSTRACT

Using a zinc finger nuclease (ZFN) that creates a site-specific DNA double-strand break (DSB) at the endogenous *ADH1* locus, we provide genetic evidence that histone H2AX and DSB-induced small RNAs modulate DNA repair by both non-homologous end-joining (NHEJ) and homologous recombination (HR) in the model plant *Arabidopsis thaliana*.

Keywords: Zinc finger nuclease (ZFN), DNA double-strand break (DSB) repair, H2AX, small RNA, *Arabidopsis thaliana*

1. Introduction

DNA double-strand breaks (DSBs) are lethal lesions and need to be repaired for cell survival. Both plants and vertebrates share conserved mechanisms for sensing DNA DSBs and initiating the repair process. One such mechanism is the rapid phosphorylation of histone H2AX (γ -H2AX), which is dependent on phosphatidylinositol-3-OH (PI3) kinases such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad-3-related (ATR) [1, 2]. Accumulation of γ -H2AX at DNA breaks is thought to recruit downstream components needed for repair, including factors involved in either the non-homologous end joining (NHEJ) or homologous recombination (HR) pathways [3]. It was recently shown that γ -H2AX foci are required for DNA DSB repair responses in *Arabidopsis* [4]. However, it is unclear whether γ -H2AX is required for both NHEJ and HR repair in plants. Two recent publications reported that DSB-induced small RNAs (diRNAs) function in DNA repair in plant and vertebrate cells [5, 6]. The diRNAs' role in DNA repair was demonstrated in plant cells by measuring HR [5]. However, NHEJ is the predominant repair pathway in plant and vertebrate somatic cells, and the relationship between NHEJ and diRNA-mediated DNA repair remains unclear. According to both studies, DSB-induced γ -H2AX accumulation was not affected in cells depleted of diRNAs. Thus, γ -H2AX seems to function independent of the diRNAs.

Study of DNA DSB repair in plants has largely relied on inducing random DSBs in the genome by physical or chemical means such as UV light or ionizing radiation [7, 8]. DNA DSBs can also be induced at a given locus by sequence-specific nucleases (SSNs) [9]. There are four major SSNs, namely meganucleases [10, 11], zinc finger nucleases (ZFNs) [12, 13], TAL effector nucleases (TALENs) [14, 15] and CRISPR-Cas9 [16-18]. Current applications of SSNs have been primarily focused on genome editing in diverse organisms, including plants [9]. As

SSNs can induce DNA DSBs at a specific locus, they are also very useful tools for studying DNA DSB repair, especially when coupled with high-throughput DNA sequencing. In plants, high-throughput DNA sequencing has been used to survey the repair profiles of DNA DSBs induced by transposons [19], ZFNs [20], TALENs [21] and CRISPR-Cas9 [22]. By coupling site-specific ZFNs and high-throughput DNA sequencing, we recently showed that DNA repair pathways can be manipulated to achieve desired genome modifications in plants [23]. Here, we took a similar genetic approach and examined the roles of γ -H2AX and the small RNA pathway in the repair of DSBs at the endogenous *ADH1* locus of *Arabidopsis*. The experimental approach allowed us to simultaneously look at DNA DSB repair by NHEJ and HR.

2. Materials and Methods

2.1. Plasmids and plant materials

The ADH1-ZFN expression plasmid was constructed by Gateway LR recombination between the entry vector pENTR-ADH1-ZFN [24] and the destination vector pZHY051 [21]. The resulting plasmid, pZHY273, expresses the *ADH1-ZFN* gene from the 35S constitutive promoter. The HR donor plasmid has been published previously [23]. T-DNA insertional lines were obtained from the *Arabidopsis* Biological Research Center (ABRC) at Ohio State University, Columbus, OH. The mutant lines include *ath2axa* (SALK_007006), *ath2axb* (SALK_012255), *nrd1a-3* (SALK_128428) [25] and *dcl3-1* (SALK_005512) [26]. The *ath2axa ath2axb* double mutant was generated by crossing the two single mutants. All mutants were genotyped for homozygosity with the primers listed in Table S1.

