THE MAINTENANCE OF GENOME INTEGRITY IN PLANTS: NOVEL CHALLENGES IN BASIC AND APPLIED RESEARCH

EDITED BY: Alma Balestrazzi, Ayako N. Sakamoto and Kaoru Okamoto Yoshiyama
PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-819-2
DOI 10.3389/978-2-88963-819-2

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THE MAINTENANCE OF GENOME INTEGRITY IN PLANTS: NOVEL CHALLENGES IN BASIC AND APPLIED RESEARCH

Topic Editors:
Alma Balestrazzi, University of Pavia, Italy
Ayako N. Sakamoto, National Institutes for Quantum and Radiological Science and Technology (Japan), Japan
Kaoru Okamoto Yoshiyama, Tohoku University, Japan

Citation: Balestrazzi, A., Sakamoto, A. N., Yoshiyama, K. O., eds. (2020). The Maintenance of Genome Integrity in Plants: Novel Challenges in Basic and Applied Research. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-819-2

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frontiers in Plant Science

EDITORIAL
published: 15 May 2020
doi: 10.3389/fpls.2020.00585

Editorial: The Maintenance of Genome Integrity in Plants: Novel Challenges in Basic and Applied Research

Alma Balestrazzi[1]*, *Kaoru O. Yoshiyama*[2] *and Ayako N. Sakamoto*[3]

[1] Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy, [2] Life Sciences, Tohoku University Aoba-ku, Sendai, Japan, [3] Department of Radiation-Applied Biology Research, National Institutes for Quantum and Radiological Science and Technology, Takasaki, Japan

Keywords: genome integrity, DNA repair, DNA damage, UV, double strand breaks, cell cycle

Editorial on the Research Topic

The Maintenance of Genome Integrity in Plants: Novel Challenges in Basic and Applied Research

Plants are sessile organisms endowed with astonishing genomic plasticity. A plethora of undesired events challenge DNA integrity and a deeper knowledge of the mechanisms underlying DNA repair and maintenance of genome stability will help improving our understanding of how plants cope with hostile environments in this era of global climate change. This Research Topic provides a comprehensive overview of the current progress on the study of the genotoxic stress response in plants at the cellular and molecular level.

Roldán-Arjona et al. provide an exhaustive review of the recent advances in the study of base excision repair (BER). Plants share several BER factors with other organisms, although possessing unique features elucidated by biochemical and genetic studies. The review underlines the gap of knowledge regarding the identification of DNA polymerases involved in gap filling, the interplay between BER factors and chromatin remodeling mechanisms, and the BER pathways within mitochondria/chloroplasts. Similarly, Sakamoto et al. reports on the state of the art of translesion synthesis (TLS), one of the pathways used to overcome stalled replication. TLS polymerases are generally low-fidelity enzymes, prone to induce mutations. The plant DNA polymerase ζ, η, κ, θ, and λ and Reversionless1 (Rev1), involved in the TLS, have been studied at the genetic level using mutants which display enhanced sensitivity to DNA-damaging agents. There is evidence that TLS polymerases act in parallel with the Rad5-dependent pathway involved in the repair of the stalled replication fork. Current studies point at plants as an ideal model for assessing the role, regulation, and interaction of TLS polymerases since, differently from animals, these functions can be disrupted in plants without severe reduction of fertility.

The contribution of NER (nucleotide excision repair) to the removal of UV-induced DNA lesions has been clarified by Al Khateeb et al.. They showed in *Arabidopsis thaliana* that loss of function mutants of *AtUVSSA* (*UV Stimulated Scaffold protein A*), *AtUSP7* (*Ubiquitin Specific Peptidase 7*), and *AtTFIIS* (*RDO2, Reduced Dormancy 2*) genes exhibit increased UV sensitivity. This finding highlights the conserved role of such NER components in the DNA damage response (DDR) triggered by UV radiation.

The increasing number of studies dealing with DNA damage accumulated in the embryo genome and the repair capacity of the seed has been extensively reviewed by Waterworth et al.. These authors underline how DDR factors participating in genome maintenance represent promising targets for the genetic improvement of crop germination performance in the field, in particular under stress conditions.

OPEN ACCESS

Edited by:
Marc Libault, University of Nebraska-Lincoln, United States

Reviewed by:
Christopher Edward West, University of Leeds, United Kingdom

***Correspondence:**
Alma Balestrazzi
alma.balestrazzi@unipv.it

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *05 March 2020*
Accepted: *17 April 2020*
Published: *15 May 2020*

Citation:
Balestrazzi A, Yoshiyama KO and Sakamoto AN (2020) Editorial: The Maintenance of Genome Integrity in Plants: Novel Challenges in Basic and Applied Research. Front. Plant Sci. 11:585. doi: 10.3389/fpls.2020.00585

Meiotic DNA recombination requires crossing-over and chromosome segregation. Crossing-over begins with double-strand breaks (DSBs) induction. Disrupted Meiotic cDNA1 (DMC1) is a conserved recombinase that searches for and invades homologous sequences to aid in the repair of meiotic DSBs. Szurman-Zubrzycka, Baran et al. identified a DMC1 homolog in barley and isolated two independent *dmc1* mutant lines. Analysis of the *dmc1* plants revealed that chromosome bridges, chromosome fragments, micronuclei, and abnormal tetrads were more common during meiosis, compared with the parental variety. Thus, DMC1 is required for DSB repair, crossing-over, and proper chromosome disjunction.

RAD54, a chromatin remodeling factor, forms DNA repair foci in nuclei after DNA damaging treatments. Hirakawa and Matsunaga monitored the RAD54 foci in γ-irradiated *A. thaliana* root cells. The foci were detected in the epidermis, cortex, endodermis, and stem cells in the meristematic zone but not in the quiescent center. The foci were more frequent in the non-S-phase cells. More than half of the foci were attached to the nuclear envelope (NE) in the wild type but the number decreased under INM (Inner Nuclear Membrane)-protein-deficient background, suggesting that the NE plays a role in genome stability.

Plants acquire tolerance to chilling following exposure to low, non-freezing temperatures (cold acclimation). Wang et al. investigated the expression of DNA damage-inducible protein 1 (CsDDI1) and other DDR genes in cold-acclimated cucumber. Inhibition of H_2O_2 biosynthesis down-regulated *CsDDI1* gene transcription, suggesting H_2O_2 plays a crucial role in triggering cold adaption. CsDDI1 over-expression in *A.thaliana* provides increased tolerance to chilling, lower level of reactive oxygen species, higher catalase and superoxide dismutase activities, and expressions of defense genes. This suggests that the *CsDDI1* gene increases chilling tolerance in plants by enhancing the antioxidant defense system.

The model bryophyte *Physcomitrella patens* exhibits particularly high frequencies of gene targeting, making it an interesting model to study the mechanism of gene targeting. Guyon-Debast et al. reported on the role of the XPF/ERCC1 complex in *P. patents*. Knockout *xpf* and *ercc1* mutants grow under normal condition, however they show high UV-B and MMS (methyl methanesulfonate) sensitivity, indicating that the XPF/ERCC1 complex is involved in the repair of UV- and MMS-induced DNA damage. Using different constructs, they suggest that the complex is required for the homologous recombination between end-out or end-in construct and genome loci. These findings provide clues to improve gene targeting efficiency in other plants.

Aluminum (Al) toxicity is a worldwide problem limiting crop productivity in acidic soils. In *A. thaliana*, Al causes DSBs in roots and ATR, a key factor in DDR, regulates root growth inhibition induced by Al. Szurman-Zubrzycka, Nawrot et al. demonstrated the role of ATR in response to Al toxicity in barley, the most Al-sensitive species among the cereals. They developed barley *atr* mutants, tolerant to Al, in which cell cycle progression was not arrested despite DNA damage accumulation. This knowledge would be useful for growing barley in Al-containing soil.

Nisa et al. reviewed the recent advances in DDR signaling in plants, focusing on the mechanisms leading to cell cycle arrest. They showed that the DDR-triggered cell cycle arrest is induced by SOG1-dependent and SOG1-independent pathways. The former induces cell cycle arrest through several mechanisms, including degradation of the mitotic Cyclin Dependent Kinase B2;1 (CDKB2;1), induction of the CDK inhibitors, and activation of MYB3R repressors. The latter may involve the E2F-RBR1 (RetinoBlastoma Related 1) complexes that function as SOG1 antagonist. They also reviewed recent findings on the relationship between DDR and biotic/abiotic stress responses. Accumulating evidence indicates that DDR is activated in response to pathogen infection or salicylic acid treatment as well as in Al-mediated growth inhibition and chilling stress.

AUTHOR CONTRIBUTIONS

AB commented the following articles: Roldán-Arjona et al., Sakamoto et al., Al Khateeb et al., and Waterworth et al.. AS commented the following articles: Szurman-Zubrzycka, Baran et al., Hirakawa and Matsunaga, and Wang et al.. KY commented the following articles: Guyon-Debast et al., Szurman-Zubrzycka, Nawrot et al., and Nisa et al.. All authors and read and revised the complete editorial.

FUNDING

This work was supported by research fellowship from the Japan Society for the Promotion of Science (17K07455 to KY, and 17K00561 and 19K12332 to AS) and by Sumitomo foundation (Grant No. 181147 to KY). This work was also supported by the Italian Ministry of Education, University and Research (MIUR): *Dipartimenti di Eccellenza* Program (2018–2022) - Dept. of Biology and Biotechnology "L. Spallanzani", University of Pavia (to AB).

ACKNOWLEDGMENTS

AS thanks Enago (www.enago.jp) for the English language review.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 April 2019
doi: 10.3389/fpls.2019.00516

UVSSA, UBP12, and RDO2/TFIIS Contribute to Arabidopsis UV Tolerance

*Wesam M. Al Khateeb[1], Annan A. Sher[2], Jeffery M. Marcus[2] and Dana F. Schroeder[2]**

[1] Department of Biological Sciences, Yarmouk University, Irbid, Jordan, [2] Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

Plant DNA is damaged by exposure to solar radiation, which includes ultraviolet (UV) rays. UV damaged DNA is repaired either by photolyases, using visible light energy, or by nucleotide excision repair (NER), also known as dark repair. NER consists of two subpathways: global genomic repair (GGR), which repairs untranscribed DNA throughout the genome, and transcription-coupled repair (TCR), which repairs transcribed DNA. In mammals, CSA, CSB, UVSSA, USP7, and TFIIS have been implicated in TCR. Arabidopsis homologs of CSA (AtCSA-1/2) and CSB (CHR8) have previously been shown to contribute to UV tolerance. Here we examine the role of Arabidopsis homologs of UVSSA, USP7 (UBP12/13), and TFIIS (RDO2) in UV tolerance. We find that loss of function alleles of *UVSSA, UBP12,* and *RDO2* exhibit increased UV sensitivity in both seedlings and adults. UV sensitivity in *atcsa-1, uvssa,* and *ubp12* mutants is specific to dark conditions, consistent with a role in NER. Interestingly, *chr8* mutants exhibit UV sensitivity in both light and dark conditions, suggesting that the Arabidopsis CSB homolog may play a role in both NER and light repair. Overall our results indicate a conserved role for UVSSA, USP7 (UBP12), and TFIIS (RDO2) in TCR.

Keywords: Arabidopsis, transcription coupled repair, UV, CSA, CSB, UVSSA, UBP7, TFIIS

OPEN ACCESS

Edited by:
Alma Balestrazzi,
University of Pavia, Italy

Reviewed by:
Michael G. Kemp,
Wright State University, United States
Alfred Batschauer,
University of Marburg, Germany

***Correspondence:**
Dana F. Schroeder
Dana.Schroeder@umanitoba.ca

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *26 February 2019*
Accepted: *03 April 2019*
Published: *24 April 2019*

Citation:
Al Khateeb WM, Sher AA, Marcus JM and Schroeder DF (2019) UVSSA, UBP12, and RDO2/TFIIS Contribute to Arabidopsis UV Tolerance. Front. Plant Sci. 10:516. doi: 10.3389/fpls.2019.00516

INTRODUCTION

Unable to move, plants must adapt to their surroundings. An important and unavoidable component of a plant's environment is solar radiation, which includes both beneficial visible light and damaging ultraviolet (UV) rays. UV radiation harms a variety of cellular components including DNA. UV damaged DNA, primarily pyrimidine photodimers, is repaired by photolyases, using the energy from visible light (light repair), and by nucleotide excision repair (NER) (dark repair) (Pang and Hays, 1991; Molinier, 2017).

Nucleotide excision repair is a conserved multistep pathway involving damage recognition, strand unwinding, excision, repair synthesis, and ligation. Damage recognition is via one of two NER sub-pathways. Global genomic repair (GGR) identifies UV damage in DNA throughout the genome, while transcription coupled repair (TCR) initiates repair of transcribed strands.

Abbreviations: CHR8, chromatin remodeling 8; CSA/B, Cockayne syndrome A/B; GGR, global genomic repair; NER, nucleotide excision repair; RDO2, reduced dormancy 2; RNAP, RNA polymerase II; TCR, transcription coupled repair; TFIIS, transcription elongation factor IIS; UBP and USP, ubiquitin specific protease; UV, ultraviolet irradiation; UVSSA, UV stimulated scaffold protein A.

TCR has been well studied in humans, where deficiencies in this process can result in Cockayne Syndrome and UV-sensitive syndrome (Gregersen and Svejstrup, 2018). UV damaged DNA arrests progression of RNA polymerase II (RNAP), resulting in stabilization of RNAP – Cockayne Syndrome B (CSB) interaction. CSB then recruits the Cockayne Syndrome A (CSA)-DDB1-cullin 4 complex, which ubiquitinates CSB, followed by UV Stimulated Scaffold protein A (UVSSA) and Ubiquitin Specific Peptidase 7 (USP7), which stabilize CSB. Subsequently, core NER components, such as TFIIH and the XPG and XPF endonucleases, are recruited, and resulting in damage excision and repair. Re-initiation of transcription following repair is thought to involve the TFIIS elongation factor (Geijer and Marteijn, 2018).

In plants, UV damage in transcribed strands is preferentially repaired, and this process is regulated by the circadian clock (Fidantsef and Britt, 2012; Oztas et al., 2018). The Arabidopsis homologs of CSA, CSB, USP7, and TFIIS have previously been identified and described. *Arabidopsis thaliana* has two CSA homologs, AtCSA-1/ CSAat1A (At1g27840) and AtCSA-2/ CSAat1B (At1g19750) (Kunz et al., 2005). Despite the fact that these two proteins are 92% identical, they are both required for tolerance to UV and MMS and repair of transcribed strands. The CSA homologs interact with DDB1A, localize to the nucleus, and form heterotetramers (Biedermann and Hellmann, 2010; Zhang et al., 2010). The Arabidopsis CSB homolog is SWI2/SNF2 protein Chromatin Remodeling 8 (CHR8, At2g18760) (Kunz et al., 2005; Singh et al., 2010). *CHR8* RNAi lines result in UV sensitivity, but do not exhibit ionizing radiation or intrachromosomal recombination rate phenotypes, consistent with a role in NER (Shaked et al., 2006). UBP12 (At5g06600) and UBP13 (At3g11910) are the Arabidopsis USP7 homologs and have been implicated in plant immunity, flowering, seed, and root development, as well as jasmonate signaling (Ewan et al., 2011; Cui et al., 2013; Derkacheva et al., 2016; Jeong et al., 2017; An et al., 2018). The Arabidopsis TFIIS homolog is Reduced Dormancy 2 (RDO2, At2g38560), which is required for regulation of seed dormancy by *Delay of Germination 1 (DOG1)* (Léon-Kloosterziel et al., 1996; Grasser et al., 2009; Liu et al., 2011; Mortensen and Grasser, 2014). RDO2/TFIIS has also been implicated in mRNA processing in plants, including in response to light (Dolata et al., 2015; Antosz et al., 2017; Godoy Herz et al., 2019). In this study we identify the Arabidopsis UVSSA homolog and examine the roles of UVSSA, UBP12/13, and RDO2 in UV tolerance.

MATERIALS AND METHODS

Phylogenic Tree Construction

Gymnosperm UVSSA homologs were accessed via the PLAZA gymnosperm site[1] (Proost et al., 2015) while all other homologs were identified via KEGG (Kyoto Encyclopedia of Genes and Genomes[2]). UVSSA amino acid sequences were aligned in CLUSTAL Omega (Sievers et al., 2011) using the default settings and saved in NEXUS format for phylogenetic analysis. The aligned amino acid sequences were then analyzed by maximum parsimony as implemented in PAUP* version 4.0b8/4.0d78 using the default settings unless otherwise specified (Swofford, 2002). One million maximum parsimony heuristic search replicates were performed with random sequence addition, tree bisection and reconnection branch swapping on only the best trees, multiple trees saved at each step, and retention of all best trees. In addition, 1 million random sequence addition fast addition bootstrap search replicates were performed with retention of all groups consistent with 50% bootstrap consensus.

