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A Role for Small RNAs in DNA Double-Strand Break Repair

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SUMMARY

Eukaryotes have evolved complex mechanisms to repair DNA double-strand breaks (DSBs) through coordinated actions of protein sensors, transducers, and effectors. Here we show that ~21-nucleotide small RNAs are produced from the sequences in the vicinity of DSB sites in *Arabidopsis* and in human cells. We refer to these as diRNAs for DSB-induced small RNAs. In *Arabidopsis*, the biogenesis of diRNAs requires the PI3 kinase ATR, RNA polymerase IV (Pol IV), and Dicer-like proteins. Mutations in these proteins as well as in Pol V cause significant reduction in DSB repair efficiency. In *Arabidopsis*, diRNAs are recruited by Argonaute 2 (AGO2) to mediate DSB repair. Knock down of Dicer or Ago2 in human cells reduces DSB repair. Our findings reveal a conserved

fashion. It can cause deletions or insertions at the break site because of the modification of DNA ends before joining (Lieber, 2010). In contrast, HR is considered error free, but it requires resection of the DSB and a sister chromatid as template for repair (Moynahan and Jasin, 2010; San Filippo et al., 2008; Sasaki et al., 2010). Single-strand annealing (SSA) is a particular type of HR that takes place when DSB resection occurs at repetitive sequences, providing complementary single strands that can then anneal (Ciccia and Elledge, 2010; Hartlerode and Scully, 2009). These repair pathways all require well-regulated and coordinated enzymatic actions of protein sensors, transducers, and effectors in the DSB signaling cascade (Ciccia and Elledge, 2010; Huen and Chen, 2008; Polo and Jackson, 2011).

Small RNAs have emerged as key players in various aspects of biology. Three major classes of small RNAs have been discovered in eukaryotes: microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs). miRNAs and siRNAs are processed by RNase III domain-containing Dicer

into dsRNAs (Xie et al., 2004), which are in turn processed by DCL3 into 24 nucleotide (nt) hc-siRNAs (Xie et al., 2004). Hc-siRNAs are associated with AGO4 subfamily members and direct DNA methylation by the de novo DNA methyltransferase DRM2 through a pathway known as RNA-directed DNA methylation (RdDM) (Law and Jacobsen, 2010; Matzke et al., 2009). Functionally analogous to plant hc-siRNAs, piRNAs are specific to animals and most often specifically generated in the germ line to inactivate transposons (Malone and Hannon, 2009). In addition to these three classes of small RNAs, other types of small RNAs have also been found in various organisms. *Tetrahymera thermophila* contains scan RNAs (scnRNAs) that are involved in developmentally regulated DNA elimination (Mochizuki and Gorovsky, 2004; Yao and Chao, 2005). More recently, high-throughput approaches have identified promoter-associated short RNAs (PASRs) (Kapranov et al., 2007), termini-associated short RNAs (TASRs) (Kapranov et al., 2007; Kapranov et al., 2010), and transcription-initiation RNAs (tiRNAs) (Taft et al., 2009) in animals.

In light of the expanding universe of small RNAs and their increasingly diverse biological roles, we explored whether small RNAs could play a role in DSB repair. Using well-established reporter assays for DSB repair in *Arabidopsis thaliana* and human cells, we found that DSBs trigger the production of small RNAs from the sequences in the vicinity of DSB sites and these small RNAs are required for efficient DSB repair. Our results reveal an unsuspected and conserved role for small RNAs in the DSB repair pathway.

RESULTS

DCLs Are Required for Efficient DSB Repair in *Arabidopsis*

We used an *Arabidopsis* transgenic line carrying the DGU.US reporter in which expression of the endonuclease I-*Sce*I, induced by crossing, introduces a single DSB in the genome (Mannuss et al., 2010; Orel et al., 2003). Repair of the DSB through SSA mechanism restores β-glucuronidase (GUS) expression, which provides a visible and quantitative readout of DSB repair events (Figure 1A). Confirming published results (Mannuss et al., 2010; Orel et al., 2003), crossing the DGU.US reporter (R) line with a DSB-triggering (T) line that expresses I-*Sce*I induced DSB repair with a repair rate about two orders of magnitude higher than that of uncrossed R line (Figures 1B, 1D, and 1E). We then introduced, via crossing, this reporter system into an *Arabidopsis atr* mutant background to assay the DSB repair rate in this mutant (Figure S1, available online). *ATR* encodes a phosphatidyl inositol 3-kinase-like protein kinase (PI3 kinase) that primarily responds to stalled replication forks and also acts with ATM (Ataxia Telangiectasia Mutated, another PI3 kinase) in DSB response (Amiard et al., 2010; Culligan et al., 2004; Culligan and Britt, 2008; Culligan et al., 2006; Jazayeri et al., 2006). We found that the repair efficiency was greatly reduced in the *atr* mutant as determined by GUS staining or PCR detection of repaired DNA (Figures 1B, 1D, and 1E).

DCLs are required for small RNA biogenesis in *Arabidopsis* (Xie et al., 2004). We next examined whether DCLs are involved in DSB repair. We introduced the GUS reporter system into *dcl2*, *dcl3*, and *dcl4* mutant backgrounds. Intriguingly, compared with those in the wild-type controls, the repair rates were reduced by 42%, 90%, and 44% in the *dcl2*, *dcl3*, and *dcl4* mutant backgrounds, respectively (Figures 1C, 1D, and 1E), suggesting that DCL2, DCL3, and DCL4 play partially redundant roles in DSB repair and that DCL3 is the major player.