2.2. Plant growth and protoplast transformation

All plants were grown in BM2 soil in Conviron® growth chambers with 12 hours light and 12 hours dark at 22°C and 75% humidity. *Arabidopsis* protoplasts were prepared from rosette leaves of 4-week old plants. Protoplast transformation was conducted by following a previously published protocol [23]. Fifteen micrograms of the ADH1-ZFN expression plasmid and/or donor plasmid was used for each transformation. Fifteen micrograms of YFP-expression plasmid (pZHY162) [23] was added to the donor only or ZFN only samples to transform the protoplasts with equal amounts of DNA and to estimate transformation efficiency.

2.3. Plant genomic DNA extraction and NHEJ analysis

Plant genomic DNA was extracted from transformed protoplasts using the CTAB method [27]. For NHEJ analysis, DNA sequence flanking the ADH1-ZFN target site was PCR amplified by primers ADH1F and ADH1R2 (Table S1) and subsequently digested with the NlaIII restriction enzyme (NEB).

2.4. RNA extraction and RT-PCR

Total RNA was extracted from rosette leaves of Col WT, *ath2axa*, *ath2axb* and *ath2axa ath2axb* double mutant plants with TRIzol reagent from Invitrogen. The extracted RNA was used as a template for amplifying *AtH2AXA*, *AtH2AXB* and *Actin2* with the OneStep RT-PCR kit from Qiagen. The primer sequences are included in Table S1.

2.5. 454 pyrosequencing and data analysis

454 pyrosequencing of amplicons and data analysis were done according to our published protocol [23]. The barcoded primers used in this study are listed in Table S1.

3. Results and Discussion

3.1. A protoplast system for studying DNA DSB repair in *Arabidopsis* mutants

We previously looked at DNA DSB repair in *Arabidopsis* lines with mutations in genes involved in NHEJ and sister-chromatid-based HR. Our approach involved introducing a DSB at the *ADH1* locus in protoplasts and then assessing repair outcomes using high-throughput DNA sequencing [23]. The success of that study prompted us to look at DSB repair in other *Arabidopsis* lines with mutations in genes whose role in DNA repair is less well understood, namely mutants of *H2AX* and small RNA pathway genes. There are two homologous *H2AX* genes in *Arabidopsis*, *AtH2AXa* and *AtH2AXb* [1]. Both *H2AX* genes are expressed constitutively and may function redundantly [4, 28]. *Arabidopsis* T-DNA insertion lines for both *H2AX* genes were obtained and crossed to generate double mutants (Fig. S1A). Reverse transcription (RT)-PCR assays performed to assess mRNA expression suggested that both mutants are likely null alleles (Fig. S1B). This observation is supported by previous work showing that γ -H2AX protein was undetectable in the double mutant [28]. In *Arabidopsis*, the production of diRNAs requires multiple siRNA pathway components, such as RNA polymerase IV (Pol IV), Dicer-like 3 (DCL3) and Argonaute 2 (AGO2) [5, 29]. We chose to work on *Arabidopsis* lines with mutations in *NRPD1a* (which encodes a major subunit of Pol IV) and *DCL3*, and we obtained lines with T-DNA insertions in both genes [25, 26].

In our previous study, we crossed mutant lines of interest with an *Arabidopsis* line harboring an *ADH1-ZFN* line expressed from an estrogen-inducible promoter [23, 24]. In order to quickly examine additional *Arabidopsis* mutants for their role in DSB repair, we decided to directly transform protoplasts derived from mutants with a construct expressing the ADH1-ZFN from the

constitutive 35S promoter (Fig. 1A). The ZFN targets the third exon of the *ADH1* gene (Fig. 1B), and the donor plasmid allows for the insertion of 68 bp through HR-mediated gene targeting (GT) (Fig. 1B). Similar to our previous approach [23], high-throughput 454 pyrosequencing was used to reveal the repair profiles in different mutant backgrounds.