Plant Material and Growth Conditions

The following T-DNA alleles were used in this study: SALK_030558 (*AtCSA-1*) (Lee et al., 2010), SALK_000799 and SAIL_273_G11 (*CHR8*), SAIL_58_C12 and SALK_061538 (*UVSSA*), GABI_742C10 (*UBP12*) (Cui et al., 2013), and SALK_027259 (*RDO2*) (Grasser et al., 2009; Liu et al., 2011). Col-0 was used as the wild type control for the SALK and GABI lines (Alonso et al., 2003; Kleinboelting et al., 2012), while Col-3 was used as the control for the SAIL lines (Sessions et al., 2002). All plant material was obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, United States) or the Nottingham Arabidopsis Stock Centre (NASC) (Nottingham, Loughborough, United Kingdom). Alleles were genotyped with the primers listed in **Supplementary Table S1** along with T-DNA specific primers LBb1.3: ATTTTGCCGATTTCGGAAC (SALK lines), LB3SAIL: TAGCATCTGAATTTCATAACCAATCTCGATACAC (SAIL lines), and GK_8409: ATATTGACCATCATACTCATTGC (GABI line). For plant growth, seeds were sterilized and plated on Linsmaier and Skoog (LS) media (Caisson, Smithfield, UT, United States) with 0.6% sucrose and 0.8% Phytoblend (Caisson). After 2–3 days of stratification at 4°C, plates were moved to an incubator with fluorescent bulbs (100 μM photons m^{-2} s^{-1}) and grown under long day conditions (16 h light/8 h dark) at 20°C and 50% relative humidity. For adult growth, 14 day old plants were transplanted into soil (Sunshine mix no. 1, Sun Gro, Bellevue, WA, United States) and grown under the same conditions.

RNA Extraction and RT-PCR

Ribonucleic acid was extracted from approximately fifty 7-day-old seedlings per genotype with the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions including a DNase treatment. RNA was quantified with a Nanodrop spectrophotometer (Thermo Scientific) and 1 μg used to synthesize cDNA, using the Maxima First Strand cDNA synthesis kit (Fermentas, Waltham, MA, United States). For semi-quantitative RT-PCR, *CHR8, UVSSA, AtCSA-1,* and *UBP12* were amplified for 30 cycles and *RDO2* for 26 cycles using the primers indicated (**Supplementary Table S1**) and the *Actin* loading control amplified for 22 cycles. For quantitative real time PCR, cDNA was diluted 40 fold and PCR performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, United States), a CFX Connect Real time PCR detection system

[1] https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/
[2] http://www.kegg.jp/

(Bio-Rad), and the primers listed in **Supplementary Table S1**. *EF1α* (At5g60390) (Jain et al., 2006; Hossain et al., 2012) was used to normalize sample loading and three technical replicates were analyzed per sample.

Adult Growth Analysis

The following data was collected from plants transplanted to soil: flowering time (day the first bud is detected), rosette diameter at 4 weeks, number of shoots and silique length at 6 weeks.

UV Sensitivity Assays

Seeds were plated, stratified, and grown vertically in the conditions above for 3 days, then seedlings irradiated with 1000 J m^{-2} UV-C (corresponding to 65 s exposure to shortwave UV lamp XX-15S, UVP/LLC, Upland, CA, United States). Plates were rotated 90° and incubated in either long day or dark conditions for the indicated number of days, then scanned. Image J was used to measure root and hypocotyl length.

For adult UV assays, 21 day old plants in soil were irradiated with 500 J m^{-2} UV-C, incubated in the dark for 3 days, then returned to long day conditions. Three days later, individual leaves were scored as either undamaged (green) or damaged (yellow or brown), and % damaged leaves (number of damaged leaves/total leaves) was calculated for all plants.

Statistical Analysis

All experiments were performed at least twice and representative experiments shown. Two-tailed student's *t*-tests ($p \leq 0.05$) were used to assess statistical significance.

RESULTS

In this study we identify the Arabidopsis UVSSA homolog. Arabidopsis UVSSA (encoded by At3g61800) is 39% and 28% identical to rice and human UVSSA, respectively. Clear UVSSA homologs are found throughout the animal and plant kingdoms including angiosperms, gymnosperms, ferns, and moss. One million maximum parsimony phylogenetic search replicates for UVSSA homolog amino acid sequences recovered a single most parsimonious tree (score 2561) (**Figure 1**) that is topologically congruent with well supported hypotheses of plant evolutionary history (Morris et al., 2018). Conserved domains in UVSSA proteins include ENTH/VHS in the N terminus and DUF2043 in the C terminus (**Figure 2**). ENTH/VHS domains are multi-helical with an alpha-alpha 2-layered structural fold, while DUF2043 is an approximately 100 amino acid long UVSSA-specific domain, which includes three conserved cysteines and a CP(y/l)HG motif (Marchler-Bauer et al., 2017). AtUVSSA has a potential bipartite NLS in the C terminus and SUBAcon predicts nuclear localization (score 0.994) (De Castro et al., 2006; Hooper et al., 2014), consistent with a role in DNA repair.

Public gene expression data was examined for Arabidopsis *UVSSA* and the other TCR gene homologs: *AtCSA-1*, *CHR8* (*CSB* homolog), *UBP12* and *UBP13* (*USP7* homologs), and *RDO2* (*TFIIS* homolog). With respect to absolute levels of expression (**Supplementary Figure S1A**), *UBP12, UBP13,* and *RDO2* are expressed throughout the plant, consistent with the broad role of these genes in development (Grasser et al., 2009; Cui et al., 2013; Derkacheva et al., 2016), while *AtCSA-1, CHR8,* and *UVSSA* are expressed at lower levels (Schmid et al., 2005). With respect to relative levels of expression (**Supplementary Figure S1B**), *CHR8* and *UVSSA* are enriched in mature pollen, while *RDO2, AtCSA-1, CHR8,* and *UVSSA* are up-regulated more than two-fold in dry seed, perhaps contributing to maintenance of seed genome integrity (Waterworth et al., 2015).

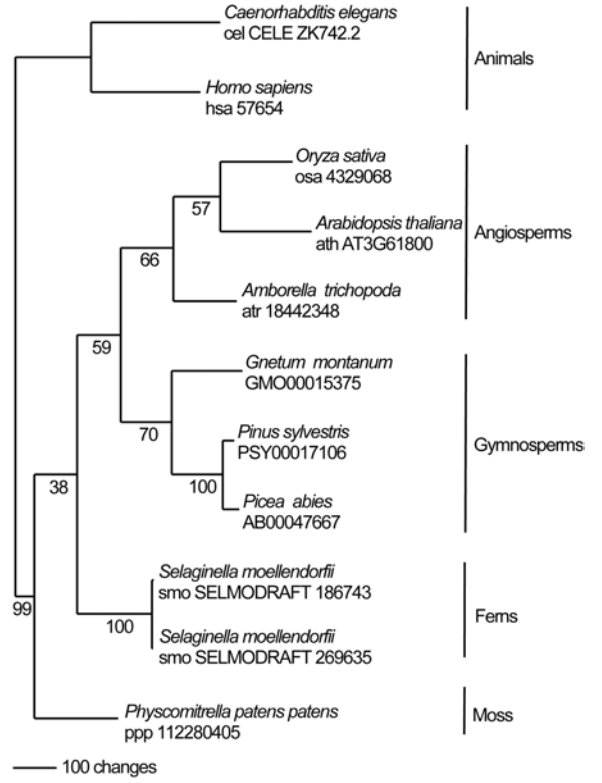

FIGURE 1 | The single most parsimonious phylogenic tree (score 2561) based on analysis of amino acid sequences of UVSSA homologs. Maximum parsimony bootstrap support is indicated under each node. Gymnosperm UVSSA homologs are from the PLAZA gymnosperm site (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/) (Proost et al., 2015) while all other homologs were accessed via KEGG (Kyoto Encyclopedia of Genes and Genomes) (http://www.kegg.jp/).

Public expression data was also examined to determine the effect of potentially mutagenic stress on expression of these genes. *CHR8* was found to be upregulated by genotoxic stress induced by bleomycin and mitomycin C treatment, consistent with previous reports (Molinier et al., 2005), in both the shoot and root, but the other genes were not, while UV-B treatment did not result in major changes to the levels of any of the genes (**Supplementary Figure S2**; Kilian et al., 2007).

In order to examine the role of these genes in Arabidopsis UV tolerance, T-DNA insertion mutants were obtained. Previously described alleles of *AtCSA-1* (SALK_030558) (Lee et al., 2010) and *RDO2* (SALK_027259) (Grasser et al., 2009; Liu et al., 2011) were utilized. *UBP12* allele GABI_742C10 (*ubp12-2*) has previously

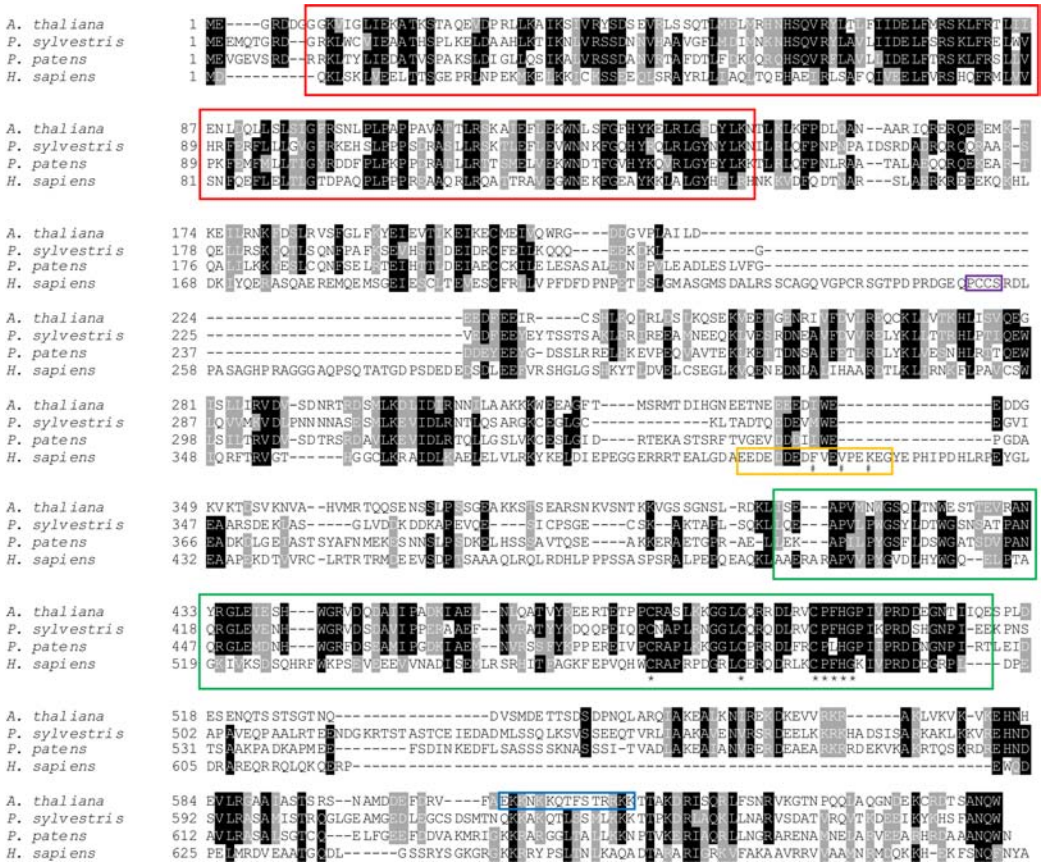

FIGURE 2 | Amino acid alignment of UVSSA from representative angiosperm (*Arabidopsis thaliana*), gymnosperm (*Pinus sylvestris*), moss (*Physcomitrella patens patens*), and animal (*Homo sapiens*) species. Sequences were aligned using NCBI COBALT (Papadopoulos and Agarwala, 2007) and formatted using Boxshade. Amino acids showing identity (black) and similarity (gray) are indicated. Conserved ENTH/VHS (red) and DUF2043 (green) domains are boxed, with asterisks indicating conserved cysteines and CP(y/l)HG motif in the DUF2043 domain (Marchler-Bauer et al., 2017). The blue box indicates potential bipartite NLS in Arabidopsis UVSSA identified using Prosite (De Castro et al., 2006). Regions required for interaction of human UVSSA with USP7 (purple) and TFIIH (yellow) are shown and key residues indicated with #s (Higa et al., 2016, 2018; Okuda et al., 2017).

been shown to result in reduced levels of both *UBP12* and *UBP13*, thus acts as a weak double mutant (Cui et al., 2013). In previous studies RNAi lines of *CHR8* were shown to exhibit UV sensitivity (Shaked et al., 2006). Here we examine two T-DNA alleles of *CHR8*, *chr8-1* (SALK_000799) and *chr8-2* (SAIL_273_G11) (**Figure 3A**). For *UVSSA*, two T-DNA alleles were examined, *uvssa-1* (SAIL_58_C12), located 38 bp upstream of the start codon, and *uvssa-2* (SALK_061538), located in the first intron past the start codon.

We examined the effect of these alleles on gene expression using semi-quantitative RT-PCR. Primers flanking the *chr8-1* and *chr8-2* insertion sites detected no *CHR8* transcript, indicating these are null alleles (**Figure 3B**). Semi-quantitative RT-PCR with T-DNA insertion flanking primers also confirmed loss of transcript in the *atcsa-1, ubp12,* and *rdo2* lines (**Supplementary Figure S3**). For *UVSSA*, we utilized primers in the first and second coding exons, since the effect of T-DNA insertion on coding sequences was our primary concern. *uvssa-2* results in a null allele, but in *uvssa-1* both the predicted band and a larger band were detected (**Figure 3C**). The size of the larger band was consistent with that of the unspliced transcript, so we hypothesized that *uvssa-1* insertion affected intron splicing [note the *uvssa-1* samples did not result in larger gDNA-size bands of *CHR8*, thus were not gDNA contaminated (data not shown)]. Real-time qPCR with an intron-specific primer was used to quantify the effect of the *uvssa-1* allele on splicing, and large amounts of the unspliced product were detected (**Figure 3D**). Due to the presence of an in frame stop codon in the intron, this transcript results in a truncated 77 amino acid product. *uvssa-1* also resulted in increased levels of correctly spliced *UVSSA*. Thus *uvssa-1* would be predicted to result in increased levels of both full length and truncated UVSSA.

Mutant alleles of the TCR genes were grown in long day conditions with their respective controls and their developmental phenotypes examined. *ubp12-2* mutants exhibited decreased rosette size, early flowering (days), and decreased apical dominance (increased number of shoots) (**Supplementary Figure S4**), consistent with previously described phenotypes (Cui et al., 2013; Derkacheva et al., 2016). The other mutant alleles did not exhibit any developmental

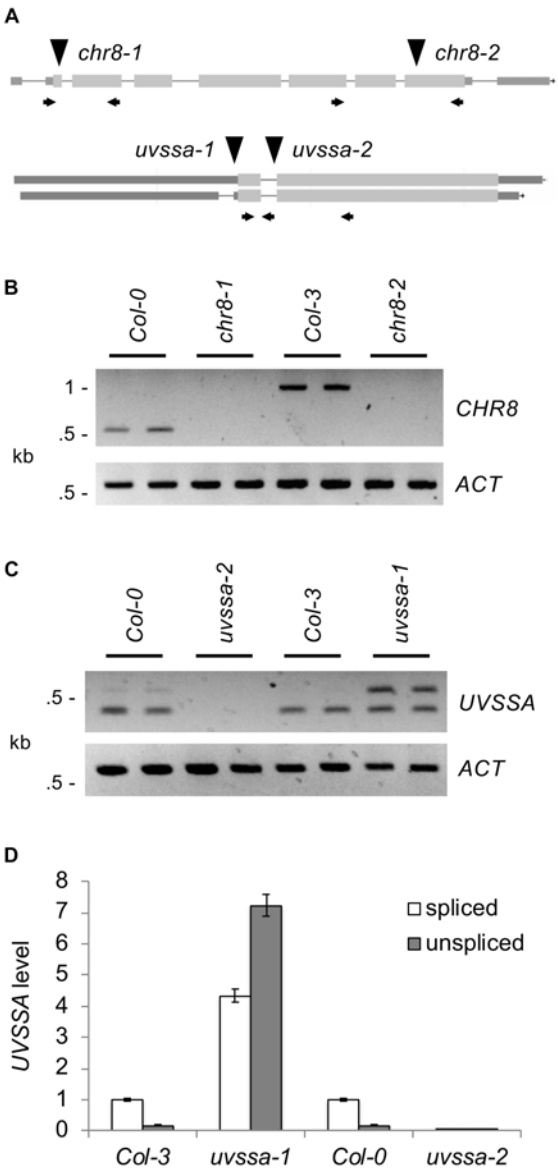

FIGURE 3 | Analysis of *CHR8* and *UVSSA* alleles. **(A)** Map of *CHR8* (*At2g18760*) and *UVSSA (At3g61800)* genes. Lines indicate introns and boxes exons, with coding regions shaded light gray and untranslated regions dark gray. For *CHR8*, transcript *At2g18760.4* is shown because it is the best match to light grown seedling RNA-Seq data in JBrowse (Buels et al., 2016). For *UVSSA*, both known transcripts, *At3g61800.1* (above) and *At3g61800.2* (below), are shown. The location of T-DNA alleles *chr8-1* (SALK_000799), *chr8-2* (SAIL_273_G11), *uvssa-1* (SAIL_58_C12), and *uvssa-2* (SALK_061538) are indicated with triangles. Arrows indicate primers used in RNA analysis. **(B)** Semi-quantitative RT-PCR analysis of *CHR8* and *ACTIN* expression in *chr8-1* and *chr8-2* and their respective wild-type backgrounds, using *CHR8* primers flanking each allele. **(C)** Semi-quantitative RT-PCR analysis of *UVSSA* and *ACTIN* expression in *uvssa-1* and *uvssa-2* and their respective wild-type backgrounds, using *UVSSA* primers in the first and second coding exons. For **(B,C)**, two technical replicates per sample are shown. **(D)** qPCR analysis of spliced (primers in first coding exon and spanning exon 1–2 junction) and unspliced (primers in first coding exon and following intron) *UVSSA* level. *UVSSA* level was normalized using *EF1α* and expressed as relative to Col-3 spliced product level. Error bars indicate SE of three technical replicates.

phenotypes with the exception of a slight increase in apical dominance in *chr8-2*. *RDO2* mutants have been described as early flowering (Grasser et al., 2009), however, additional analysis indicates this phenotype is observed with respect to number of leaves, rather than number of days, at flowering (Mortensen and Grasser, 2014), consistent with our results.