DSBs Trigger Production of Small RNAs from Sequences Flanking the DSB Sites

The requirement of DCLs for efficient DSB repair suggested the involvement of small RNAs in this process. Thus, we sought to examine whether small RNAs were produced from the sequences around the DSB site by northern blot analysis. When a probe that recognizes the ~450 nt sequence flanking the I-*Sce*I site (Figure 1A) was used, small RNAs were barely detectable in the R or T line but were readily detected in the progenies of RxT cross (Figure 2A). These small RNAs were named diRNAs for DSB-induced small RNAs. Deep sequencing analyses revealed that diRNAs were specifically derived from both sense and antisense strands of the sequence around the I-*Sce*I site (Figure 2B). Confirming the northern blot results, ~40 times more diRNA reads were obtained from the RxT sample compared to those from the uncrossed R line (Figure 2B), whereas the expression profiles of endogenous small RNAs (miRNAs and siRNAs) were comparable in these two samples (Table S1). Moreover, we found that the production of diRNAs was dependent on *ATR*. In the *atr* mutant background, the diRNAs were barely detectable either by northern blot (Figure 2C) or deep sequencing (Table S2).

We extended the diRNA analysis to another DSB repair reporter (DU.GUS) system (Orel et al., 2003). In this system, the DSB site generated by I-*Sce*I digestion is repaired by the HR pathway that involves gene conversion through synthesis-dependent strand annealing (SDSA). We detected the production of diRNAs in the DU.GUSxT plants by deep sequencing (Figure 2D). The amount of diRNAs produced in the DU.GUSxT plants was approximately one-fifth of that detected in the RxT plants (Figures 2B and 2D). This is in correlation with the lower DSB repair frequency in the DU.GUS line, which has been reported to be about five times lower than that in the DGU.US line (Orel et al., 2003).Taken together, these observations demonstrate that DSBs induce the production of diRNAs specifically around the DSB sites.

diRNAs Play a Role in DSB Repair

To investigate the role of diRNAs in DSB repair, we first examined the production of diRNAs in the *dcl* mutants that had reduced repair rates (Figures 1C, 1D, and 1E). Compared to the wild-type controls, *dcl2*, *dcl3*, and *dcl4* accumulated many fewer diRNAs (Figure 3A and Table S2). In particular, the production of diRNAs was reduced by 98% in the *dcl3* mutant, consistent with its predominant effect on the repair efficiency (Figures 1C, 1D, and 1E). DCL2, DCL3, and DCL4 are known to process dsRNAs into 22 nt, 24 nt, and 21 nt siRNAs, respectively (Qi et al., 2005; Xie et al., 2004). It is intriguing that the production of both 21 nt and 24 nt siRNAs was greatly reduced in each of the *dcl* mutants (Figure 3A). This suggests that diRNAs might be processed from their dsRNA precursors through coordinated actions of DCL2, DCL3, and DCL4. Coordinated actions of DCLs

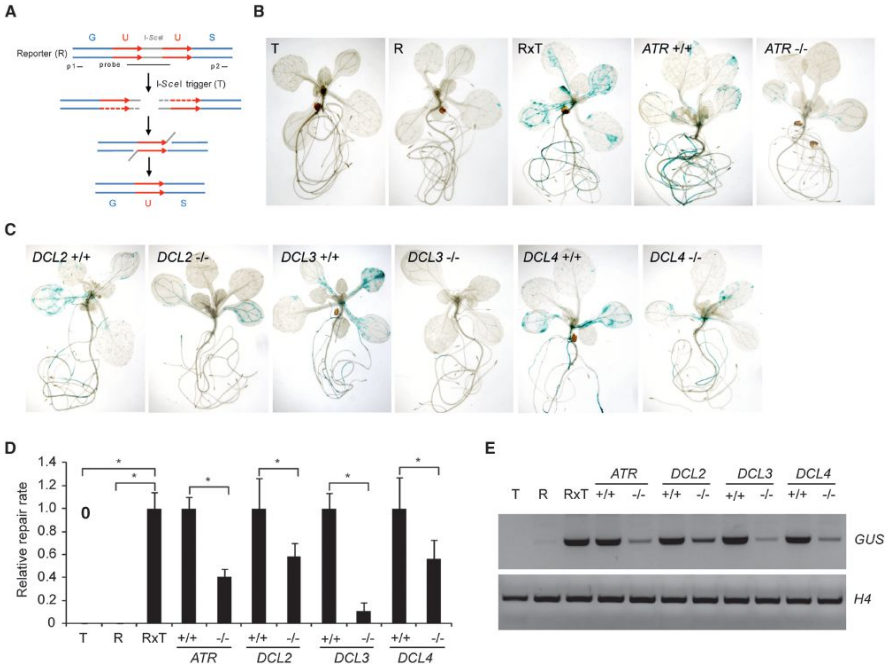

Figure 1. DCLs Are Required for Efficient DSB Repair in *Arabidopsis*

(A) Schematic representation of DSB repair in the DGU.US reporter system. The DGU.US reporter (R) line harbors an I-*Sce*I site located within the direct repeats (U) of a nonfunctional *GUS*. Crossing R with the DSB-triggering (T) line that expresses the I-*Sce*I endonuclease introduces a single DSB in the genome of F1 progenies (RxT). Repair of the DSB restores the functional *GUS*.