We first tested the protoplast system by transforming the *ADH1*-ZFN and donor plasmids individually or together into protoplasts derived from the Col wild type (WT) and five mutant lines (Fig. 1C and 1D). For all genotypes, the transformation efficiency exceeded 90%, based on expression of a YFP construct that was co-transformed with the donor or ZFN (Fig. 1D; data not shown). As expected, transformation of *ADH1*-ZFN plasmid resulted in targeted mutations, and co-transformation of the donor plasmid resulted in GT at the *ADH1* locus (Fig. 1D).

3.2. Assessing DNA repair outcomes at the *ADH1* locus

Recent studies have found that small RNAs are induced by DNA DSBs [5, 6]. We decided to assess DNA repair outcomes in different genetic backgrounds where small RNA pathway genes are knocked out. In order to obtain detailed profiles of DNA DSB repair outcomes at the *ADH1* locus in different genetic backgrounds, we sequenced PCR amplicons spanning the ZFN target site using 454 pyrosequencing. We first extracted DNA from transformed protoplasts and then PCR-amplified the target region with barcoded primers (Table S1). For each treatment, the amplicons from two biological replicates were then subjected to 454 pyrosequencing. For all samples, the number of valid sequencing reads ranged from 16,904 to 22,062 (Table 1). Further analysis of these sequencing reads revealed many different types of NHEJ-induced events, including deletions, insertions, deletions plus insertions, and nucleotide substitutions (Table 1). In addition, both GT and imprecise GT events were recovered from most samples (Table 1; see below for more detailed analysis and discussion).

3.3. H2AX and diRNAs play a positive role in NHEJ

The 454 sequencing data revealed a NHEJ-mediated mutation frequency of ~13% at the *ADH1* locus in the WT background (Fig. 2A). Mutation frequencies in the mutant backgrounds were significantly lower, the lowest being only ~50% of the WT. It is unlikely that H2AX and diRNAs negatively regulate the precision of NHEJ. A more likely explanation for the lower frequencies is that more DSBs remain unrepaired in the mutants than in WT. If this is the case, then H2AX and diRNAs play a positive role in NHEJ, which is consistent with their roles in DNA repair in plants [4, 5]. An assay for quantification of unrepaired DSBs across these different genotypes could validate this hypothesis. Further, since NHEJ-mediated mutagenesis was greatly affected in the *ath2axb* mutant but only slightly in the *ath2axa* mutant, the former likely plays a more important role in modulating NHEJ than the latter. Mutations in the siRNA pathway genes (*NRPD1a* and *DCL3*) also resulted in lower mutation frequencies, ~6% and ~9% respectively. We further analyzed different types of NHEJ-induced mutations, namely deletions, insertions, deletions plus insertions and nucleotide substitutions. The mutation profiles were comparable among all genotypes, except *nrpd1a*, in which there seems to be more insertions and less deletion-plus-insertion events (Fig. S2A). Assessment of the length of microhomology (MH) used to create the deletions revealed no evidence that DNA repair shifted from classic NHEJ to alternative MH-based NHEJ in the mutants. Thus, neither H2XA nor diRNAs appear to influence pathway choice as previously observed for Ku70/80 or Lig4 in plants [23, 30]. However, when we looked at length of insertions at the DSB site, we found a consistent pattern: small 2-9 bp insertions were less frequent in all mutant backgrounds (Fig. 2B). As with the mutation frequency (Fig. 2A), this suggests that slowdown of NHEJ repair due to the lack of

γ H2AX or diRNAs promotes larger DNA insertions, but somehow does not promote larger deletions. The fundamental mechanism underlining the phenomenon remains to be explored.