The UV tolerance of the mutant alleles of the TCR genes was then assessed. Since TCR is a sub-pathway of NER, or dark repair, we assessed UV tolerance in seedlings following dark incubation after UV treatment. As previously described (Shaked et al., 2006; Biedermann and Hellmann, 2010; Zhang et al., 2010), *AtCSA-1* and *CHR8 (CSB)* loss of function resulted in increased UV sensitivity in the dark (**Figures 4A–C** and **Supplementary Figures S5A,B**). The *UVSSA* loss of function allele, *uvssa-2*, also resulted in increased UV sensitivity in the dark (**Figure 4E**). The *uvssa-1* allele, which results in increased levels of both truncated and full length UVSSA, did not exhibit either increased or decreased UV tolerance following 2 or 3 days of dark incubation (**Figure 4D** and **Supplementary Figure S5C**). *ubp12-2* also exhibited increased UV sensitivity in the dark (**Figure 4F** and **Supplementary Figure S5D**). *rdo2* exhibited increased dark UV sensitivity in hypocotyls (but not roots) after 2 days of incubation, but not after 3 days (**Figure 4G** and **Supplementary Figure S5E**). We also examined UV sensitivity in adult plants following dark incubation and found that, as in seedlings, *atcsa-1, chr8, uvssa-2, ubp12,* and *rdo2* mutants exhibit UV sensitivity, while *uvssa-1* does not (**Figure 5**).

To examine the specificity of the UV sensitivity of these alleles, they were also incubated in light (long day) following UV treatment. *atcsa-1, uvssa-2,* and *ubp12* were not UV sensitive in the light (**Figures 4A,E,F**), consistent with the dark specific role of NER. Surprisingly, both *chr8* alleles displayed UV sensitivity following light incubation (**Figures 4B,C**), exhibiting the expected dose dependence, with the more severely truncated *chr8-1* allele demonstrating a stronger root phenotype in both light and dark. This result suggests that CHR8 plays a role in light repair, distinct from the other components of the TCR pathway.

DISCUSSION

In this study, we examined the UV sensitivity of mutant alleles of Arabidopsis homologs of genes implicated in mammalian TCR. As previously reported, we find *atcsa-1* mutants exhibit increased dark specific UV sensitivity (Biedermann and Hellmann, 2010). Our *atcsa-1* dark root phenotype is not as strong as that of mutants in other TCR components such as CSB/CHR8 and UVSSA, this may be due to redundancy with AtCSA-2/CSAat1B.

The Arabidopsis homolog of mammalian CSB [also known as Excision Repair Cross-Complementing 6 (ERCC6)] and yeast Rad26 is CHR8 (Kunz et al., 2005; Singh et al., 2010). In this study, we utilized *CHR8* T-DNA lines and observed increased UV sensitivity following dark incubation, consistent with previous studies using *CHR8* RNAi lines (Shaked et al., 2006). Also, unique among the TCR mutants we examined, *chr8* alleles exhibited increased UV sensitivity following light incubation. Mammalian CSB has been implicated in regulation of transcription and

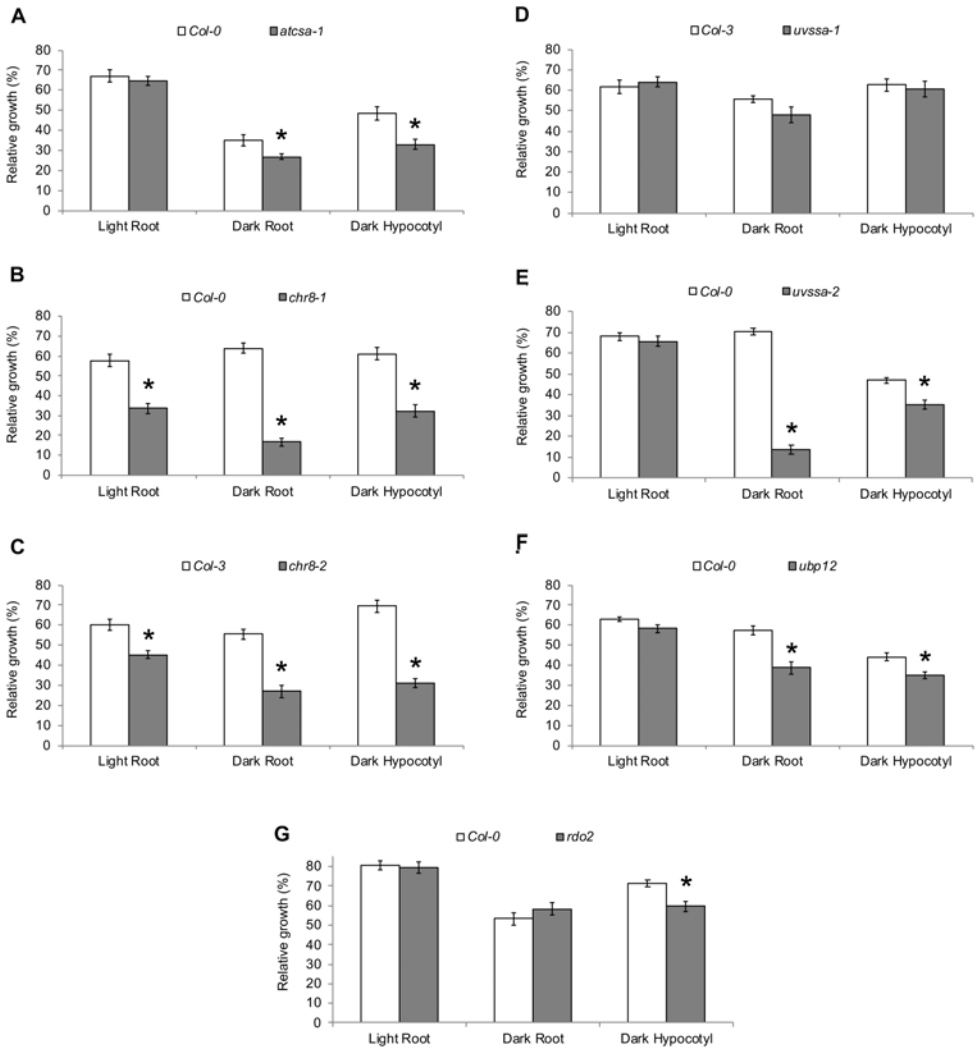

FIGURE 4 | UV tolerance of mutants in TCR genes. Relative growth of roots and hypocotyls of **(A)** *atcsa-1*, **(B)** *chr8-1*, **(C)** *chr8-2*, **(D)** *uvssa-1*, **(E)** *uvssa-2*, **(F)** *ubp12*, and **(G)** *rdo2* after 1000 J m^{-2} UV treatment, followed by 2 days of long-day (light) or dark incubation. Data are expressed as length relative to unirradiated control of the same genotype. Values are means ± SE (n = 20), *$p \leq 0.05$ of mutants vs wild type.

base excision repair in addition to TCR (Stevnsner et al., 2008; Boetefuer et al., 2018), so one of these roles may contribute to the *chr8* light UV sensitivity phenotype.

In humans, mutation of *UVSSA* results in defective TCR and UV sensitive syndrome (Cleaver, 2012). Loss of the *C. elegans* UVSSA homolog also results in increased UV sensitivity (Babu and Schumacher, 2016). While UVSSA is conserved throughout the animal kingdom (Nakazawa et al., 2012), it is absent from Drosophila. However, Drosophila also lack CSA and CSB homologs, and do not appear to perform TCR (Sekelsky, 2017). Yeast also lack UVSSA, although both *S. cerevisiae* and *S. pombe* have CSB homologs and perform TCR (Li and Li, 2017; Xu et al., 2017). Here we show that UVSSA is found throughout the plant kingdom, with conserved ENTH/VHS and DUF2043 domains. Recently, the region corresponding to amino acid 400–415 of human UVSSA was found to be well conserved in animals and required for TFIIH interaction (Okuda et al., 2017). Although this region is still acidic in plants, it not well conserved with human UVSSA and plants lack F408 and V411, which are required for TFIIH interaction and TCR in humans (Okuda et al., 2017), as well as K414, which is mono-ubiquitinated (Higa et al., 2018). In addition, residues 251–254 of human UVSSA have been shown to be required for USP7 interaction, CSB stability, and TCR (Higa et al., 2016), yet this sequence is also not conserved in plants. Nonetheless our data show that lack of UVSSA results in dark specific UV sensitivity in Arabidopsis, consistent with a role in NER.

Arabidopsis USP7 homologs UBP12 and UBP13, like other ubiquitin specific proteases, play important roles in plant development and environmental response (Zhou et al., 2017). UBP12/13 interact with LHP1 and deubiquitinate RGFR1 and

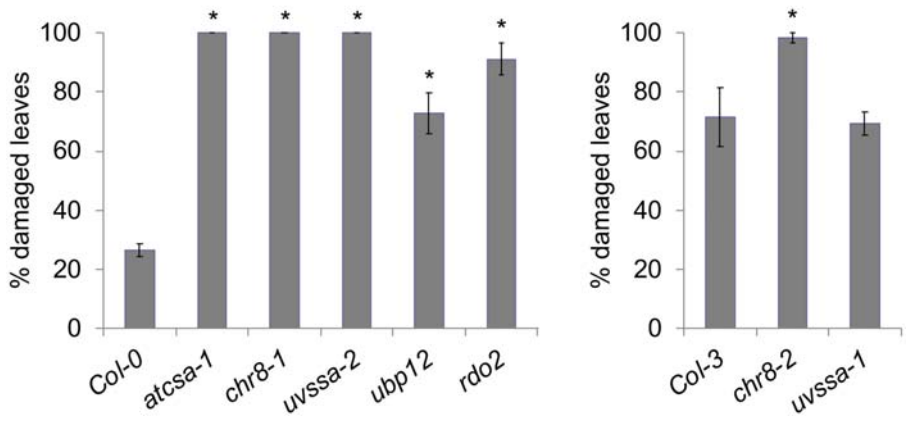

FIGURE 5 | UV tolerance in adult plants. Percentage damaged leaves after 500 J m^{-2} UV treatment, followed by 3 days of dark incubation. Values are means ± SE (n = 6), $^*p \leq 0.05$ of mutants vs respective wild type.

MYC2 (Derkacheva et al., 2016; Jeong et al., 2017; An et al., 2018). Our results here indicate that UBP12 (and UBP13) are involved in UV tolerance, suggesting they may also deubiquitinate UVSSA and CSB, as has been proposed for mammalian USP7 (Geijer and Marteijn, 2018). UBP12 and UBP13 act redundantly, and double null alleles are inviable due to pollen defects (Ewan et al., 2011; Derkacheva et al., 2016). Here we use an allele of *UBP12, ubp12-2,* which also results in a partial decrease in *UBP13* level, and resulting in a weak double mutant (Cui et al., 2013). However, because this is a weak (non-null) double mutant, we may be underestimating the role of UBP12/13 in UV tolerance.

In mammals, in addition to acting during transcript elongation, TFIIS has been shown to facilitate transcription re-initiation following RNAP arrest, and is recruited to the stalled polymerase in a CSB and CSA dependent manner (Donahue et al., 1994; Kalogeraki et al., 2005; Fousteri et al., 2006; Dutta et al., 2015). In yeast, loss of TFIIS only results in increased UV sensitivity in a GGR-deficient background, however, the same is true of CSB homolog Rad26 (Wong and Ingles, 2001). In mammals, reduction of TFIIS resulted in reduced RNA synthesis recovery, but had no effect on UV sensitivity (Jensen and Mullenders, 2010). In this study we detected a UV sensitive phenotype in TFIIS deficient Arabidopsis (*rdo2*), however, it was milder than observed for the other TCR mutants and not detectable 3 days after seedling UV treatment. Interestingly, the UV sensitive phenotype of both *rdo2* and *atcsa-1* was stronger in hypocotyls than in roots, at 2 days than at 3 days, and in adults than in seedlings, suggesting the role of these genes in UV tolerance may vary with tissue, time, and phenotype assessed (growth versus tissue death).

CONCLUSION

In this study, we have identified the Arabidopsis UVSSA homolog and shown that Arabidopsis UVSSA, USP7 (UBP12/13), and TFIIS (RDO2) homologs contribute to UV tolerance, along with CSA and CSB (CHR8) homologs, suggesting conservation in the mechanisms of TCR.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

WAK, AS, and DS performed the experiments. JM conducted the phylogenetic analysis. DS wrote the first draft of the manuscript. All authors contributed to revised manuscript and approved the final version, designed the experiments and analyzed the data.

FUNDING

This study was supported by the Natural Sciences and Engineering Research Council of Canada.

ACKNOWLEDGMENTS

We are grateful to Janelle Lazaro and Linda Al Rayes for technical assistance and Triparna Lahari for helpful discussion. Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center and the Nottingham Arabidopsis Stock Centre.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00516/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 09 May 2019
doi: 10.3389/fpls.2019.00588

The XPF-ERCC1 Complex Is Essential for Genome Stability and Is Involved in the Mechanism of Gene Targeting in *Physcomitrella patens*

*Anouchka Guyon-Debast[1], Patricia Rossetti[2], Florence Charlot[1], Aline Epert[1], Jean-Marc Neuhaus[2], Didier G. Schaefer[2] and Fabien Nogué[1]**

[1] Institut Jean-Pierre Bourgin, INRA, AgroParisTech, CNRS, Université Paris-Saclay, Versailles, France, [2] Laboratoire de Biologie Moléculaire et Cellulaire, Institut de Biologie, Université de Neuchâtel, Neuchâtel, Switzerland

The XPF-ERCC1 complex, a highly conserved structure-specific endonuclease, functions in multiple DNA repair pathways that are pivotal for maintaining genome stability, including nucleotide excision repair, interstrand crosslink repair, and homologous recombination. XPF-ERCC1 incises double-stranded DNA at double-strand/single-strand junctions, making it an ideal enzyme for processing DNA structures that contain partially unwound strands. Here, we have examined the role of the XPF-ERCC1 complex in the model bryophyte *Physcomitrella patens* which exhibits uniquely high gene targeting frequencies. We undertook targeted knockout of the *Physcomitrella ERCC1* and *XPF* genes. Mutant analysis shows that the endonuclease complex is essential for resistance to UV-B and to the alkylating agent MMS, and contributes to the maintenance of genome integrity but is also involved in gene targeting in this model plant. Using different constructs we determine whether the function of the XPF-ERCC1 endonuclease complex in gene targeting was removal of 3′ non-homologous termini, similar to SSA, or processing of looped-out heteroduplex intermediates. Interestingly, our data suggest a role of the endonuclease in both pathways and have implications for the mechanism of targeted gene replacement in plants and its specificities compared to yeast and mammalian cells.

Keywords: XPF-ERCC1, gene targeting, *Physcomitrella patens*, DNA repair, recombination

OPEN ACCESS

Edited by:
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Reviewed by:
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***Correspondence:**
Fabien Nogué
fabien.nogue@inra.fr

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *07 March 2019*
Accepted: *18 April 2019*
Published: *09 May 2019*

Citation:
Guyon-Debast A, Rossetti P, Charlot F, Epert A, Neuhaus J-M, Schaefer DG and Nogué F (2019) The XPF-ERCC1 Complex Is Essential for Genome Stability and Is Involved in the Mechanism of Gene Targeting in Physcomitrella patens. Front. Plant Sci. 10:588. doi: 10.3389/fpls.2019.00588

INTRODUCTION

The XPF-ERCC1 complex is a highly conserved heterodimeric structure-specific endonuclease (SSE) composed of the XPF catalytic subunit and the ERCC1 DNA binding subunit that is involved in DNA repair and maintenance of chromosome stability (Dehé and Gaillard, 2017; Faridounnia et al., 2018). The protein sequences of the different ERCC1 homologs (ERCC1 in *Drosophila melanogaster*, Rad10 in *Saccharomyces cerevisiae* and Swi10 in *Schizosaccharomyces pombe*) and XPF homologs (MEI-9 in *D. melanogaster*, Rad1 in *S. cerevisiae* and Rad16 in *S. pombe*) are highly conserved as well as their capacity for heterodimerization, which insures stability and functionality of the complex. Consistent with the importance of heterodimerization of the two proteins for their function is that individual mutants in the *ERCC1* and *XPF* genes exhibit similar phenotypes

(Gregg et al., 2011). The endonuclease activity of the XPF-ERCC1 complex is responsible for DNA cleavage near junctions between single-stranded and double-stranded DNA, where the single strand departs 5′ to 3′ from the junction.

XPF-ERCC1 is essential for nucleotide excision repair (NER) of DNA, a mechanism that removes DNA damage induced by ultraviolet (UV) radiation and by mutagenic chemicals, or chemotherapeutic drugs that form bulky DNA adducts. In mammalian cells, null mutants of the *ERCC1* or *XPF* genes are lethal and weaker mutations can result in xeroderma pigmentosum (XP), trichothiodystrophy (TTD), and Cockayne syndrome (CS), genetic disorders that are typical of mutations in genes required for NER (Gregg et al., 2011). However, there is evidence that the ERCC1 and XPF proteins have functions distinct from NER. Indeed, it was demonstrated that XPF-ERCC1 participates in the Fanconi Anemia Pathway of DNA interstrand crosslinks repair (Bhagwat et al., 2009) and recently the XPF-ERCC1 complex has been shown to be involved in a sub-pathway of long-patch base excision repair (BER) involving 5′ gap formation (Woodrick et al., 2017).