(B and C) Representative GUS staining images for DSB repair analysis in the T, R, and RxT plants and RxT plants in the indicated mutant backgrounds (−/−) or their corresponding wild-type (+/+) backgrounds. See Figure S1 for the crossing scheme.

(D) The relative repair rate in the indicated plants determined by GUS staining. For each genetic background, at least 30 plants from three independent experiments were stained and blue sectors were counted. The DSB repair rates in the mutant plants (−/−) are presented in relation to those of the corresponding wild-type (+/+) controls (arbitrarily set to 1.0). Error bars indicate standard error of the mean (SEM), and the asterisks indicate a significant difference between the indicated groups (t test, p value < 0.001).

(E) Detection of repaired DNA in the indicated plants by PCR using primers p1 and p2 depicted in (A), *Histone H4* was also amplified and used as the internal control. See also Figure S1.

have been previously shown for the biogenesis of long miRNAs in rice (Wu et al., 2010).

The generation of diRNAs from both sense and antisense strands at approximately equal frequency (Figure 2B) suggested that they were processed from dsRNA precursors, the production of which usually requires RDRs (Xie et al., 2004). RDR2 is required for the production of hc-siRNAs (Xie et al., 2004), whereas RDR6 plays a role in the biogenesis of *trans*-acting siRNAs (Peragine et al., 2004; Vazquez et al., 2004). We found that mutations in *RDR2* and *RDR6* caused 87% and 82% reductions in diRNA production, respectively (Figure 3A and Table S2). However, these mutations had no significant effects on the repair efficiency (Figures 3B and 3C and Figure S3), which could indicate a redundancy between RDR2 and RDR6 or that other RDRs are involved. Alternatively, these findings could also imply that there might be a threshold of diRNA abundance required for its function in DSB repair and that the levels of diRNAs present in the *rdr2* and *rdr6* mutants are sufficient.

We next examined whether Pol IV and Pol V are required for diRNA biogenesis and DSB repair. Pol IV and Pol V are both involved in the *Arabidopsis* RdDM pathway. Pol IV is required for hc-siRNA biogenesis (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005), whereas Pol V generates nascent scaffold

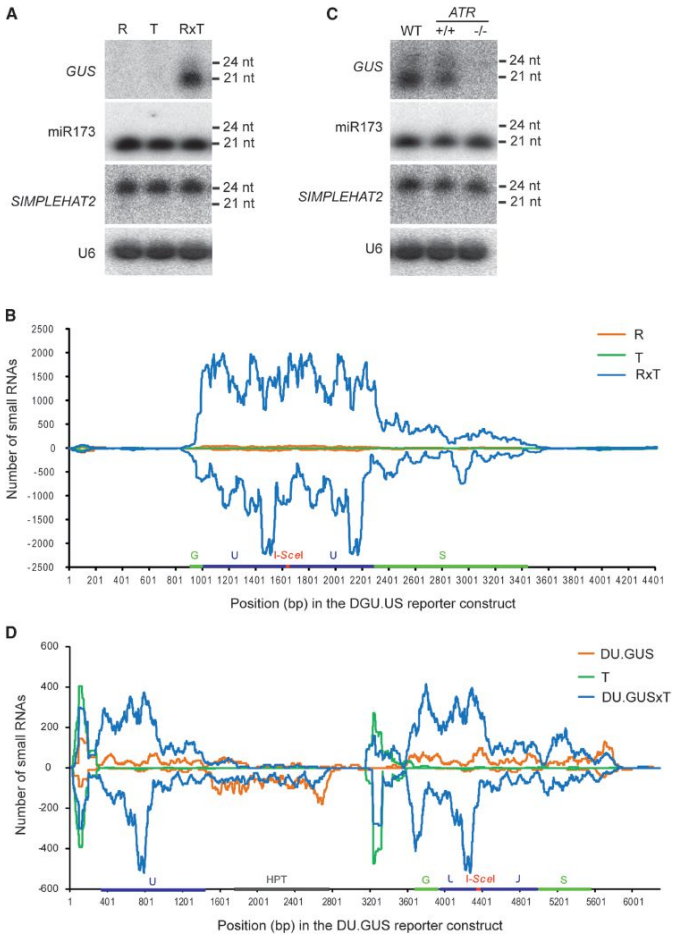

Figure 2. DSB Induces the Production of diRNAs Specifically Around the DSB Site in *Arabidopsis*

(A) Detection of small RNAs in the F1 progenies (RxT), as well as the parental plants (R and T) by northern blot analysis. The probe used for detection of GUS-derived diRNAs is depicted in Figure 1A. miR173, *SIMPLEHAT2* hc-siRNAs, and U6 were probed as controls. nt is an abbreviation for nucleotide.

(B) Deep sequencing analysis of diRNAs generated from DGU.US reporter construct. The y axis represents the number of normalized small RNA reads per 10 million sequences, numbers in (+) and (−) values represent the reads of small RNAs derived from sense and antisense strands, respectively. The nucleotide positions (bp) of the components in the DGU.US construct are shown.