3.4. H2AX and diRNAs positively contribute to repair by HR

We next looked at GT, which in plant somatic cells occurs largely by synthesis-dependent strand annealing (SDSA) (Fig. 3A) [9, 31, 32]. The SDSA model indicates GT can result from HR or a combination of HR and NHEJ (Fig. 3A). In the latter case, some GT events will have mutations in chromosomal sequences corresponding to one of the two homology arms, which we term imprecise GT (Fig. 3A). When we analyzed the 454 data, we indeed found evidence of both precise and imprecise GT, the latter being more predominant in our experimental system (Fig. 3B). When compared to the WT, both precise and imprecise GT were significantly decreased (>3 fold) in all mutants except for *ath2axb*. The impairment in HR in the diRNA pathway mutants is consistent with recent reports [5, 6, 33]. **For example, it was recently reported that that AGO2 helps recruit RAD51 for HR repair in human cells [33]. It will be interesting to know whether a similar mechanism works in plants.**

Interestingly, although the frequency of precise GT dropped significantly, the frequency of imprecise GT remained unaffected in the *ath2axb* mutant (Fig. 3A), suggesting distinct roles for the two H2AX homologs in HR in *Arabidopsis*. Such an observation prompted us to further analyze imprecise GT events. Mutations due to imprecise NHEJ allow us to measure the relative length of gene conversion (GC) tracks (Fig. 3A) for each imprecise GT event. The relative lengths of GC tracks in diRNA pathway mutants and the WT are comparable (Fig. 3C). However, much longer GC tracks were observed when *AtH2AXa*, but not *AtH2AXb*, is mutated (Fig. 3C).

Taken together, these results suggest that AtH2AXa has a specific role in controlling imprecise HR.

4. Conclusion

Using a protoplast assay system, we surveyed repair outcomes of DNA DSBs induced by a ZFN at the *Arabidopsis ADH1* locus in different mutant backgrounds. Our experimental approach allowed us to simultaneously examine both NHEJ and HR repair in *Arabidopsis* somatic cells. We demonstrated for the first time that H2AX proteins indeed play a positive role in DNA DSB repair in plants. Further, our results suggest that siRNAs contribute to HR repair in plants, as previously demonstrated in human cells [33]. In addition, we provide genetic evidence that siRNAs positively contribute to NHEJ repair. We provide a model to summarize our findings (Fig. 4). In *Arabidopsis* somatic cells, diRNAs and γ H2AX are involved in DNA DSB repair through both the NHEJ and HR pathways. The diRNA response is either independent of γ H2AX or downstream of it. Our finding for H2AX's involvement in DNA repair is consistent with previous reports that *H2AX* knockout and knockdown plants display certain levels of genotoxic sensitivity [4, 28]. Strikingly, two H2AX isoforms in *Arabidopsis* seem to have both redundant and divergent functions in modulating NHEJ and HR repair. Although only one genomic locus was studied here, we think the results can be generally applied to other loci based on our previous work where three genomic loci were studied with a similar strategy [23]. Since the data presented here are only genetic evidences, we cannot rule out the possibility that changes of NHEJ and HR repair in these mutants are due to indirect effects. Nevertheless, this study points to an exciting future research topic on elucidation of the roles of diRNA and γ H2AX in DNA DSB repair in plants by using molecular and biochemical approaches.

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Figure legends

Fig. 1. The protoplast transformation system for studying DNA DSB repair. . (A) The *ADH1*-ZFN target site. The DNA recognition sequences for each ZFN are underlined. The *Nla*III restriction site is marked in bold. (B) ZFN-mediated GT at the *ADH1* locus. A schematic of the *ADH1* locus and the ZFN cleavage site are shown. The donor contains a left homology arm (LHA) of 161 bp and a right homology arm (RHA) of 144 bp. GT will result in an insertion of 68 bp (Ins) at the ZFN target site. (C) The protoplast-based experimental system. Protoplasts derived from *Arabidopsis* rosette leaves are transformed with both *ADH1*-ZFN expression and gene targeting (GT) donor plasmids. Transformed protoplasts are subsequently collected for DNA extraction and 454 pyrosequencing. (D) Detection of ZFN-mediated NHEJ mutagenesis and GT at the *ADH1* locus. The undigested upper bands are indicative of mutations (upper panel). GT was detected by PCR using primers ZY070-F and ZY073-R1 (panel B). Amplification of the

locus with the ZY070-F and ZY070-R primer set was used as a PCR control (lower panel). This experiment was done twice independently with similar results.