In addition to involvement in NER, BER and interstrand crosslink repair, there is evidence for a role of XPF-ERCC1 in double strand break (DSB) repair (Ahmad et al., 2008). Resolution of a DSB can be done by non-homologous end joining or by homology-directed repair (HDR) (Symington and Gautier, 2011). The XPF-ERCC1 complex and its *S. cerevisiae* homolog, the RAD1-RAD10 complex have been shown to participate to non-homologous repair of DSB and the RAD1 protein of *S. cerevisiae* and the mammalian ERCC1 protein play a major role in Alt-EJ (also known as MMEJ), a Ku-independent sub-pathway of NHEJ, that is error-prone, and that utilizes short stretches of homology to join two broken DNA ends (Ma et al., 2003; Ahmad et al., 2008). The RAD1-RAD10/XPF-ERCC1 complex participates also to intra or extra chromosomal HR between sequence repeats in *S. cerevisiae* (Klein, 1988; Schiestl and Prakash, 1988, 1990; Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Prado and Aguilera, 1995), in mammalian cells (Sargent et al., 1997, 2000; Al-Minawi et al., 2008) and in plants (Dubest et al., 2002, 2004). The function in HR of the RAD1-RAD10 endonuclease, is the removal of non-homologous 3′ termini of single-stranded overhangs of broken ends to facilitate single-strand annealing (SSA), an error-prone sub-pathway of HR (Bardwell et al., 1994; Pâques and Haber, 1997). Interestingly, the function of the RAD1-RAD10/XPF-ERCC1 complex has been shown to be also important for HR mediated gene targeting (GT) in *S. cerevisiae* and in mammalian cells (Schiestl and Prakash, 1988; Saffran et al., 1994; Adair et al., 2000; Niedernhofer et al., 2001; Langston and Symington, 2005; Rahn et al., 2011). There are two general methods for gene targeting, based on the two arrangements of donor DNA that can be used for gene targeting, called ends-in and ends-out (Hastings et al., 1993). They differ in whether the double-strand break is within the region of homology (ends-in) or at the ends (ends-out) leading to targeted gene replacement (TGR). Analysis of the capacity for GT using ends-in or ends-out substrates of recombination of mutants affected in the XPF-ERCC1 complex suggest that this complex could have different roles during gene targeting and that these roles could differ from one species to another.

In flowering plants, the study of the mechanisms of recombination is far behind that of mammals and yeast (Waterworth et al., 2011) and the low level of gene targeting efficiency, even if it can be increased to 1% by using a CRISPR-Cas9 based strategy (Wolter et al., 2018), makes the deciphering of this important mechanism difficult. However, in the plant kingdom the moss *Physcomitrella patens* is an exception to this rule and exhibits rates of gene targeting (GT) comparable to *S. cerevisiae* allowing advances in the understanding of DNA metabolism in plants (Schaefer and Zrÿd, 1997; Kamisugi et al., 2005, 2006; Odahara et al., 2007; Trouiller et al., 2007, 2006; Schaefer et al., 2010; Kamisugi et al., 2012; Wendeler et al., 2015; Collonnier et al., 2017; Odahara and Sekine, 2018; Wiedemann et al., 2018). It must be noticed that the mechanisms underlying targeted DNA integration are not necessarily conserved between *P. patens* and *S. cerevisiae* (Schaefer et al., 2010) and these differences can probably account for the differences that can be observed concerning the nature of the final products of ends-out constructs integration in the two species. First, ends-out construct integration producing single copy gene conversion (TGR), similar to what is observed in yeast, can be found in *Physcomitrella* but the majority (more than 80%) of the targeted integration events comprises head-to-tail concatemers of the transforming DNA fragment (Schaefer, 2002; Kamisugi et al., 2006), that could result from episomally replicating DNA (Murén et al., 2009). Second, a significant proportion (around 50%) of gene targeting events in *Physcomitrella* result in insertion of the ends-out construct adjacent to the target locus (targeted gene insertion-TGI). Typically, TGI comprises an HR event at one end of the integrant, accompanied by an apparent NHEJ event at the other (Schaefer, 2002; Kamisugi et al., 2005, 2006; Collonnier et al., 2017). This profile of integration is rarely observed in *S. cerevisiae* but frequent for GT events in flowering plants and animal cells (Adair et al., 1998; Hanin et al., 2001).

To investigate the potential role of the XPF-ERCC1 complex in DSB repair and gene targeting mechanisms in *Physcomitrella* we produced mutants for the *PpERCC1* and *PpXPF* homologous genes. Our data show that loss of ERCC1 and/or XPF functions generates a strong mutator phenotype and hypersensitivity to UV-B and methyl methanesulfonate (MMS) induced DNA lesions, suggesting an active role of this complex in the moss NER and BER repair pathways. Using different constructs we further revealed that the moss XPF-ERCC1 complex is required for HR between both ends-out or ends-in constructs and genomic loci.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Physcomitrella patens (Hedw.) B.S.G. "Gransden" was used in this study. Individual strains were vegetatively propagated as lawns of protonemal filaments on rich agar $PpNH_4$ medium ($PpNO_3$ medium supplemented with 2.7 mM NH_4-tartrate) overlaid with cellophane, or cultured as "spot inocula" on minimal medium ($PpNO_3$) for phenotypic analyses and sporogenesis as previously

described (Trouiller et al., 2006). Protoplast isolation, PEG mediated transformation and selection of transformed plants were performed according to (Schaefer et al., 2010).

Gene Identification and Isolation

Genomic DNA and total RNA were isolated from *Physcomitrella* as previously described (Trouiller et al., 2006). *Physcomitrella* genomic sequences encoding the *ERCC1* and *XPF* genes were identified by BLAST search[1]. The available gene models were used for the design of PCR primers to amplify cognate genomic sequences, which were cloned in the TOPO®-TA (life technologies, United States) or pBluescript (Stratagene, United States) plasmids. PCR primers used are listed in **Supplementary Table S1**. In order to obtain a correct gene model for each sequence, full-length cDNA were amplified from *Physcomitrella* polyribosome-derived RNA by RT-PCR (Kamisugi et al., 2012) and sequenced. Predicted polypeptide sequences were aligned with the orthologous genes from other eukaryotes using CLUSTALW.

Generation of Deletion Mutants

The KO vector pERCC1 delta contains a 654 bp 5′-targeting fragment (chrom6: 19338880–19339533) and an 865 bp 3′-targeting fragment (chrom6: 19341999–19342863) flanking a LoxP-HygroR-LoxP marker in vector pBHRF (Schaefer et al., 2010). The KO vector pXPF delta contains a 974 bp 5′-targeting fragment (chrom18: 8489087–8490060) and a 1096 bp 3′-targeting fragment (chrom18: 8493588–8494683) flanking a LoxP-NeoR-LoxP marker in vector pBNRF (Schaefer et al., 2010). Moss protoplasts were transformed with pERCC1 delta digested with *Avr*II and *Pac*I, or with pXPF delta digested with *Xba*I and *Pac*I. Transformed plants carrying targeted gene replacement were identified by PCR genotyping and subsequent deletion of the selection marker was obtained by transient Cre recombinase expression (Trouiller et al., 2006). The double *ercc1/xpf* mutant was generated by retransforming a *PpErcc1*Δ deletion line with pXPF delta and selecting for targeted gene replacement at the *PpXPF* locus among neo^R plants.

Analysis of Gene Expression in Mutants

Transcript abundance in selected knockout lines was determined by RT-PCR. Total RNA was extracted using TRIZOL-reagent (Invitrogen) from 100 mg of protonemal tissue. Contaminating DNA was removed by DNaseI treatment with RNase Free DNase set (Qiagen) using spin columns of the Rneasy plant mini kit (Qiagen). RT-PCR was performed with RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) on 500 ng RNA according to the supplier's instructions. Quality control of DNA or RNA was performed using primers PpAPT#14 + PpAPT#19 (**Supplementary Table S1**). Detection of *ERCC1* and *XPF* mRNA in mutant lines was performed by RT-PCR using primers indicated in **Supplementary Table S1**.

[1]https://phytozome.jgi.doe.gov

UV-B and MMS Sensitivity Assays

For UV-B sensitivity protoplasts of wild-type, *ercc1*Δ, *xpf*Δ, or *xpfK*Δ/*ercc1*Δ strains were spread (ca. 25000/plate) and regenerated on protoplast agar medium ($PpNH_4$ + 0.5 g/L glucose + 6.6% mannitol). Plates were immediately exposed to UV-B light (60 J/m^2/s) from a 312 nm TFX lamp. We calculated the flux with a UV-Elektronik GmbH dosimeter. The. Plates were immediately transferred to darkness for 24 h after treatment then to standard growth conditions for protoplast regeneration. Survival was determined as described previously (Kamisugi et al., 2012). For Methyl methanesulfonate (MMS) (Sigma-Aldrich) sensitivity, protoplasts were spread on protoplast agar medium freshly supplemented with different concentration of MMS and further regenerated under standard growth conditions. Cultures were transferred to $PpNH_4$ medium without MMS after 6 days and survival was determined 2 weeks later by microscopic observation.

Evaluation of Spontaneous Mutation Frequency

To assess the mutator phenotype of *ercc1*Δ and *xpf*Δ mutants, we measured the level of spontaneous loss of function of the Adenine Phosphoribosyl Transferase gene (*PpAPT*) which confers resistance to 2-fluoroadenine (2-FA), as previously described (Trouiller et al., 2006). Several million of plants were regenerated from protoplasts of wild-type, *ercc1*Δ and *xpf*Δ mutants and then transferred to medium supplemented with 10 mM 2-FA (Fluorochem). After 2 weeks, the number of resistant plants was counted. Results were analyzed using the Fisher's exact test.

Gene Targeting Assays

The different APT based gene targeting constructs used in this study are described in **Figures 3**, **4**. GT efficiencies were assessed after transformation with vector PpAPT-KO2 (Schaefer et al., 2010) and selection for Hygro^R plants followed by selection for 2-FA^R plants among them, as performed in Charlot et al. (2014), GT frequencies were assessed after transformation with vector PpAPT-KO2 or PpAPT-KO9 and direct selection of 2-FA^R plants, a selection that only identifies targeted integration events in *PpAPT*. To evaluate the importance of the form of the transforming DNA, transformation was performed with PpAPT-KO9 digested with *Eco*RI + *Hin*dIII (ends-out) or with *Xba*I (ends-in) and direct selection for 2-FA^R plants. To study the impact of heterologous sequences at the ends of the transforming DNA, transformation was performed with PpAPT-KO9 digested with *ApaL*I or with PpAPT-KO2 linearized within the hygromycin resistance cassette with *AsiS*I (long ends-in): in both cases transformation was followed by direct selection for 2-FA^R clones. Experiments were repeated three to eight times and statistically analyzed using the Fisher exact test.

Analysis of Transformed Plants

In order to analyze large numbers of transformed plants for the nature of gene targeting events, PCR-based genotyping assays were used. Primers PpAPT#2 and PpAPT#20 located outside

the genomic fragments present in the cassette (**Supplementary Table S1** and **Figure 3**) were used to detect monocopy insertions. For detection of targeted gene replacement (TGR) and targeted gene insertion (TGI) integrations the 5′ and 3′ junctions of the integrations were characterized using primers PpAPT#2 + ProRev and PpAPT#20 + TerFwd (**Supplementary Table S1** and **Figure 3**) respectively.

Cytology

Binocular observations were made with a Nikon SMZ1000.

RESULTS

Identification of PpXPF and PpERCC1 and Generation of Deletion Mutants

The XPF-ERCC1 heterodimeric complex is a highly conserved structure endonuclease (Schwartz and Heyer, 2011). Sequence homology searches of the *P. patens* genome with the Arabidopsis homologs identified a single putative homolog for XPF (*PpXPF*, Pp3c18_11670) and ERCC1 (*PpERCC1*, Pp3c6_29610). PpXPF and PpERCC1 full length cDNAs were isolated and sequenced: this analysis confirmed the predicted structures (9 exons, **Figure 1A**) and protein sequences found in the database (Phytozome 12.0). A phylogenetic analysis, including plants, algae, animals and fungi established that *PpXPF* and *PpERCC1* effectively belong to the XPF and ERCC1 plant clades among eukaryotic SSE of the XPF/MUS81 family (**Supplementary Figure S1**). The moss PpXPF protein is composed of 1047 amino acids and shares 50.3, 35.5, and 30.4% sequence identity with AtXPF, HsXPF, and SpRad16, respectively (**Supplementary Figure S2**). The homology between PpXPF and the XPF/RAD1 homologs is distributed throughout their length, but it is especially strong at the C-terminal end, in the region involved in the formation of the heterodimeric protein complex. Noticeably, the nuclease domain (restriction endonuclease type-II like PR011335) containing the specific motif ERKXXXD required for nuclease activity (Ciccia et al., 2008) and the RuvA_2-like domain (IPR010994) containing the duplicated Helix-hairpin-Helix (HhH) motif (PFAM:HHH_5) functioning as a scaffold for complex formation with ERCC1 (Tripsianes et al., 2005) can be identified in PpXPF (**Supplementary Figure S2**). The moss PpERCC1 protein is composed of 427 amino acids and shares 44.6, 27.6, and 23.3% sequence identity with AtERCC1, HsERCC1, and SpSwi10, respectively (**Supplementary Figure S3**). Sequence conservation is especially high within the central RAD10 domain (IPR004579) and the C-terminal RuvA 2-like domain, which are involved in DNA binding and complex formation in ERCC1 homologs (Manandhar et al., 2015). These features are consistent with our assumption that PpXPF and PpERCC1 encode the proteins forming the moss heterodimeric XPF/ERCC1 complex involved in several aspects of DNA repair.

To investigate PpXPF and PpERCC1 functions we generated *xpf* and *ercc1* deletion mutants, named *xpf*Δ and *ercc1*Δ respectively, using targeted gene disruption followed by Cre/lox mediated elimination of the resistance cassette (**Figure 1A** and section "Materials and Methods"). Deletion of exons 1–8 in *PpERCC1* and of exons 1–6 in *PpXPF* was confirmed by PCR genotyping and sequence analysis (data not shown). A double knock-out mutant named *xpf*Δ/*ercc1*Δ was produced by re-transformation of an *ercc1*Δ mutant with the pXPF delta vector. RT-PCR analysis established that the full-length transcripts were no longer produced in these mutants (**Figure 1B**). For all further experiments, we used two independent *xpf*Δ, *ercc1*Δ, or *xpf*Δ/*ercc1*Δ strains and both alleles of the same mutants show similar phenotypes.

The *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ Mutants Show No Developmental Defects

In Bryophytes, the life cycle is dominated by the haploid gametophyte. Haploid spores of *P. patens* germinate to form a juvenile filamentous network of tip growing cells, the protonema. One week after germination, initials of leafy shoots called buds differentiate from protonemal branch initials and further develop by meristematic growth into the leafy gametophores. After 1 month, each individual plant is composed of several dozens of gametophores. Short day length and low temperature induces the differentiation of the reproductive organs at the shoot apex. Finally fertilization of the egg cell by flagellated antherozoids give rise to the epiphytic diploid sporophyte in which meiosis takes place to produce new haploid spores, reviewed in Bonhomme et al. (2013) and Kofuji and Hasebe (2014).

The phenotype of the *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ mutants was assessed throughout the entire life cycle. Protonemal growth, bud differentiation and leafy shoots development were similar in the three mutants compared to the wild-type and the *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ strains are fertile (**Figure 1C** and **Supplementary Figure S4**). These findings show that the XPF/ERCC1 complex plays no direct role in either vegetative or reproductive moss development.