(C) Northern blot detection of diRNAs in RxT in Col-0 background (WT), *atr* mutant (−/−) and its corresponding wild-type (+/+) backgrounds.

(D) Deep sequencing analysis of diRNAs generated from DU.GUS reporter construct. The y axis represents the number of normalized small RNA reads per 10 million sequences, numbers in (+) and (−) values represent the reads of small RNAs derived from sense and antisense strands, respectively. The nucleotide positions (bp) of the components in the DU.GUS construct are shown. See Figure S2 for the diagram of DU.GUS reporter construct.

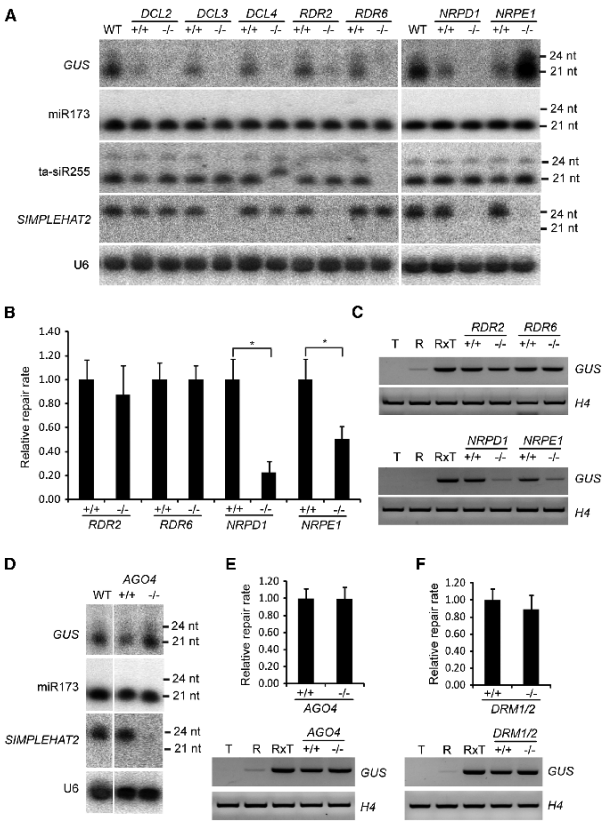

Figure 3. A Role for diRNAs in DSB Repair in *Arabidopsis*

(A) Northern blot analysis of diRNA accumulation in the indicated mutant (−/−) and the corresponding wild-type (+/+) backgrounds. miR173, ta-siR255, and S*IMPLEHAT2* hc-siRNAs were also probed and used for verification of respective mutant backgrounds. U6 was detected and used as a loading control. nt is an abbreviation for nucleotide.

(B) The relative DSB repair rates in the indicated plants determined by GUS staining. For each genetic background, at least 30 plants from three independent experiments were stained and blue sectors were counted. The repair rates in the mutant plants (−/−) are presented in relation to those of the corresponding wild-type (+/+) controls (arbitrarily set to 1.0). Error bars indicate SEM, and asterisks indicate a significant difference between the indicated groups (t test, p value < 0.001).

(C) Detection of repaired DNA in the indicated plants by PCR.

(D) Detection of diRNAs in the *ago4* mutant (−/−) and the wild-type control (+/+) plants.

(E) The repair rates in the *ago4* mutant (−/−) and its corresponding wild-type control (+/+) determined by GUS staining (upper panel) and PCR (lower panel).

(F) The repair rates in the *drm1*/*drm2* double mutant (−/−) and its corresponding wild-type control (+/+) determined by GUS staining (upper panel) and PCR (lower panel). See also Figures S3 and S4.

transcripts upon which RdDM effector complexes are assembled but has no direct role in hc-siRNA biogenesis (Wierzbicki et al., 2008). Intriguingly, diRNA production was greatly compromised in *nrpd1* (*NRPD1* encodes the largest subunit of Pol IV) but increased in *nrpe1* (*NRPE1* encodes the largest subunit of Pol V) (Figure 3A and Table S2). Repair rates were reduced by 80% and 50% in the *nrpd1* and *nrpe1* mutant backgrounds, respectively (Figures 3B and 3C and Figure S3). These data indicate that Pol IV and Pol V are involved in DSB repair through regulating diRNA biogenesis and functioning, respectively.

diRNA-Mediated DSB Repair Does Not Involve the RdDM Pathway

DCL3, Pol IV, and Pol V are all components in the RdDM pathway (Law and Jacobsen, 2010). The dependence of DSB repair on these genes raised a possibility that diRNAs function through RdDM to mediate DSB repair. To test this possibility, we investigated whether AGO4 (the major RdDM effector protein that binds hc-siRNAs) (Qi et al., 2006; Zilberman et al., 2003) and DRM2 (the de novo DNA methyltransferase that catalyzes RdDM) (Cao and Jacobsen, 2002; Matzke et al., 2009) are involved in DSB repair. In the *ago4* mutant, the accumulation of diRNAs was not reduced but instead mildly increased (Figure 3D and Table S2), and DSB repair efficiency was also not affected (Figure 3E). Similarly, mutation in *DRM1/2* did not have an obvious effect on DSB repair (Figure 3F).