Fig. 2. Non-homologous end-joining (NHEJ) DNA repair in different genotypes as revealed by 454 sequencing. (A) ZFN-induced NHEJ-mediated mutagenesis at the *ADHI* locus in different genotypes as revealed by 454 pyrosequencing. (B) Analysis of NHEJ-mediated insertions at the *ADHI* locus among different genotypes. Means were calculated from two independent experiments, and the error bars indicate standard deviations. Statistically significant differences are indicated by an asterisk ($P < 0.05$, t-test).

Fig. 3. Homologous recombination (HR)-based GT in different genotypes. (A) The synthesis-dependent strand annealing (SDSA) model shows the mechanism by which either precise GT or imprecise GT can be achieved. The gray regions denote sequences (68 bp in this study) to be inserted into the genome through HR. The unfilled rectangles denote mutations created through imprecise NHEJ. In the case of imprecise GT, the distance from the site of the NHEJ-induced mutation to the nearest boundary of the other homology arm is denoted as ‘relative length of gene conversion (GC) track.’ (B) Frequencies of GT in different genotypes. (C) Comparison of relative length of GC tracks based on imprecise GT in different genotypes. Means of two independent experiments are shown. The error bars indicate standard deviations. Statistically significant differences are indicated by an asterisk ($P < 0.05$, t-test).

Fig. 4. A model for the roles of diRNAs and the two H2AX isoforms in repair of a DSB in *Arabidopsis* somatic cells. diRNAs: Sensing a DNA DSB triggers the production of diRNAs, which involves RNA polymerase IV (NRPD1 and NRPD2), RNA-dependent RNA polymerases

(RDRs) and DCL3. diRNAs then form complex with AGO2 to participate in DNA DSB repair.

The diRNAs may act independent of γ -H2AX (1) or downstream of γ -H2AX (2). They appear to act upstream of DNA repair pathways, and do not appear to impact repair pathway choice.

H2AX: A DNA DSB rapidly leads to the phosphorylation of two isoforms of H2AX (γ -H2AX), namely AtH2AXa and AtH2AXb. This process is largely dependent on ATM and slightly dependent on ATR. γ -H2AX may function both as a signal and platform for recruiting downstream components for DNA DSB repair. Although both AtH2AXa and AtH2AXb are redundant in DNA repair through the NHEJ and HR pathways, they seem to have divergent functions as well. For example, AtH2AXa has a specific role in regulating imprecise HR and AtH2AXb seem to regulate NHEJ and precise HR. Note that although NRDP2, RDRs and AGO2 are not included in this study, they are included in this model for the purpose of illustrating the possible diRNA pathway.

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Table 1. Summary of 454 sequencing results at *ADH1*.

Experiment	Valid reads	Deletions	Insertions	Mismatches	Deletions plus insertions	GT	Imprecise GT
<i>Col</i> Rep1	20952	1587	508	237	295	52	102
<i>ath2axa</i> Rep1	19887	1183	395	245	268	15	37
<i>ath2axb</i> Rep1	20046	728	224	239	133	11	103
<i>ath2axa ath2axb</i> Rep1	20323	616	298	147	114	11	29
<i>nprd1a</i> Rep1	16904	746	528	184	35	0	11
<i>dcl3</i> Rep1	18263	533	120	289	146	17	0

<i>Col</i> Rep2	22062	1685	579	255	360	65	108
<i>ath2axa</i> Rep2	21179	1247	514	274	327	5	33
<i>ath2axb</i> Rep2	20972	690	243	249	138	12	121
<i>ath2axa ath2axb</i> Rep2	21450	724	338	193	135	2	27
<i>nprd1a</i> Rep2	18418	788	589	249	42	3	24
<i>dcl3</i> Rep2	19115	546	177	279	155	14	1

Figure

Fig. 1

Fig. 2

Fig. 3

Fig. 4