The *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ Mutants Are Impaired in Repairing Exogenous and Endogenous DNA Damage

The XPF/ERCC1 complex is involved in several DNA repair pathways in eukaryotes: it is a major factor of NER and also contributes to ICL, SSA, and HR. We therefore evaluated the sensitivity of the mutants to DNA damage induced by UV-B and methyl methanesulfonate (MMS). To assess the sensitivity of the *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ strains to UV-B light, which generates DNA damage essentially repaired by the NER pathway, we monitored the ability of UV-B-treated protoplasts to regenerate into plants. Our data show that both *xpf*Δ and *ercc1*Δ mutants display an extremely high UV-B sensitivity which is not further increased in the double *xpf*Δ/*ercc1*Δ strain (**Figure 2** and **Supplementary Figure S5**). With a lethal dose 50 around 8 mJ/cm^2, these mutants are ca. 10-fold more sensitive than the *rad51-1-2 double mutant* strain (Schaefer et al., 2010; Charlot et al., 2014) and 20-fold more sensitive than the WT (**Figure 2**). Such a strong sensitivity to UV-B provides evidence for a key

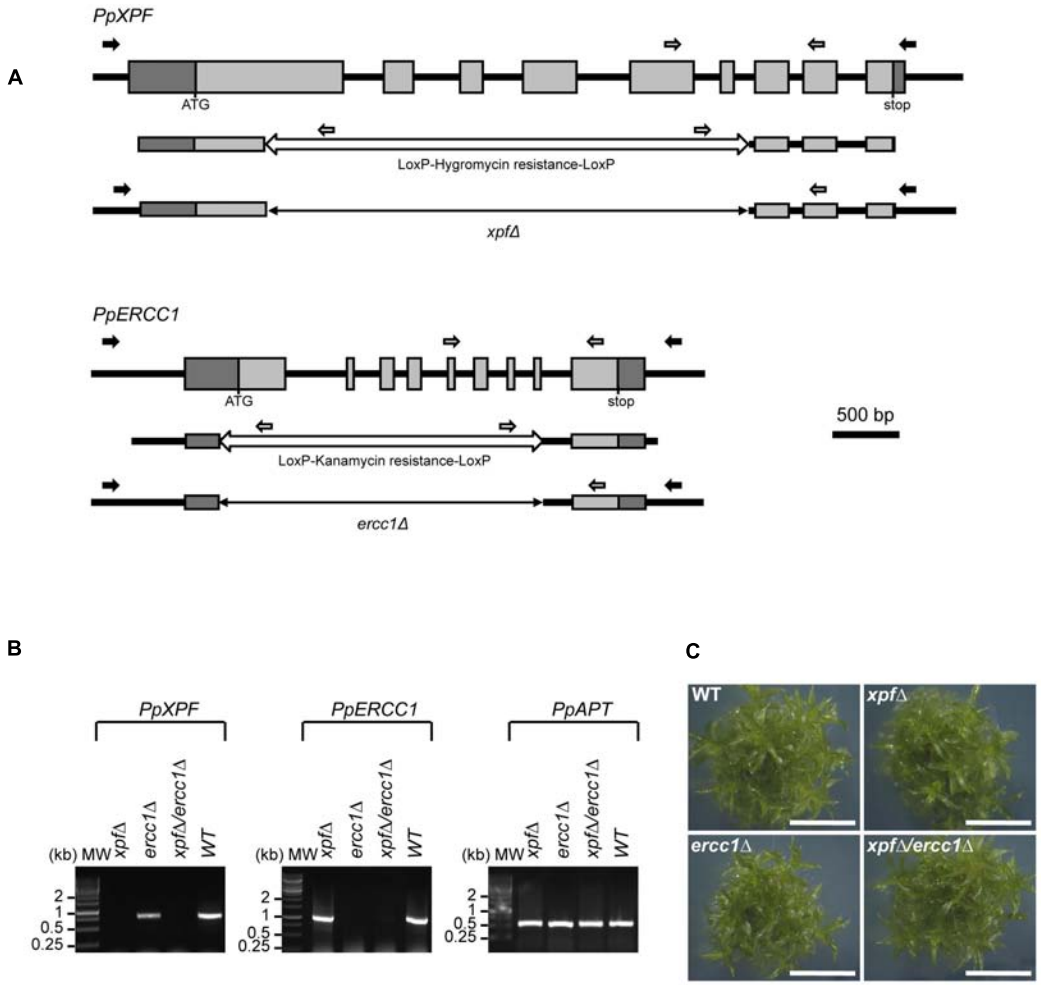

FIGURE 1 | Structure and targeted disruption of *Physcomitrella XPF* and *ERCC1* genes. **(A)** Structure of the WT, *PpXPF*, and *PpERCC1* loci (top line), of the KO vector (middle) and of the deleted locus in the *xpf*Δ and *ercc1*Δ mutants (bottom). Exons are represented by gray boxes, with 5′- and 3′-UTR sequences in darker gray. The region deleted by cre-lox excision of a selection cassette is shown as a double arrow line. Arrows indicate the position of the primers used to genotype the plants by PCR (black and white) and RT-PCR (gray). **(B)** RT-PCR analysis of *XPF* and *ERCC1* transcripts in wild-type and mutants plants. RNA was isolated from protonemal tissue of wild-type and mutants lines for cDNA synthesis and PCR amplification using gene-specific primers. The *PpAPT* transcript has been used as control. Primers are listed in **Supplementary Table S1**. **(C)** Morphology of plants of wild type of *xpf*Δ, *ercc1*Δ single mutants and *xpf*Δ/*ercc1*Δ double mutant. The picture was taken after 3 weeks of growth. scale bar = 4 mm.

involvement of the moss XPF/ERCC1 complex in the repair of UV-induced DNA damage, most likely by the NER pathway. We further evaluate the implication of the XPF/ERCC1 complex in the repair of DNA damage induced by the alkylating agent MMS, which is believed to stall replication forks. In this assay, fresh protoplasts are regenerated for 6 days on MMS containing medium, and their ability to regenerate into plants is evaluated after a further 14-days of growth on standard medium. Our analyses show that the *xpf*Δ, *ercc1*Δ, and *xpf*Δ/*ercc1*Δ strains display a similar increased sensitivity to MMS compared to the WT (**Figure 2** and **Supplementary Figure S5**). This finding indicates that the moss XPF/ERCC1 complex contributes to the repair of DNA damages induced by an MMS treatment. Further experiments are needed to characterize the exact nature of these damages.

Finally to evaluate the level of contribution of the XPF/ERCC1 complex to the repair of endogenous DNA damage, we measured the level of spontaneous loss of function of the *PpAPT* reporter gene (Trouiller et al., 2006) and assess the mutator phenotype of these mutants. More than 2 million protoplasts of WT, *xpf*Δ and *ercc1*Δ strains were regenerated and selected for their resistance to 2-fluoroadenine. No 2-FA resistant plants were recovered in the WT, and a total of 5 and 4 2-FA resistant plants were identified in the *xpf*Δ and *ercc1*Δ mutant, respectively (**Table 1**). We previously showed that the *APT* mutation rate in WT was around 10^{-8} (Charlot et al., 2014), this analysis shows that the *APT* mutation rate is increased approximately 100-fold in the *xpf*Δ (2.5×10^{-6}) and *ercc1*Δ (1.8×10^{-6}) strains. These data support a direct involvement of the moss XPF-ERCC1 complex

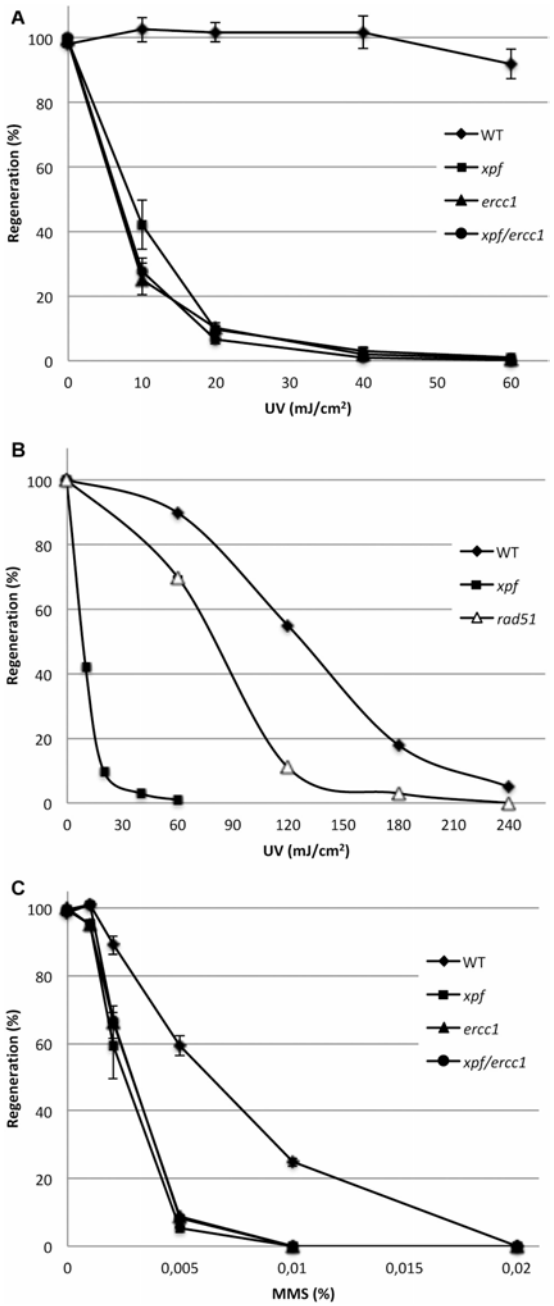

FIGURE 2 | Sensitivity of the wild-type (WT) and of the *xpf*, *ercc1*, and *xpf/ercc1* mutants toward genotoxic agents. **(A)** Survival curves of the WT, *xpf* and *ercc1* ($n = 4$), and *xpf/ercc1* strains ($n = 2$) in response to low doses of UV-B light (scale bar = standard deviation). **(B)** The survival curves of WT, *xpf* and *rad51* strains in response to higher UV-B doses are shown (references: this study and Charlot et al., 2014). **(C)** Survival rates of protoplasts after exposure to MMS: survival is expressed as the percentage of regenerated protoplasts relative to untreated samples. The experiment was performed two times except for *xpf/ercc1* (scale bar = standard deviation).

in repairing naturally occurring DNA damage to prevent the accumulation of mutations in the genome. Taken together the above data demonstrate that the moss XPF-ERCC1 complex plays an important role in the repair of both endogenous and exogenous DNA damage.

TABLE 1 | PpXPF and PpERCC1 are required to repair endogenous DNA damage.

Genotype	Regenerants ($\times 10^3$)[a]	2-FA resistant	Rate in 10^6
WT	107200	2	0.02
*xpf*Δ	1963	5 ($p = 3.8 \times 10^{-8}$)[b]	2.54
*ercc1*Δ	2216	4 ($p = 2.4 \times 10^{-6}$)[b]	1.89

[a]*Regenerants number was evaluated 5 days after protoplasts isolation, before transfer on 2FA.* [b]*Differences between WT and mutants were compared using Fisher's exact test.*

Gene Targeting Using an Ends-Out Construct Is Reduced in the *xpf*Δ and *ercc1*Δ Mutants

In yeast and mouse ES cells, the RAD1-RAD10 (or XPF-ERCC1) complex is involved in gene targeting using ends-out constructs (Niedernhofer et al., 2001; Langston and Symington, 2004, 2005). In order to investigate the involvement of the moss XPF-ERCC1 complex in gene targeting, we determined gene targeting rates in wild-type, *xpf*Δ and *ercc1*Δ mutants cells after transformation with an ends-out targeting substrate with homologous ends designed to inactivate the *PpAPT* gene and containing an hygromycin resistance cassette (PpAPT-KO2) (**Figure 3**). The gene targeting efficiency (GTE), determined as the frequency of 2-FA resistant plants amongst transformed plants (Hyg^R) (**Table 2**), reaches 69.5% in the wild-type and is reduced by 1.7 and 1.5-folds in the *xpf*Δ, and *ercc1*Δ mutants respectively (**Table 2**, Fisher's test $p \leq 0.003$). These experiments demonstrate that PpXPF and PpERCC1 contribute to but are not essential for gene targeting.

Gene Targeting Decrease in the *xpf*Δ and *ercc1*Δ Mutants Is Not Due to the Heterologous Selectable Marker

The linearized ends-out targeting constructs PpAPT-KO2 used in the previous experiment does not contain non-homologous tails at its 3′ ends. For this reason the decrease of GT in the *xpf*Δ or *ercc1*Δ mutants cannot be attributed to the role of the PpXPF-PpERCC1 complex in removing 3′ overhangs of non-homologous sequences as described for SSA or for targeted integration of ends-in targeting constructs in CHO cells. Nevertheless, in yeast and mouse the endonuclease complex has been shown to be also involved in the resolution of the large loop of mismatches that forms between the targeted gene and the central region of heterology corresponding to the selectable marker (Niedernhofer et al., 2001; Langston and Symington, 2004, 2005). In order to determine if the moss XPF-ERCC1 complex has a role in handling the heterology generated by the presence of the selectable marker (hygromycin resistance cassette in this case), GT frequencies (GTF) of an ends-out targeting substrate with homologous ends but lacking a selection marker (**Figure 3**, PpAPT-KO9 digested by *Eco*RI/*Hin*dIII),

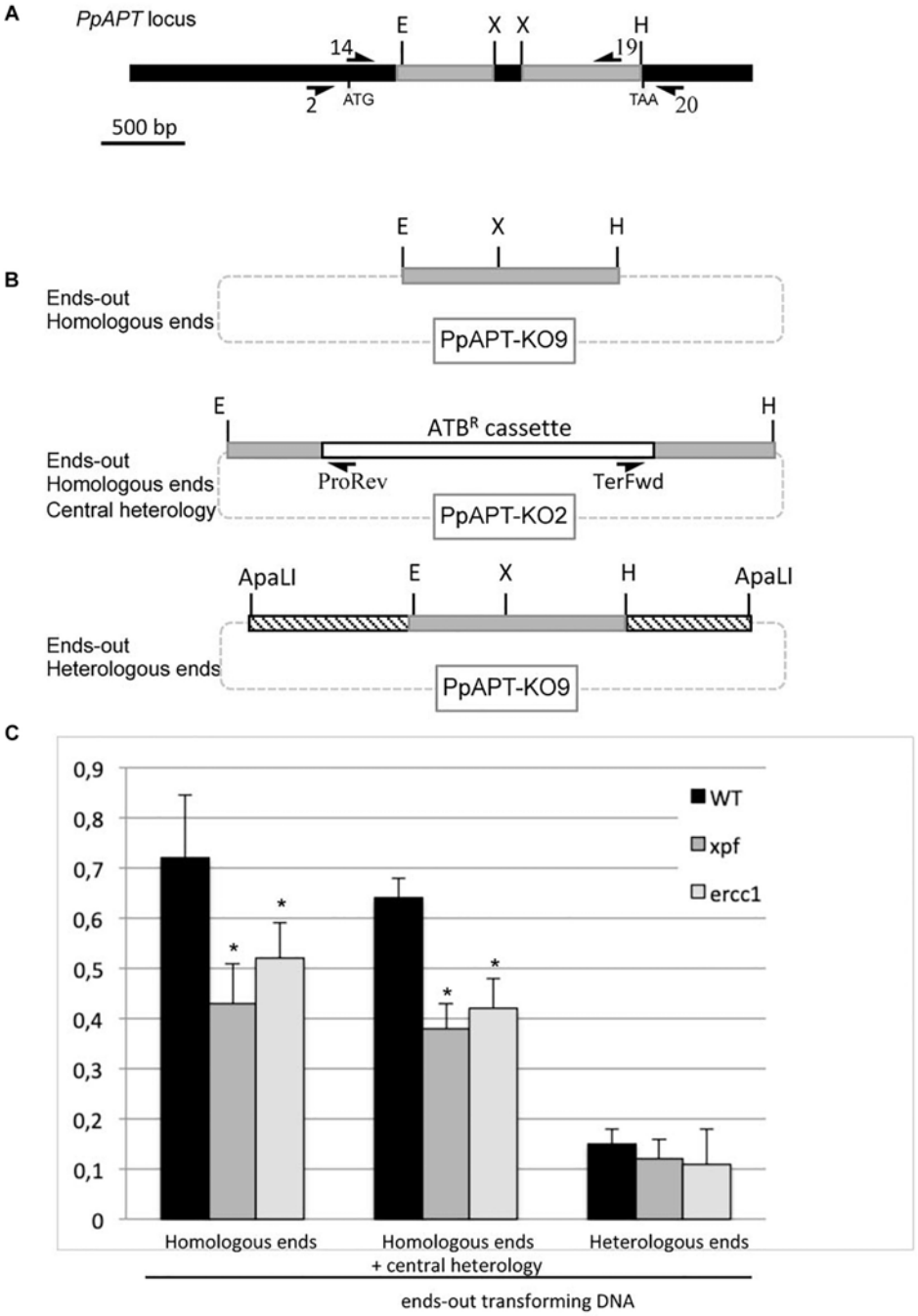

FIGURE 3 | Gene targeting frequency in WT and *xpf* and *ercc1* mutants using ends-out type vectors. **(A)** PpAPT WT locus. Regions targeted using ends-out or ends-in vectors are in gray. Primers used in this study are referenced in **Supplementary Table S1**. Different forms of the transforming DNA used. **(B)** Digestion of PpAPT-KO9 with *Eco*RI + *Hind*III give rise to ends-out transforming DNA, with double strand breaks at the edges of *APT* sequences. Digestion of PpAPT-KO2 with *Eco*RI + *Hind*III give rise to ends-out transforming DNA, with double strand breaks at the edges of *APT* sequences and a central heterologous region. Digestion of PpAPT-KO9 with *ApaL*I generates ends-out transforming DNA with heterologous sequence at the DSB (dashed bars: heterologous stretches, thin lines: plasmid sequences, dotted lines: plasmid sequences absent from the transforming DNA). **(C)** Gene targeting frequency of the *APT* gene in the wild type, and *xpf* and *ercc1* mutants using different ends-out types transforming DNA. Asterisks indicate significant differences with the WT (Fisher exact test p-value ≤ 0.01).

were measured. This substrate confers 2-FA resistance upon targeted integration at the *PpAPT* locus, generating a 159 bp deletion. The gene targeting frequency was determined as the frequency of 2-FA resistant plants amongst regenerated protoplasts (**Table 2** and **Supplementary Table S2**). GTF observed in the wild type using this ends-out targeting

TABLE 2 | Comparison of transformation and gene targeting efficiencies using an ends-out type targeting construct containing an heterologous selectable marker.