The reduced DSB repair efficiency observed in *dcl3*, *nrpd1*, and *nrpe1* could be caused by dysregulation of genes involved in DNA damage response in these mutants. To test this possibility, we used quantitative RT-PCR (qRT-PCR) to measure the expression levels of several genes (*MRE11*, *RAD50*, *NBS1*, *ATM*, *ATR*, *RAD51*, *RPA1*, *BRCA1*, *BRCA2*, *RAD54*, *RECQ4A*, *RAD5A*, and *RPA2b*) that play key roles in DNA damage response. We found that the expression levels of all the examined genes were comparable in wild-type and mutant plants (Figure S4).

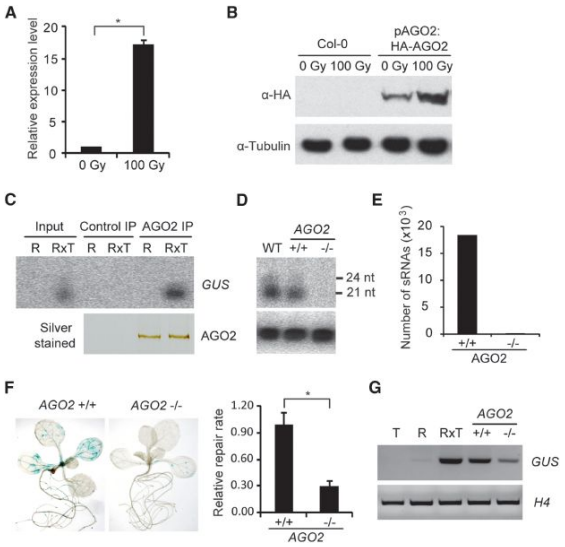

Figure 4. AGO2 Is an Effector Protein of diRNAs

(A and B) AGO2 expression was induced by γ-irradiation as measured at both mRNA and protein levels by qRT-PCR (A) and western blot (B) analyses, respectively. Error bar indicates SEM and the asterisk (*) indicates a significant difference between the indicated samples (t test, $p < 0.001$). In (B) tubulin was detected in parallel and used as a loading control.

(C) Detection of diRNAs in the immunopurified AGO2 complex from the RxT plants by northern blot analysis. The silver-stained gel shows comparable amounts of AGO2 complexes were used for RNA extraction.

(D and E) Northern blot (D) and deep sequencing (E) analyses of diRNAs in the *ago2* mutant (−/−) and the corresponding wild-type (+/+) plants. In (E), reads per 10 million sequences are shown after being normalized with reads of endogenous miRNAs.

(F) The effect of mutation in *AGO2* on the DSB repair rate was determined by GUS staining. Representative images are shown in the left. Relative repair rate was calculated and shown in the right. For each genetic background, at least 30 plants from three independent experiments were assayed. Error bars indicate SEM, and the asterisk indicates a significant difference between the indicated groups (t test, p value < 0.001).

(G) Detection of repaired DNA by PCR in the indicated plants. See also Figure S5.

These results argue against the possibility that diRNAs mediate DSB repair through the RdDM pathway or through the regulation of genes involved in DSB response.

AGO2 Is an Effector Protein of diRNAs

To dissect the mechanism through which diRNAs act in DSB repair, we sought to identify the effector protein that recruits diRNAs. It has been previously reported that the expression of *AGO2* can be induced by γ-irradiation, a potent DSB inducer (Culligan et al., 2006). Consistent with the published results, we found that expression of *AGO2*, but not the expression of other *AGO*s, was highly induced in plants upon γ-irradiation at both mRNA and protein levels (Figures 4A and 4B and Figure S5A). This suggested AGO2 as a potential candidate effector that recruits diRNAs. To test this, we immunopurified AGO2 complexes from the RxT plants as well as the uncrossed R line (Figure 4C) and examined whether they contained diRNAs. Northern blot analysis detected diRNAs in the AGO2 complexes isolated from the RxT plants but not in those from the uncrossed R line (Figure 4C). Confirming the northern blot results, deep sequencing analysis revealed that *GUS*-matching diRNAs accounted for ~3% of the AGO2-bound small RNAs, and there was a 6.2-fold enrichment of diRNAs in AGO2 relative to those in the total extracts (Figures S5B and C). In agreement with the role of AGO2 in recruiting diRNAs, the *ago2* mutant had dramatically reduced accumulation of diRNAs (Figures 4D and 4E) and reduced repair rate (Figures 4F and 4G). These results indicate that AGO2 is an effector protein of diRNAs and plays a role in DSB repair.

We reasoned that some diRNAs might be produced from endogenous loci upon γ-irradiation. We immunoprecipitated AGO2 complexes from plants with or without the treatment of γ-irradiation and analyzed the coimmunoprecipitated small RNAs by deep sequencing. We identified 150 loci that produced two times more small RNAs in the γ-irradiated plants than in the unirradiated plants (Table S3). As γ-irradiation triggers DSB randomly in the chromosomes, we were unable to determine the DSB sites in the γ-irradiated plants. It remains to be confirmed whether these induced small RNAs were DSB associated.

diRNAs Are Not Involved in the Phosphorylation of H2AX

Phosphorylation of histone H2AX, referred to as γ-H2AX, is one of the earliest events in the response to DSBs. γ-H2AX plays a key role in recruiting repair and chromatin remodeling factors at the sites of DNA damage (Fillingham et al., 2006; Paull et al., 2000; Podhorecka et al., 2010) and has emerged as a highly specific and sensitive molecular marker for monitoring DSBs and their repair (Amiard et al., 2010; Kinner et al., 2008). In *Arabidopsis*, phosphorylation of H2AX is dependent on both ATM and ATR in response to DSBs caused by ionizing radiation, and ATM has a dominant role (Friesner et al., 2005).

We examined whether diRNAs are required for the phosphorylation of H2AX at DSB sites induced by γ-irradiation. We performed γ-H2AX immunofluorescence staining with nuclei isolated from *Arabidopsis* leaves. As expected, γ-H2AX foci were not detectable in the nuclei of unirradiated plants. When plants were irradiated with 25 Gy, 100% of the nuclei from

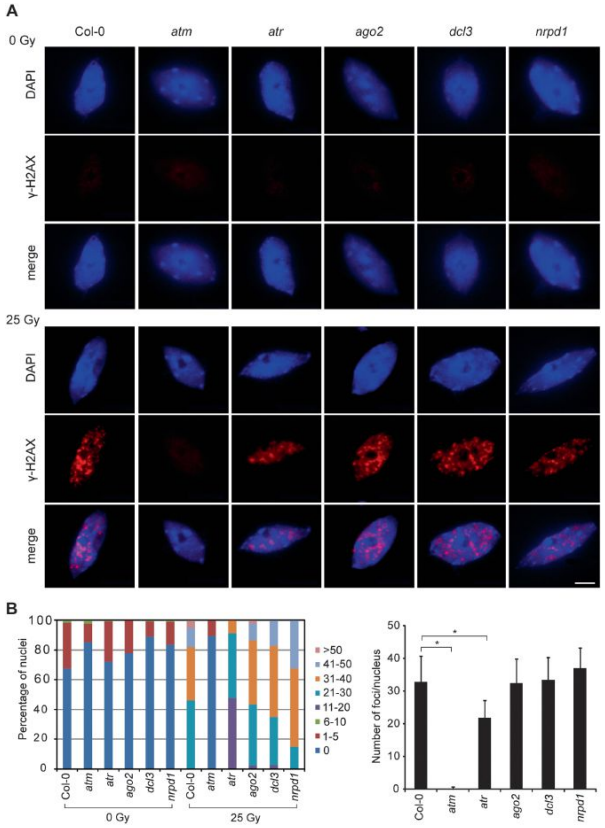

Figure 5. diRNAs Are Not Involved in the Phosphorylation of H2AX

(A) Detection of γ-H2AX by fluorescent immunostaining in the nuclei of wild-type (Col-0) and the indicated mutant plants without (0 Gy, upper panels) or with (25 Gy, lower panels) the treatment of 25 Gy γ-irradiation. DNA was stained with DAPI (blue), and γ-H2AX foci were colored in red. Representative pictures are shown. The scale bar = 5 μm.

(B) Graphic representation of the number of γ-H2AX foci detected in the unirradiated or irradiated wild-type (Col-0) and mutant plants. The numbers of foci in at least 30 nuclei were counted for each sample and used to generate the diagrams. In the right panel, error bars indicate SEM, and the asterisks (*) indicate a significant difference between the indicated groups (t test, p value < 0.001).

See also Figure S6.

wild-type plants showed γ-H2AX foci with a mean of 31 foci per nucleus (Figures 5A and 5B). The proportion of nuclei showing γ-H2AX foci and the number of foci per nucleus were dramatically reduced in the irradiated *atm* mutant plants and slightly but significantly decreased in the irradiated *atr* mutant plants (Figures 5A and 5B). However, the amounts of γ-H2AX foci in irradiated *nrpd1*, *dcl3*, and *ago2* mutant plants were comparable to those in irradiated wild-type plants (Figures 5A and 5B). Immunofluorescence staining with mitotic root tip nuclei showed that mutations in *AGO2* and *DCL3* did not decrease the numbers of γ-H2AX foci in M-phase or interphase nuclei (Figure S6). These data indicate that diRNAs are not involved for the generation of γ-H2AX and diRNAs most likely function downstream of H2AX phosphorylation.

Detection of diRNAs and Their Role in DSB Repair in Human Cells

Our findings point to a key role for diRNAs in the DSB repair pathway in plants. Because multiple aspects of this pathway are highly conserved, we asked whether diRNAs could also be involved in DSB repair in mammalian cells. We employed human U2OS cells carrying a DR-GFP substrate (DR-GFP/U2OS), which contains two nonfunctional *GFP* open reading frames, including one GFP-coding sequence that is interrupted by a recognition site for the I-*Sce*I endonuclease. Expression of I-*Sce*I leads to formation of a DSB in the I-*Sce*I *GFP* allele, which is repaired by HR using a nearby *GFP* lacking N- and C-terminal GFP-coding sequences, thereby producing functional green fluorescent protein (GFP) that can be readily detected by flow cytometry (Pierce et al., 1999). DSBs were induced by the expression of I-*Sce*I in DR-GFP/U2OS cells (Figure S7). Deep sequencing analyses demonstrated that, as in *Arabidopsis*, DSBs in human cells induced diRNA production from sense and antisense strands of the sequence close to the DSB site (Figure 6A). diRNAs appeared to be produced from the vicinity of the DSB but not directly around it as in *Arabidopsis*. To examine the impact of diRNA production on DSB repair in human cells, we investigated the effect of Dicer or Ago2 depletion on DSB repair efficiency. Whereas DR-GFP/U2OS cells treated with control siRNAs displayed efficient repair resulting in robust production of GFP-positive cells after I-*Sce*I expression, a significant reduction in HR of DNA DSB repair was observed after Dicer or Ago2 depletion (Figures 6B and 6C). We tested the protein expression levels of several DSB repair proteins and found that the levels