Genotypes	Regenerants[a]	Hyg^R plants	$2FA^R$ plants[b]	Gene targeting frequency (%)[c]	Gene targeting efficiency (%)[d]
Wild type	94219	868	603	0.64 ± 0.08[e]	69.5 ± 9.57[e]
xpf	124210	1091	472*	0.38 ± 0.1	43.3 ± 12.42
ercc1	22381	205	94*	0.42 ± 0.12	45.8 ± 16.32

[a]*Protoplasts were transformed with vector PpAPT-KO2 digested with BsaAI + BsrGI (ends-out) and regenerants were selected on hygromycin.* [b]*2-FA^R clones are the stable Hyg^R clones that experienced a gene targeting event and thus survived after subculture on 2-FA medium. Differences between wild type and the mutants were compared using Fisher's exact test. *Correspond to p-value <0.01.* [c]*GTF (in %) express the frequency of 2-FA resistant among the population of regenerants.* [d]*GTE (in %) express the frequency of 2-FA resistant among the population of stably transformed plants (Hyg^R).* [e]*Average and standard deviation were determined from at least two independent experiments.*

construct is slightly but significantly higher (Fisher exact test p-value = 0.01) compared to GTF observed using the ends-out targeting construct containing the selection marker that creates a central region of heterology (**Figure 3**). GTFs observed using this ends-out targeting construct in the *xpf*Δ and *ercc1*Δ mutants were reduced by 1.7 and 1.4-fold respectively compared to wild type (**Figure 3**). This reduction is very similar to the one observed using the ends-out targeting construct containing the selectable marker. These results show that the ERCC1 and XPF proteins are necessary for gene targeting of an ends-out type targeting construct even in the absence of a large loop of mismatches in its central region.

Gene Targeting Using an Ends-In Construct Is Affected in the *xpf*Δ and *ercc1*Δ Mutants

In *S. cerevisiae* and in hamster cells (CHO), the RAD1-RAD10/XPF-ERCC1 is involved in removing long non-homologous tails from the 3′ ends of invading strands from ends-in gene targeting constructs (Schiestl and Prakash, 1988, 1990; Adair et al., 2000; Sargent et al., 2000). In Arabidopsis, XPF (RAD1) and ERCC1 (RAD10) have been shown to play a role in intermolecular recombination between plasmids by removing non-homologous 3′ ends from recombination intermediates (Dubest et al., 2002, 2004). In order to test the role of the moss XPF-ERCC1 complex in gene targeting using an ends-in targeting substrate with homologous ends GT frequencies (GTF) of an ends-in targeting construct (**Figure 4**, PpAPT-KO9 digested by *Xba*I), were measured in the wild type and *xpf*Δ or *ercc1*Δ mutants. GTF observed in the wild type using this ends-in targeting construct is slightly (1.2-fold) but significantly lower (**Supplementary Table S2**, Fisher exact test p-value = 0.014) compared to GTF observed using the ends-out targeting construct. GTF, reaches 0.59% in the wild-type and is reduced by 1.2 and 1.4-folds in the *xpf*Δ, and *ercc1*Δ mutants respectively (**Figure 4** and **Supplementary Table S2**, Fisher's test p-value ≤ 0.05). Use of an ends-in targeting substrate with heterologous ends strongly decrease the GTF in the wild type and the two mutants (**Figure 4** and **Supplementary Table S2**). These results show that the ERCC1 and XPF proteins are also involved in gene targeting of an ends-in type targeting construct and that presence of heterologous sequences at the 5′ and 3′ extremities of this type of construct is very detrimental for the efficiency of gene targeting.

The Nature of Targeted Integration Is Modified in the *xpf*Δ and *ercc1*Δ Mutants

If, as in yeast, *P. patens* stable transformants can result from a double recombination at both ends of the ends-out type targeting fragment leading to TGR, another type of integration, named targeted gene insertions (TGI, **Supplementary Figure S6**), can be found in *P. patens*, like in ES cells (Kamisugi et al., 2006). In order to test the role of the PpXPF-PpERCC1 complex in the formation of TGI, we measured, using a PCR based approach, the ratio of targeted gene replacement (TGR) versus targeted gene insertion (TGI) in $2FA^R$ plants obtained after transformation with the ends-out targeting substrate (PpAPT-KO2 digested *Eco*RI/*Hin*dIII, **Table 2** and **Figure 3**) in the wild-type and *xpf* backgrounds. For the wild-type, 79% of targeted transformants were identified as TGR and 21% as TGI, while in the *xpf*Δ mutant, a statistically higher number of the transformants (Fisher's exact test $P = 0.005$) were identified as TGR (93%) and 7% as TGI (**Figure 5A**). These findings show that XPF and probably the XPF-ERCC1 complex is an important factor in the mechanism leading to TGI in *P. patens*.

Another marked difference between *S. cerevisiae* and *P. patens* concerning the type of integration is the fact that insertion of concatenated copies of the donor cassettes is frequent in GT experiments in *P. patens* (**Supplementary Figure S6**; Kamisugi et al., 2006). These concatenates result probably from episomally replicating DNA (Murén et al., 2009) a characteristic that have been used recently for complementation of an auxotrophic marker in *P. patens* (Ulfstedt et al., 2017). In order to clarify the role of the PpXPF-PpERCC1 complex in the formation of targeted gene replacement with head-to-tail multicopy, we measured, using a PCR based approach, the number of monocopy TGR (**Supplementary Figure S6**) in $2FA^R$ plants obtained after transformation with the ends-out targeting substrate (PpAPT-KO9 digested *Eco*RI/*Hin*dIII, **Figure 3**) in the wild-type and mutants backgrounds. The percentage of monocopy integration in the WT and in the *xpf*Δ and *ercc1*Δ mutants was 21, 36, and 40% respectively (**Figure 5B**). Therefore the proportion of monocopy TGR is significantly more important in the *xpf*Δ and *ercc1*Δ mutants compared to the wild-type (chi-squared test $P < 0.05$).

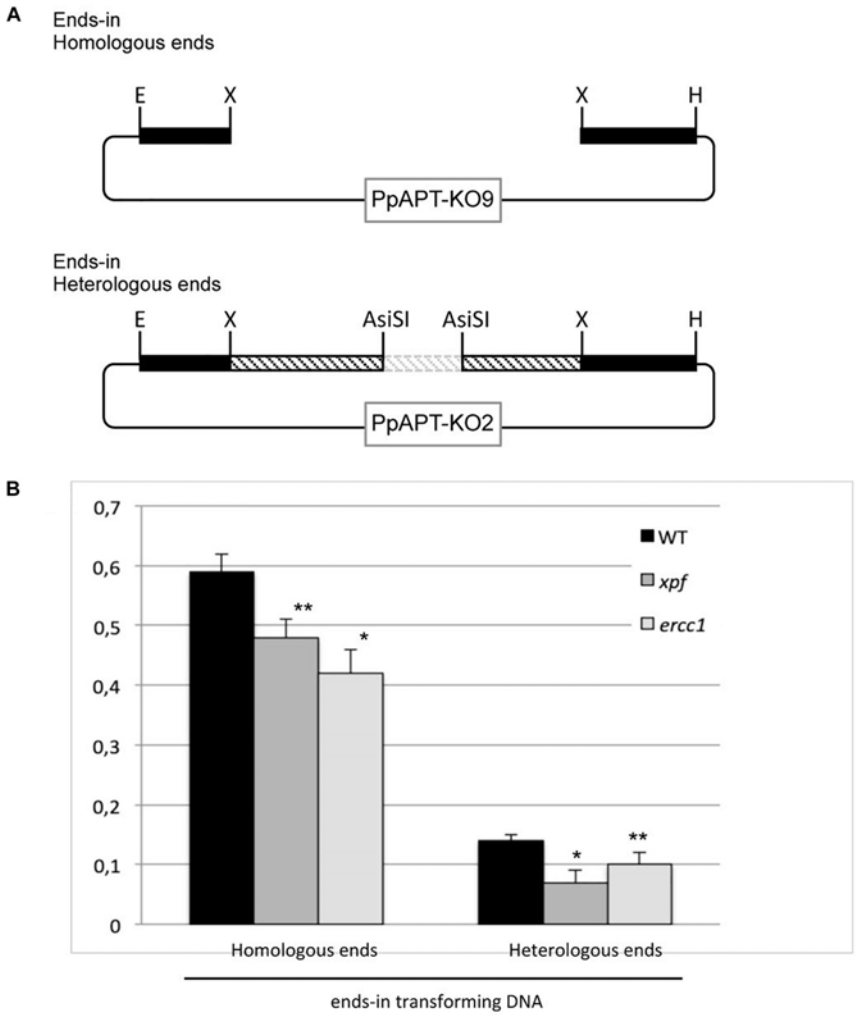

FIGURE 4 | Gene targeting frequency in WT and *xpf* and *ercc1* mutants using ends-in type vectors. **(A)** Different forms of the transforming DNA used. Digestion of PpAPT-KO9 with *Xba*I give rise to ends-in transforming DNA, with double strand breaks at the edges of *APT* sequences. Digestion of PpAPT-KO2 with *Asi*SI generates ends-in transforming DNA with heterologous sequence at the double strand breaks (dashed bars: heterologous stretches, thin lines: plasmid sequences, dotted lines: plasmid sequences absent from the transforming DNA). **(B)** Gene targeting frequency of the *APT* gene in the wild type, and *xpf* and *ercc1* mutants using different ends-in types transforming DNA. Asterisks indicate significant differences with the WT (*Fisher exact test *p*-value ≤0.01; **Fisher exact test *p*-value ≤0.05).

We can conclude from these results that the nature of the targeted integrations is altered in these mutants. The PpERCC1 and PpXPF proteins are involved in the mechanism that results in the integration of the ends-out targeting construct via TGI and in the integration of concatemers. Nevertheless, TGI type integrations and concatemers integrations can be detected in the mutants contexts, implying that other nucleases can partially complement the absence of XPF and ERCC1 for these mechanisms in *P. patens*.

DISCUSSION

We report here the identification and characterization of the *P. patens* mutant for the *XPF* and *ERCC1* genes. The *xpf* and *ercc1 P. patens* mutants are viable and show no phenotypic defect under normal conditions, in agreement with what was observed for the Arabidopsis *xpf* and *ercc1* mutants (Hefner et al., 2003; Preuss and Britt, 2003; Dubest et al., 2004), but in contrast with the situation observed in mammalian cells, where null mutants of the ERCC1 or XPF genes are lethal (McWhir et al., 1993; Núñez et al., 2000; Hsia et al., 2003; Tian et al., 2004). Like their Arabidopsis counterparts the *xpf* and *ercc1 P. patens* mutants are fully fertile suggesting that in plants, like in *S. cerevisiae*, this complex has only a minor role, if one, in meiosis which is in contrast with what is observed in Drosophila or *C. elegans* where the homologs of XPF1 have been shown to be involved in meiotic crossover formation (Sekelsky et al., 1995; Radford et al., 2005; Agostinho et al., 2013; O'Neil et al., 2013; Saito et al., 2013).

The *P. patens xpf* and *ercc1* mutants present a strong increase in sensitivity to UV-B compared with the wild type. These

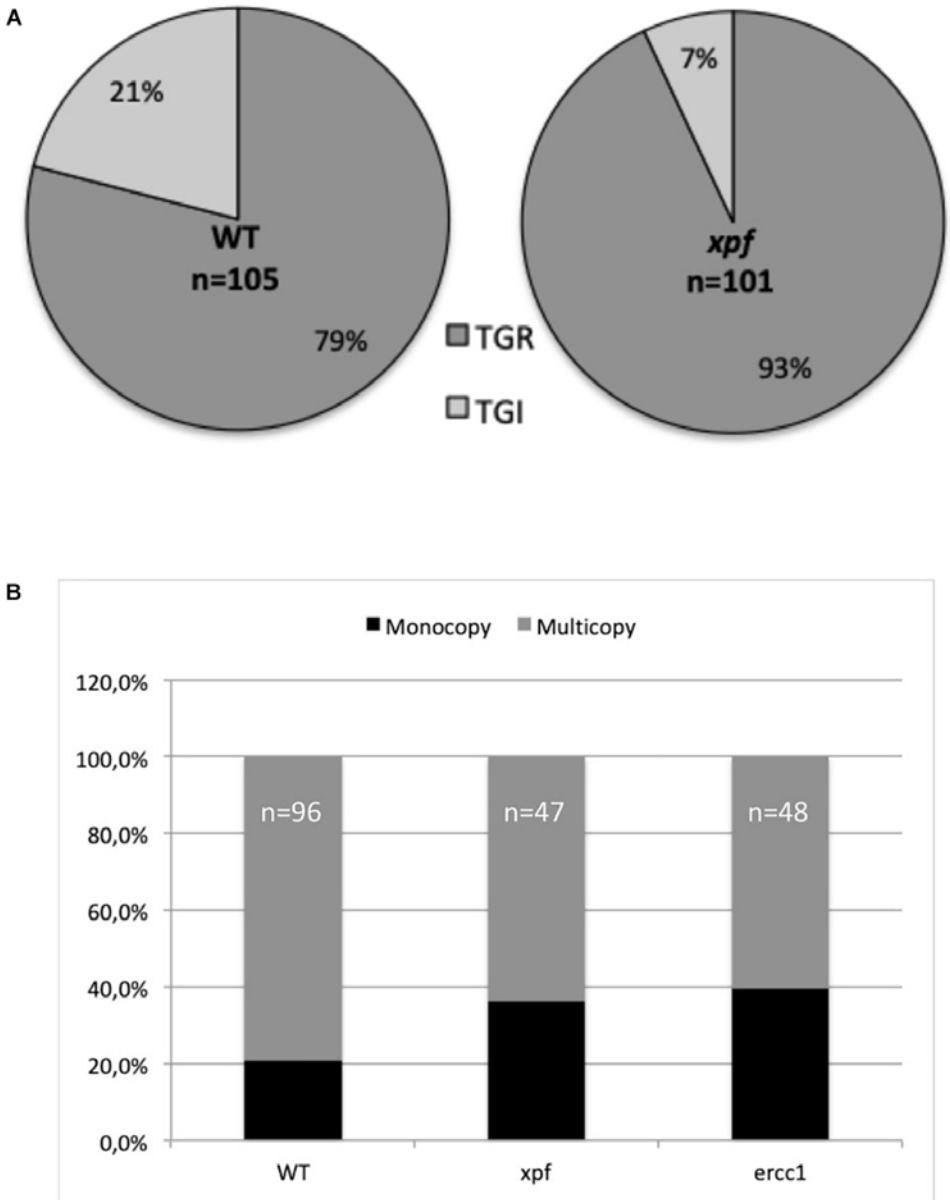

FIGURE 5 | Type of integrations at the *APT* locus using an ends-out construct for the wild-type (WT) and mutants. **(A)** Rate of targeted gene replacement (TGR) vs. targeted gene insertion (TGI) integrations was estimated using primers specific to the PpAPT-KO2 cassette and primers located on the *PpAPT* gene but outside the genomic fragments present on the donor DNA cassette in WT and *xpf* mutant (see section "Materials and Methods," **Figure 3** and Supplementary Figure S6). **(B)** Rate of monocopy vs. multicopy insertions at the targeted locus was estimated in WT and *xpf* and *ercc1* mutants, using primers located outside the sequences homologous to the gene fragments present in the PpAPT-KO9 donor DNA template (see section "Materials and Methods," **Figure 3** and Supplementary Figure S6).

results are consistent with previous studies on this complex in yeast (Prakash et al., 1993), animals (Gregg et al., 2011), and in *Arabidopsis thaliana* (Fidantsef et al., 2000; Liu et al., 2000; Hefner et al., 2003; Dubest et al., 2004; Biever et al., 2014) and confirm the requirement of the XPF-ERCC1 complex in the NER pathway in plants. Interestingly, the frequency of spontaneous mutations (mutator rate) in the *P. patens xpf* and *ercc1* mutants is very high compared to wild type (100-fold increase) and is even higher than the one found in the *rad51* mutant background, depleted for homologous recombination (Schaefer et al., 2010). This is reminiscent of what is observed in the *S. cerevisiae rad1* mutant where an increases in the frequencies of single-base-pair substitution, single-base-pair deletion and insertion of the yeast retrotransposon Ty have been described (Kunz et al., 1990). It must be noticed that the fold increase in spontaneous mutations in the *xpf*/*rad1* mutants backgrounds is significantly higher in *P. patens* compared to *S. cerevisiae* (Kunz et al., 1990; Doetsch et al., 2001). These

data demonstrate the essential role of the XPF/ERCC1 complex in genome stability in *P. patens* and could potentially reflect a more prominent role of the NER pathway in genome stability in *P. patens* compared to *S. cerevisiae*. In addition, we have shown here that XPF-ERCC1 is also important for the response to MMS and the recent observation in *P. patens* that the *rad51* mutant is more sensitive to MMS compared to WT (Goffová et al., 2019) reinforces the hypothesis that the HR machinery, and the XPF-ERCC1 complex would contribute to the repair of damages that would result from an MMS treatment, the exact nature of these damages being still unclear (Wyatt and Pittman, 2006). The role of the *P. patens* XPF/ERCC1 complex in these repair pathways could also be the cause of the important genetic instability observed in the corresponding mutants background.