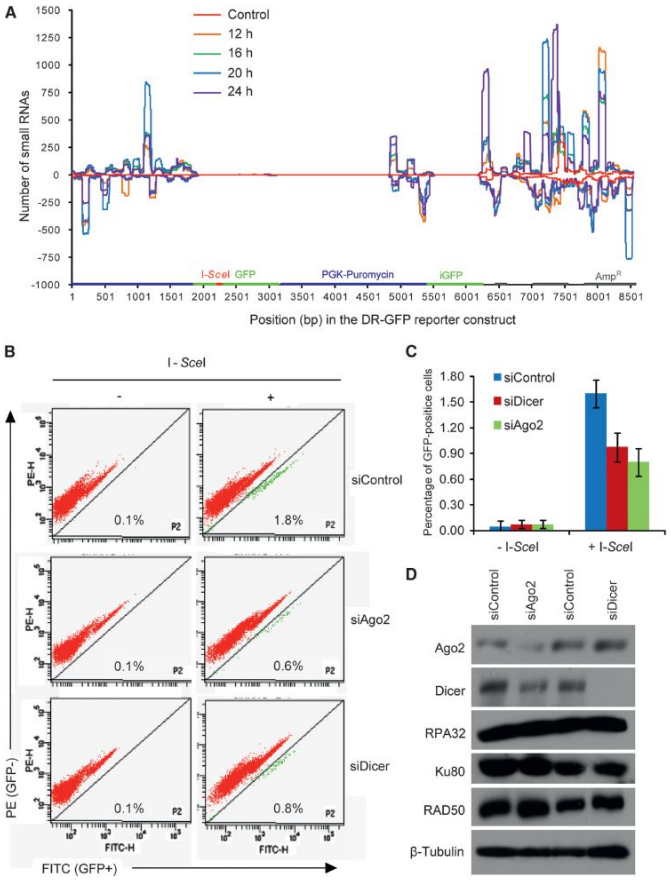

Figure 6. diRNA Production and Regulation of DSB Repair in Human Cells

(A) Deep sequencing analysis of diRNAs generated from the DR-GFP reporter construct. The y axis represents the number of normalized small RNA reads per 10 million sequences, numbers in (+) and (−) values represent the reads of small RNAs derived from sense and antisense strands, respectively. Small RNA reads from RNA isolated from DR-GFP/U2OS cells at 12, 16, 20, or 24 hr following I-*Sce*I transfection or at 20 hr following transfection with control vector (Control) are shown. The nucleotide positions (bp) of the components in the DR-GFP construct are shown.

(B and C) Relative repair rate in DR-GFP/U2OS cells after treatment with the indicated siRNAs. Forty-eight hours after siRNA treatment, DR-GFP/U2OS cells were transfected with I-*Sce*I plasmid for 48 hr and processed for flow cytometric analysis of GFP. The repair efficiency was scored as the percentage of GFP-positive cells in control, Dicer, or Ago2 siRNA-treated cells. Graph represents the mean of three independent experiments. Error bars indicate SEM.

(D) DR-GFP/U2OS cells were treated with Control, Dicer, or Ago2 siRNAs for 48 hr and analyzed by immunoblotting with the indicated antibodies.

See also Figure S7.

were comparable in control and Ago2 or Dicer siRNA-treated cells (Figure 6D). Taken together, the identification of diRNAs in both plants and human cells points to a conserved role for the small RNA pathway in DSB repair.

DISCUSSION

Damaged DNA is repaired through coordinated activation of cell cycle checkpoints and DNA repair machineries, which

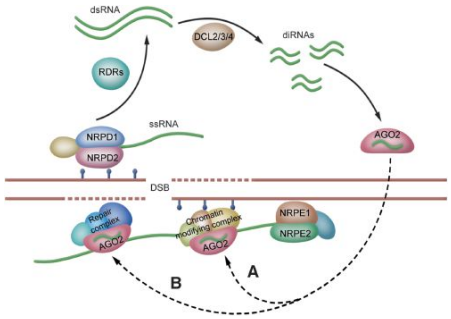

Figure 7. A Model for diRNA-Mediated DSB Repair in *Arabidopsis*

This model is proposed on the basis of genetically identified components required for diRNA-mediated DSB repair and their roles extrapolated from their known functions in the RdDM pathway in *Arabidopsis*. Single-stranded RNA transcripts (ssRNAs) are presumably generated by RNA polymerase IV (NRPD1 and NRPD2) from the sequences in the vicinity of a DSB. Redundant activities of RNA-dependent RNA polymerases (RDRs) convert the ssRNAs into double-stranded RNAs (dsRNAs), which are processed into diRNAs by coordinated actions of Dicer-like proteins (DCL2, DCL3, and DCL4). diRNAs are then incorporated into Argonaute 2 (AGO2). AGO2/diRNA complexes are localized to the DSB site through interaction with scaffold transcripts that are made by Pol V (NRPE1 and NRPE2). AGO2/diRNA complexes may recruit chromatin modifying complexes to modify local chromatin (A) or directly recruit DSB repair proteins to the DSB site (B) to facilitate DSB repair. Further experiments are required to test this model. The gray dots represent chromatin modifications.

involve protein sensors, transducers, and effectors (Ciccia and Elledge, 2010; Huen and Chen, 2008; Polo and Jackson, 2011). In this study, we established an important role for small RNAs in DSB repair, adding an unsuspected RNA component to the DSB repair signaling pathway. Importantly, we demonstrated that this layer of DSB repair regulation is conserved: diRNAs are produced in both plant and human cells and interfering with their production has severe effects on DSB repair. It is also noteworthy that in the filamentous fungus *Neurospora crassa*, QDE-2-interacting small RNAs (qiRNAs) derived from rDNA repeats have been detected in cells treated with DNA damaging agents (Lee et al., 2009). It was proposed that these small RNAs contribute to DNA damage response by inhibiting rRNA biogenesis and protein translation (Lee et al., 2009). In light of our current findings, an alternative interpretation may be considered. rDNAs are arrayed in tandem in the *Neurospora* genome (Galagan et al., 2003), which makes them perfect targets for HR. Upon exposure to DNA damaging agents, DSBs may be introduced within the rDNA repeats. This could then trigger the production of rDNA-specific small RNAs that mediate DSB repair on damaged repetitive rDNAs. Interestingly, in the fly female germline, mutations in the repeat-associated siRNA (rasiRNA, an analog of piRNA in mammals) pathway resulted in elevated γ-H2AX levels (Klattenhoff et al., 2007), raising the possibility that rasiRNAs are involved in DSB repair.

RDRs and Pol IV were found to be involved in diRNA biogenesis in *Arabidopsis* (Figure 3). Based on their roles in making dsRNA precursors of hc-siRNAs in the RdDM pathway (Law and Jacobsen, 2010; Matzke et al., 2009), we speculate that single-stranded DNA (ssDNA) generated by resection of the DSB might serve as the template for Pol IV/RDRs-mediated generation of double-stranded diRNA precursors (Figure 7). In accordance with this hypothesis, it has previously been shown that the *Neurospora* RDR (QDE-1) can produce dsRNA from ssDNA (Lee et al., 2010). We found that no diRNAs could be detected in *atr* mutant plants (Figure 2C). This could suggest that ATR-dependent phosphorylation of components of the DSB repair machinery is required for the recruitment of the diRNA biogenesis machinery to the DSB sites. Alternatively, ATR-dependent phosphorylation of components of the diRNA biogenesis machinery itself could be required for their activity or recruitment to sites of DNA damage.

diRNAs were specifically generated from the regions close to the DSB sites (Figures 2 and 6), implying that diRNAs mediate DSB repair in *cis*. We showed that DSB repair is not compromised in the *ago4* and *drm2* mutants (Figures 3E and 3F), suggesting that diRNAs do not function through changing the DNA methylation at the DSB sites to mediate DSB repair. Accumulating evidence indicates that DSBs trigger a number of histone modifications around the DSB sites and these modifications may facilitate DSB repair (Lukas et al., 2011; Polo and Jackson, 2011). We propose that diRNAs may function as guide molecules for these histone modifications at the DSB site, analogous to hc-siRNAs in the RdDM pathway (Figure 7A). Alternatively, diRNAs may play a more direct role in recruiting DSB repair complexes to DSB sites through their effector protein AGO2 (Figure 7B). The phosphorylation of H2AX around the DSB sites is one of the earliest events in response to DSBs and facilitates local recruitment and retention of DSB repair and chromatin remodeling factors (Fillingham et al., 2006; Paull et al., 2000; Podhorecka et al., 2010). We found no evidence of compromised phosphorylation of H2AX in the diRNA-deficient mutants (Figure 5 and Figure S6), demonstrating that the very early step in DSB recognition is intact and that the diRNAs most likely affect events downstream of H2AX phosphorylation.

In summary, we have demonstrated that small RNAs generated from the sequences flanking a DSB are important for efficient DSB repair. It will be very exciting to have future studies dissecting the molecular and biochemical underpinnings of diRNAs in DSB repair.

EXPERIMENTAL PROCEDURES

Plant Materials, Human Cells, and Growth Conditions

Arabidopsis mutants and lines used in this study have been previously described. See Table S4 for references and detailed information about these mutants. All plants were grown in soil or Murashige and Skoog (MS) medium at 16 hr light/8 hr dark photoperiod.

Human DR-GFP/U2OS cells (U2OS-derivative cell line harboring an integrated HR reporter construct [DR-GFP]) (Pierce et al., 1999) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

DSB Repair Reporter Assay

DGU.US-1 line (DSB reporter line) and 2X35S:I-*Sce*I-8 line (DSB-triggering line) were used for assaying DSB repair efficiency in *Arabidopsis* (Mannuss et al., 2010; Orel et al., 2003). To compare the DSB repair efficiency in a mutant