There is good evidence for a role of XPF-ERCC1 in repair of double strand break through homologous recombination (Ahmad et al., 2008). We show here that the *P. patens* XPF-ERCC1 complex is involved in gene targeting using an ends-in construct and is also, and potentially even more important, for gene targeting using an ends-out construct. Concerning the ends-in construct the role of the *P. patens* XPF-ERCC1 complex could be, as in *S. cerevisiae* and in hamster cells (CHO), to remove the non-homologous tails from the 3′ ends of invading strands (Schiestl and Prakash, 1988, 1990; Adair et al., 2000; Sargent et al., 2000), reminiscent of the function of the endonuclease complex in SSA. It is more difficult to propose this function to explain the importance of the complex in gene targeting of the ends-out construct during TGR. This role of the XPF-ERCC1 complex for ends-out gene targeting in *P. patens* is of particular interest and should be consider in the light of other observations in other models. Indeed, in Arabidopsis ERCC1 has been proposed to be involved not only in SSA recombination as measured by a plasmid assay, but also in gene conversion/crossing over in chromosomal DNA (Dubest et al., 2004) and in mouse cells this complex is essential for ends-out gene targeting in the absence of non-homologous overhangs (Niedernhofer et al., 2001), implying a more general role for this endonuclease in recombination than the removal of non-homologous DNA overhangs from recombination intermediates. The endonuclease complex has also been shown to be important for TGR using ends-out targeting constructs in *S. cerevisiae* and separate studies have reported TGR efficiency decrease, ranging from a 3- to 40-fold reduction in *rad1* and *rad10* mutants (Schiestl and Prakash, 1988, 1990; Schiestl et al., 1994; Saparbaev et al., 1996; Symington et al., 2000; Langston and Symington, 2005). When using a classical ends-out gene targeting construct one possible role for the endonuclease could be the resolution of the large loop of mismatches that forms between the targeted gene and the heterologous selectable marker (that separate de 5′ and 3′ regions of homology of an ends-out construct) during the two ends invasion process of TGR (Niedernhofer et al., 2001; Langston and Symington, 2004, 2005). We could show here that the importance of the *P. patens* XPF-ERCC1 complex for targeted integration of the ends-out construct is not affected by the presence or the absence of an heterologous selectable marker.

In order to better understand the role of the *P. patens* XPF-ERCC1 complex in TGR using an ends-out construct we have compared the nature of the TGR events found in the wild type and in the mutants. As observed previously (Kamisugi et al., 2006), we could identify several types of gene targeting event (**Supplementary Figure S6**): (i) TGR, in which the targeted locus is replaced by a single copy of the transforming DNA (HR/HR) (ii) this may involve insertion of multiple copies (concatemers) of the targeting construct and (iii) "one-end gene targeting" or TGI, that may result from an homologous recombination event at one end of the construct accompanied by an apparent non-homologous end-joining event at the other (HR/NHEJ) (iv) this process may also involve insertion of multiple copies of the targeting construct. We could show here that the XPF-ERCC1 complex is more specifically involved in the mechanism that results in the integration of the targeting construct via TGI and in the integration of concatemers. The decrease in the number of concatemers and TGI events in the *xpf* or *ercc1* mutants context could explain, at least for a part, the general decrease in gene targeting efficiency observed using a ends-out type construct in absence of the XPF-ERCC1 complex. One hypothesis to explain the role of the complex in the formation of TGI and concatemers events could be its potential role in the handling of the looped-out heteroduplex intermediates (**Supplementary Figure S6**) formed during the invasion process in presence of the concatemers that are produced before integration at the targeted *APT* locus. In this context, and taking into consideration our recent data showing the involvement of the POLQ protein in TGI events formation in *P. patens* (Mara et al., 2019), it would be interesting to check for a potential interaction between the XPF-ERCC1 complex and the Alt-EJ pathway for the targeted integration of ends-out construct in this moss. Such a cross-talk between the RAD1-RAD10 complex and the Alt-EJ repair pathway, that, like the SSA pathway, involves the removal of non-homologous 3′ tails, has already been proposed in yeast and more recently in animals (Ma et al., 2003; Sallmyr and Tomkinson, 2018).

We have shown here an essential role of the XPF-ERCC1 endonuclease complex in genetic stability of the model plant *P. patens*. Moreover, we have shown for the first time in plants, the implication of this endonuclease in gene targeting through ends-in or ends-out constructs. If the role of this complex in ends-in construct integration can be easily explained by the capacity of this endonuclease to remove non-homologous 3′ ends tails the exact role of the complex in integration of ends-out type constructs is still puzzling and further work is needed. Different functions of the XPF-ERCC1 complex and at different steps of the process of targeted integration of ends-out constructs could be involved. These functions could be shared by other organisms, like yeast and animal cells, where this endonuclease has also been shown to be important. However, more specific roles, due to specificities of the mechanism of targeted

integration in the different species, could be involved. One of these could consist in the removal of the "apparent" heterologous regions formed during concatemers production in *P. patens*. Existence of this putative mechanism in other species, and especially in flowering plants is an open question and one must take into consideration that *P. patens* has an intrinsic high level of homologous recombination that could lead to functions of the XPF-ERCC1 complex that would be specific to this moss. Nevertheless, deciphering of the shared and specific roles of the XPF-ERCC1 complex in integration of the ends-out type constructs in different species is important to better understand the action of this endonuclease in genome maintenance and could have also potential applications in order to improve the efficiency and/or the quality of gene targeting for applied research.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

FN, DS, AG-D, and J-MN designed the research. AG-D and PR performed the research with the help of FC, AE, and DS. FN, AG-D, DS, and J-MN wrote the manuscript with contributions from all the authors.

FUNDING

This work was supported by the Agence Nationale de la Recherche (ANR-09-GENM-402 and ANR-11-BTBR-0001-GENIUS). The IJPB benefits from the support of the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00588/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 17 May 2019
doi: 10.3389/fpls.2019.00653

The Plant DNA Damage Response: Signaling Pathways Leading to Growth Inhibition and Putative Role in Response to Stress Conditions

*Maher-Un Nisa, Ying Huang, Moussa Benhamed and Cécile Raynaud**

Institute of Plant Sciences Paris-Saclay, IPS2, CNRS-INRA-University of Paris Sud, Paris-Diderot and Evry, University of Paris-Saclay, Gif-sur-Yvette, France

Maintenance of genome integrity is a key issue for all living organisms. Cells are constantly exposed to DNA damage due to replication or transcription, cellular metabolic activities leading to the production of Reactive Oxygen Species (ROS) or even exposure to DNA damaging agents such as UV light. However, genomes remain extremely stable, thanks to the permanent repair of DNA lesions. One key mechanism contributing to genome stability is the DNA Damage Response (DDR) that activates DNA repair pathways, and in the case of proliferating cells, stops cell division until DNA repair is complete. The signaling mechanisms of the DDR are quite well conserved between organisms including in plants where they have been investigated into detail over the past 20 years. In this review we summarize the acquired knowledge and recent advances regarding the DDR control of cell cycle progression. Studying the plant DDR is particularly interesting because of their mode of development and lifestyle. Indeed, plants develop largely post-embryonically, and form new organs through the activity of meristems in which cells retain the ability to proliferate. In addition, they are sessile organisms that are permanently exposed to adverse conditions that could potentially induce DNA damage in all cell types including meristems. In the second part of the review we discuss the recent findings connecting the plant DDR to responses to biotic and abiotic stresses.

Keywords: cell cycle checkpoint, DNA damage, biotic and abiotic stress, genome integrity, plants

OPEN ACCESS

Edited by:
Kaoru Okamoto Yoshiyama, Tohoku University, Japan

Reviewed by:
Thomas Eekhout, VIB-UGent Center for Plant Systems Biology, Belgium
Naoki Takahashi, Nara Institute of Science and Technology (NAIST), Japan

***Correspondence:**
Cécile Raynaud
cecile.raynaud@u-psud.fr

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *01 April 2019*
Accepted: *30 April 2019*
Published: *17 May 2019*

Citation:
Nisa M-U, Huang Y, Benhamed M and Raynaud C (2019) The Plant DNA Damage Response: Signaling Pathways Leading to Growth Inhibition and Putative Role in Response to Stress Conditions. Front. Plant Sci. 10:653. doi: 10.3389/fpls.2019.00653

INTRODUCTION

Maintenance of genome integrity is essential in all living organisms. It is required for proper development and for faithful transmission of the genetic information from one generation to the next. Yet, cells are constantly subjected to DNA damage. One major source of mutations is DNA metabolism itself, both during DNA replication and DNA repair. The error rate of the replication machinery is estimated in the range of 10^{-7} to 10^{-8}. This low error rate results from the fidelity of replicative polymerases, which have an error rate between 10^{-6} and 10^{-8}, and the successful excision of 90–99% of mis-paired bases thanks to the proof-reading activity of these complexes (Kunkel, 2004). DNA repair processes can also introduce errors, with a similar rate as replication when they involve proof-reading polymerases, or with a higher rate when they involve

alternative polymerases (Kunkel, 2004; Jain et al., 2018). Finally, unrepaired lesions can block the main replicative polymerases; in that case, TransLesion Synthesis (TLS) Polymerases, take over (Uchiyama et al., 2009). They interact with each other, and are thought to form a large complex at stalled forks to allow choosing the best suited polymerase for each type of lesion (Powers and Washington, 2018). Their ability to replicate DNA passed lesions makes them error-prone: their substitution rate when replicating undamaged templates is comprised between 10^{-3} and 10^{-1} (Kunkel, 2004). In addition, DNA demethylation can also cause mutations because it requires nucleotide removal followed by Base Excision Repair (BER) (He et al., 2011).

Being sessile organisms, plants are constantly exposed to stress conditions that can also damage their DNA. Indeed, plants need light to grow photo-autotrophically, but UV light induces DNA damage, notably in the form of cyclobutane pyrimidines (CPDs). Likewise, the photosynthetic apparatus generates Reactive Oxygen Species (ROS), especially when plants are exposed to excess light, either because the intensity is very high, or when other external conditions such as heat or drought reduce the plant's capacity to consume the reducing power produced by light absorption in photosystems (Noctor and Foyer, 2016). Very few studies have estimated the frequency of DNA lesions in plant cells. In Human cells, DNA lesions caused by spontaneous hydrolysis or ROS occur at a frequency ranging from a few hundreds to over 10^5 per cell, depending on the type of damage (Bray and West, 2005). In maize, the number of apurinic/apyrimidic sites formed in root tips during the first 20 h of seed imbibition was estimated to 3.75×10^5 per genome and per cell. Thus, although detailed quantification of DNA damage occurring in plant cells is missing, DNA damage can be considered as a frequent event under normal conditions, and likely even more so in response to various stress conditions.

In spite of the high frequency of DNA damage occurring in plant cells, the estimated mutation rate is very low. Through whole genome sequencing of Arabidopsis lines propagated from single seed descent for 25–30 generations, the genome-wide average mutation rate was estimated around 7×10^{-9} per site per generation (Ossowski et al., 2010; Weng et al., 2019). This figure corresponds to less than one single mutation in the entire genome per generation, and is at least 10 times lower than the error rate of the replication machinery for a single cell cycle. This provides striking evidence for the efficiency with which DNA Damage is detected and repaired in the cell. DNA lesions can be repaired through multiple pathways that have been reviewed elsewhere and will not be described into detail here (Amiard et al., 2013; Manova and Gruszka, 2015; Spampinato, 2017). Briefly, most lesions, such as UV-induced CPDs, mismatches, etc., are sensed and repaired by dedicated machineries such as photolyases, or complexes involved in mismatch repair, BER or Nucleotide Excision Repair (NER) (Jackson and Bartek, 2009; Manova and Gruszka, 2015; Spampinato, 2017). However, if incorrectly repaired, all these lesions can hamper DNA replication or cause double strand breaks (DSBs) that require specific DNA repair pathways such as Non-homologous End Joining (NHEJ) or Homologous Recombination (HR) (Amiard et al., 2013). In that case a sophisticated signaling process called the DNA Damage Response (DDR) allows activation of cell cycle checkpoints and of specific DNA repair mechanisms (Yoshiyama et al., 2013b; Hu et al., 2016). The DDR is highly conserved between eukaryotes with some variations that will be briefly discussed below. Its ultimate outcome will depend on the severity of the DNA lesions and the efficiency of the repair process: cell cycle activity can resume if lesions are successfully repaired, but more severe DNA damage can induce endoreduplication (Adachi et al., 2011). This process corresponds to several rounds of DNA replication without mitosis, leading to an increase in nuclear DNA content; it is widely distributed in plants such as in *Arabidopsis* leaves or stems, fruits, and endosperm in cereals (Galbraith et al., 1991), and is associated with cell differentiation and enlargement (Kondorosi et al., 2000). In the context of the DDR, it is thus seen as a permanent differentiation, thereby avoiding the proliferation of cells with damaged DNA. Interestingly, endoreduplication also exists in animals although it is not as common as in plants, and can be triggered by DNA damage, and could thus be a conserved response in eukaryotes (Fox and Duronio, 2013). Finally, depending on the cell type and the severity of damage, DDR activation can result in programmed cell death (PCD) (Furukawa et al., 2010). Interestingly, plant stem cells are particularly sensitive to DNA damage and prone to enter cell death (Fulcher and Sablowski, 2009), suggesting that specific mechanisms are at work to protect meristems from accumulating mutations.

The DDR signaling pathway has received extensive attention in Mammals due to its relevance in the field of cancer research, but has also been studied into details in plants for about 15–20 years. In this review we will summarize the recent advances on the plant DDR. We will focus exclusively on the DDR signaling events and cell cycle regulation, but will not discuss the complex mechanisms involved in DNA repair that have been reviewed elsewhere (Manova and Gruszka, 2015; Spampinato, 2017). Next, we will explore the emerging connection between DDR and biotic and abiotic stress responses. Indeed, even though DDR is likely activated in response to a wide range of stress conditions and could account for some of the negative effects of stress on cell division, it has to date little been studied in the context of plant response to stress, with most studies using genotoxic to trigger the DDR.

MAIN PLAYERS IN DDR SIGNALING

ATM and ATR, the Main DNA Damage Sensors

It is now well established that the general organization of the DDR signaling cascade is conserved between plants and animals. **Figure 1** summarizes our current knowledge of the plant DDR. In animals, DDR activation relies on two protein kinases, called Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR), both of which belong to the phosphatidylinositol 3-kinase-like family (Maréchal and Zou, 2013). ATM primarily responds to DSBs whereas ATR is activated by single stranded DNA and defects in replication fork progression (Maréchal and Zou, 2013); both proteins activate downstream components of

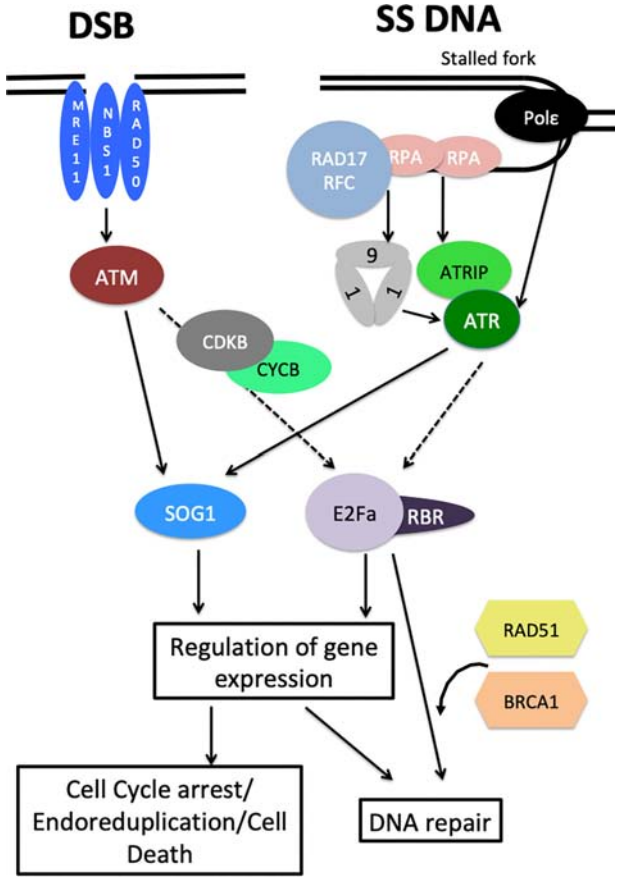

FIGURE 1 | Overview of the plant DDR. DSBs activate ATM signaling through the MNR (MRE11 NBS1 RAD50) complex while ATR is recruited to single stranded DNA by RPA proteins via ATRIP, and activated by the 9-1-1 and RAD17/RFC complexes. ATR can also be activated by DNA Polymerase ε through an unknown mechanism. Both ATM and ATR signaling converge to the SOG1 transcription factor that controls the expression of hundreds of genes involved in cell cycle regulation, cell death control, and DNA repair. E2Fa/RBR complexes also control DNA repair by regulating DNA repair genes and by recruiting RAD51 and BRCA1 at DNA damage sites. The role of E2F/RBR complexes in DDR depends on CYCB1/CDKB and ATM/ATR activity, but the exact molecular mechanisms are unknown. Dashed arrows represent putative/possibly indirect regulations.

the DDR. Arabidopsis homologs of ATR and ATM were isolated in the early 2000s (Garcia et al., 2003; Culligan et al., 2004), based on their sequence conservation with their counterparts in animal and yeast. Interestingly, Arabidopsis *atr* mutants are viable, in sharp contrast with *atr*-deficient mice that stop development at an early stage of embryogenesis (Culligan et al., 2004), which facilitated the functional dissection of ATR and ATM functions in plants. Like their animal homologs, ATM and ATR play both distinct and additive roles in response to DNA damage, both mutants being hypersensitive to DSBs induced by γ-irradiation whereas only *atr* is required for replicative stress response (Culligan et al., 2006). Recently, quantitative phosphoproteomics allowed the identification of hundreds of proteins that are differentially phosphorylated in response to genotoxic stress in an ATM/ATR dependent manner (Roitinger et al., 2015). This study highlighted the large number of ATM/ATR targets and thus their central role in coordinating DNA replication, DNA repair and gene expression in response to genotoxic stress.

Because they recognize different types of lesions, ATM and ATR are activated through different mechanisms. Like in animals and yeast, the plant ATM is activated by the MRN complex (MRE11, RAD50, and NBS1) that recognizes DSBs (Puizina et al., 2004; Waterworth et al., 2007; Amiard et al., 2010). In animals, ATR responds to a large variety of genotoxic stresses that all have in common to slow down DNA polymerases, leading to the accumulation of single stranded DNA. This single stranded DNA coated with the RPA (Replication Protein A) heterotrimeric recruits ATRIP (ATR Interacting Protein) which in turn facilitates the recruitment of ATR (Saldivar et al., 2017). ATR is then activated by a number of factors including the 9-1-1 complex (RAD9, RAD1, and HUS1), that is loaded on damaged DNA by the RAD17 replication Factor C 2-5 sub-units (RFC) (Saldivar et al., 2017). Furthermore in yeast, DNA Polymerase ε can directly contribute to ATR activation (García-Rodríguez et al., 2015), but whether this function is conserved in animals is unclear. The plant ATRIP protein has been identified (Sweeney et al., 2009), as well as the components of the 9-1-1 complex and RAD17 (Heitzeberg et al., 2004). It is worth noting that in plants, RPA sub-units are encoded by small multi-gene families that appear to have specialized functions in DNA replication or DDR signaling (Aklilu et al., 2014). In addition, the plant DNA Pol ε was shown to play a role in replicative stress sensing upstream of ATR, as observed in budding yeast (Pedroza-Garcia et al., 2017).

Both the ATR and the ATM pathways lead to the accumulation of γH2AX (a phosphorylated histone variant) at DNA damage sites (Amiard et al., 2010), which is instrumental for the recruitment of signaling and repair factors (Kinner et al., 2008). Intriguingly, plant *atr mre11* double mutants display a high frequency of anaphase bridges despite the complete absence of γH2AX accumulation, indicating that plants can repair DSBs in the absence of ATR and ATM activation (Amiard et al., 2010) but the underlying mechanisms remain to be fully elucidated.

Signaling Downstream of ATM and ATR Through the Central Integrator SOG1

In animals, the ATR and ATM branches of DDR signaling converge to activate the p53 tumor suppressor, a transcription factor that controls both DNA repair and cell cycle arrest (Yoshiyama et al., 2013b). Plant genomes lack a p53 homolog, but its functional equivalent was isolated through a genetic screen for suppressors of the growth arrest induced by γ-irradiation in the *uvh1* (UV-hypersensitive 1) mutant, that is deficient for the DNA repair endonuclease XPF (Xeroderma Pigmentosum complementation group F) (Preuss and Britt, 2003). Suppressor Of Gamma-response 1 (SOG1), is a transcription factor of the NAC (NAM, ATAF1/2, and CUC2) family and is the central regulator of the plant DDR (Yoshiyama et al., 2009). It is expressed predominantly in meristems and in vascular tissues (Yoshiyama et al., 2013a), and accounts for all the short-term transcriptional changes induced by

γ-irradiation (Yoshiyama et al., 2009). Genetic analysis revealed that *atm* and *atr* are partially redundant for the induction or endoreduplication or cell death in response to DNA damage, whereas SOG1 is strictly required (Furukawa et al., 2010; Adachi et al., 2011), which led to a model according to which SOG1 is the central integrator of DDR in plants (Hu et al., 2016). SOG1 is rapidly phosphorylated in response to DNA damage and is a direct target of ATM (Yoshiyama et al., 2013a) and ATR (Sjogren et al., 2015). This represents another difference between plant and animal DDR signaling, since in animals the CHK1 and CHK2 (Check point) kinases act as intermediates between ATR or ATM and p53, whereas genes encoding these kinases appear to be absent for plant genomes (Yoshiyama et al., 2013b). Recent genome-wide analyses of SOG1 targets confirmed the central role of SOG1 in the early transcriptional response to DSBs, placing SOG1 at the top of the regulatory DDR network (Bourbousse et al., 2018; Ogita et al., 2018). Surprisingly, quantitative phosphoproteomics allowed the identification of hundreds of proteins that are differentially phosphorylated in response to genotoxic stress in an ATM/ATR dependent manner (Roitinger et al., 2015) but failed to identify SOG1, possibly due to unfavorable peptide cleavage or to the fact that this study used mature rosettes while *SOG1* is mainly expressed in meristematic tissues (Yoshiyama et al., 2013a).

SOG1 is a transcription activator that controls the expression of DNA repair genes and cell cycle regulators (Bourbousse et al., 2018; Ogita et al., 2018). Here, we will focus on the mechanisms leading to cell cycle checkpoint activation. Depending on the phase of the cell cycle at which DNA damage occurs, cell can stop either in S phase or in G2. Replicative stress activates an intra-S checkpoint that is dependent on SOG1 and WEE1 (De Schutter et al., 2007; Cools et al., 2011; Hu et al., 2015), a protein kinase that stops the cell cycle through an inhibitory phosphorylation of Cyclin Dependent Kinases (CDK). SOG1 can also induce a G2 arrest of the cell cycle through several mechanisms. First, together with ATR, SOG1 was shown to control proteasome-dependent degradation of the mitotic CDKB2;1 (Adachi et al., 2011); second, SOG1 controls the expression of genes encoding negative cell cycle regulators such as the CDK inhibitors SMR5 and SMR7 that induce endoreduplication (Yi et al., 2014). Furthermore, the WEE1 kinase inhibits CDK activity (De Schutter et al., 2007; Cools et al., 2011; Cook et al., 2013), thereby inhibiting the G2/M transition, and SOG1 also stimulates the expression of the G2-specific CYCLINB1, a mechanism that has been proposed to delay mitosis, although it likely also reflects the specific involvement of CYCB1;1 in DNA repair (Schnittger and De Veylder, 2018). More recently, the full analysis of SOG1-dependent transcriptome changes induced by DNA damage, further revealed that SOG1 partly acts through the activation of MYB3R repressors that inhibit the expression of G2/M cell cycle genes (Bourbousse et al., 2018). MYB3R transcription factors are well known regulators of the G2/M transition, MYB3R4 being an activator, MYB3R3 and 5 repressors, and MYB3R1 behaving either as an activator or as a repressor depending on its interacting partners (Haga et al., 2011; Kobayashi et al., 2015a,b). Recently, Chen et al. (2017) demonstrated that repressor MYB3Rs (Rep-MYB3R) are essential for the growth inhibition induced by DNA damage: in response to zeocin treatment, the MYB3R3 protein accumulates in root meristems, thereby preventing cell proliferation by inducing a G2 arrest. In this work, authors showed that MYB3R3 is phosphorylated by CDKs and that this phosphorylation promotes its proteasomal degradation. Thus, reduction of CDK activity due to CDK inhibitors induction likely contributes to the accumulation of Rep-MYB3Rs in response to DNA damage. Together, these observations shed new light on the mechanisms underlying the SOG1-dependant repression of CDKB2;1 accumulation. Indeed, SOG1 positively regulates activators of the Anaphase Promoting Complex/Cyclosome (APC/C) (Bourbousse et al., 2018). The down-regulation of CDKB2;1 in response to DNA damage could thus result from the concomitant degradation of the protein by the APC/C and repression of the CDKB2;1 gene by Rep-MYB3Rs. Very recently, the ANAC044 and ANAC085 transcription factors, the two SOG1 closest relatives that are also SOG1 targets (Ogita et al., 2018), were reported to promote rep-MYB3R accumulation in response to DNA damage (Takahashi et al., 2019). Genetic analysis showed that ANAC044 and ANAC085 function in the same pathway as SOG1 to control cell cycle arrest through rep-MYB3R accumulation but not activation of *SMR* genes or DNA repair genes. To date, it remains unclear how ANAC044 and ANAC085 modulate Rep-MYB3R protein levels, as they do not directly target *Rep-MYB* genes, but this pathway could involve the regulation of proteins involved in the degradation of Rep-MYBs such as F-box proteins (Takahashi et al., 2019). **Figure 2** summarizes how DDR triggers cell cycle arrest either in S phase or in G2 phase, and can lead to cell differentiation and endoreduplication.

EF2/RBR Complexes: New Players in the Plant DDR

Despite this central role of SOG1, recent studies have revealed SOG1-independent pathways in the plant DDR. The first evidence for SOG1-independent DDR response came from the genetic analysis of *wee1 sog1* double mutants, that showed enhanced sensitivity to replicative stress compared to the *sog1* mutant, providing evidence for a SOG1-independent mechanism that could lead to WEE1 activation (Hu et al., 2015). This hypothesis is further supported by the analysis of Arabidopsis mutants with partial deficiency in the replicative DNA Polymerase ε that suffer from constitutive replicative stress (Pedroza-Garcia et al., 2016, 2017). ATR and WEE1 are both essential for the survival of *abo4-1* mutants that are partially deficient for the Pol ε catalytic subunit, whereas the *abo4-1 sog1* double mutants are viable. Consistently, some DDR responsive genes are induced in a SOG1-independent manner in the *abo4-1 sog1* double mutants (Pedroza-Garcia et al., 2017). The underlying molecular mechanism remains unknown, but may involve E2F-RBR1 (RetinoBlastoma Related 1) complexes. These transcription regulators are well known both in plants and animals for controlling S-phase entry: RBR1 binds and inhibits E2F transcription factors thereby preventing the expression of S-phase genes (Berckmans and De Veylder, 2009). Upon activation of CYCD-CDKA complexes and cell cycle entry, RBR1

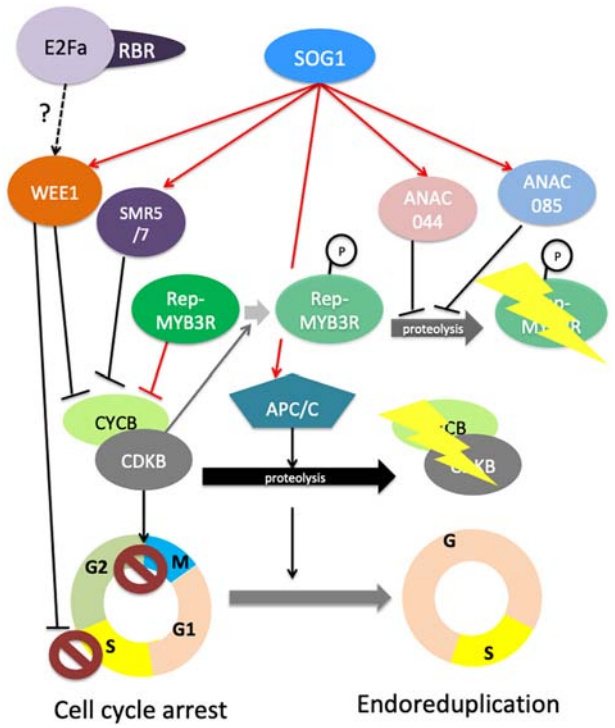

FIGURE 2 | Cell Cycle regulation in response to DNA damage. Under normal conditions, protein accumulation of repressive MYB3Rs (rep-MYB3R) is restricted to S-phase during which they repress the transcription of G2 genes including *CYCBs* and *CDKBs*. Rep-MYB3R accumulation is kept low during G2 to M through phosphorylation of these transcription factors by CYCB/CDKB complexes, leading to their degradation by the proteasome (shaded shapes and arrows). Upon DNA damage, SOG1 regulates the intra S and G2/M checkpoint by targeting the core cell cycle genes *WEE1*, *SMR5* and *7* and *APC/C* sub-units, and by indirectly controlling the accumulation of rep-MYB3Rs. How SOG1 acts on MYB3Rs degradation remains to be fully elucidated, but this pathway involved direct up-regulation of the genes encoding the ANAC044 and ANAC085 transcription factors. These two proteins influence repressive MYB3R accumulation through a mechanism that remains to be elucidated, although reduction of CYCB/CDKB complexes accumulation and activity likely contributes to this process by reducing MYB3R phosphorylation. WEE1 can inhibit S-phase progression by inhibiting the activity of CYCA/CDKA complexes. WEE1 and SMR5 and 7 can also inhibit the activity of CYCB1/CDKB complexes directly, whereas MYB3R and APC/C control the accumulation of the complex. Together, all these mechanisms contribute to lowering the activity of mitotic CDKs, leading to G2 arrest or endoreduplication. In addition to these SOG1-dependent mechanisms, E2Fa/RBR complexes likely contribute to the activation of cell cycle checkpoints possibly by regulating *WEE1* or *CYC* and *CDK* genes, but their role remains to be fully elucidated. On this figure, red arrows indicate direct transcriptional regulations whereas black arrows indicate indirect regulations.

is phosphorylated and E2F transcription factors function together with their Dimerization Partners (DP) proteins to activate the expression of genes involved in DNA replication, leading to the onset of S-phase (Gutzat et al., 2012). Besides its role in cell cycle regulation, E2Fa had been previously shown to control the expression of RNR (RiboNucleotide Reductase), an enzyme involved in deoxyribonucleotide biosynthesis that is strongly activated by DNA damage (Roa et al., 2009). Furthermore, E2Fa was shown to form foci at DNA damage sites (Lang et al., 2012). Two recent studies further substantiated the role of RBR1 and E2Fs in the plant DDR: a temperature sensitive *rbr1* mutant was shown to be hypersensitive to DNA damage, and to accumulate enhanced levels of DNA lesions in response to genotoxic stress (Biedermann et al., 2017), while *RBR1* silencing triggered DNA damage accumulation and cell death onset in root tips even in the absence of exogenous stress (Horvath et al., 2017). Intriguingly, RBR1 represses the expression of several DDR genes in a E2Fa-dependent manner (Biedermann et al., 2017; Horvath et al., 2017), and RBR1 deficiency could thus have been expected to improve the DNA repair capacity of the plant. However, authors also demonstrated that RBR1 localizes to DNA damage foci (Biedermann et al., 2017) together with E2Fa, and recruits the DNA repair proteins RAD51 (RADIATION SENSITIVE 51) and BRCA1 to the DNA damage site (Biedermann et al., 2017; Horvath et al., 2017). Thus E2F-RBR1 could play a dual role in the DDR (i) by controlling the expression of DDR genes, possibly to up-regulate their expression during S-phase and thereby enhance the repair activity at this specific phase of the cell cycle that triggers extensive DNA damage, and (ii) more directly by controlling the DNA repair process itself at specific sites (**Figure 1**). Moreover E2F/RBR complexes contribute to cell cycle checkpoint activation during DDR: loss of RBR results in enhanced cell death in response to genotoxic stress, suggesting that E2F/RBR complexes function antagonistically to SOG1 to restrict PCD (Biedermann et al., 2017). Further, since ATR and WEE1, but not SOG1 are required for the survival of Pol ε deficient mutants that display constitutive replicative stress, RBR/E2F complexes may play a role in the control of the intra-S checkpoint, possibly by controlling *WEE1* or *CDK/CYC* expression (**Figure 2**). In line with this hypothesis, RBR was found to target *WEE1* and a large number of core cell cycle regulators as well as many DNA repair genes (Bouyer et al., 2018). How E2F-RBR complexes are regulated upon DNA damage remains to be fully clarified. Formation of RBR foci upon DNA damaged was reported to depend both on CYCB1/CDKB and ATM/ATR activity (Biedermann et al., 2017; Horvath et al., 2017). Whether RBR is directly phosphorylated by ATM, ATR and CYCB1/CDBK1 complexes, or whether the kinases function sequentially remains to be established. Neither RBR nor CYCB1/CDKB have been identified as putative ATM/ATR targets (Roitinger et al., 2015). Further work will thus be needed to fully dissect this part of the DDR signaling cascade.

Besides RBR1, another regulator called SNI1 (Suppressor of Npr1 Inducible 1) was recently reported to antagonize E2Fs, and was proposed to have a dual function in the DDR by connecting cell cycle checkpoint activation and DNA repair mechanisms (Wang et al., 2018). SNI1 is a subunit of SMC5/6 complex (Structural Maintenance of Chromosome), which is conserved in all eukaryotes (De Piccoli et al., 2009). Over-expression of *SNI1* rescues the phenotype of E2Fa/DPa over-expressers that is characterized by increased endoreduplication level and retarded growth (De Veylder et al., 2002), likely because it represses E2F target genes through the recruitment of histone deacetylases (Wang et al., 2018). Reciprocally, loss of E2Fs abolishes the induction of cell death observed in the

root tip of *sni1* mutants. Interestingly, loss of genes involved in HR had been previously reported to suppress cell death in *sni1* mutants (Durrant et al., 2007; Wang et al., 2010; Song et al., 2011). Since RBR1 and E2F are recruited to a small number of foci associated with heterochromatin, and not to all DNA damage sites, it is thus tempting to speculate that RBR1/E2F complexes and SNI function in heterochromatin-specific DNA repair mechanisms. Indeed, in human cells, the choice between DSB repairs pathway is greatly influenced by chromatin compaction, heterochromatin being more prone to Non-homologuous End Joining (NHEJ) possibly to avoid HR between repeats (Lemaître and Soutoglou, 2014).

All the above-mentioned studies have been conducted in Arabidopsis, using DNA damaging agents. However, understanding and characterizing the contribution of plant DDR pathways in more physiological conditions could provide valuable insight into the plant response to various environmental stresses.

ROLE OF THE PLANT DDR IN ABIOTIC STRESS RESPONSES

Although studies connecting the plant DDR to abiotic stress responses remain scarce, maintenance of genome integrity is likely to play a role in plant stress tolerance. In agreement with this hypothesis, whole genome sequencing of two species of *Eutrema*, a recently evolved genus of alpine Brassicaceae, revealed that several genes involved in DNA repair, cell cycle regulation or DDR are duplicated, thereby providing a potential mechanistic basis for the adaptation of these plants to the harsh alpine environment (Guo et al., 2018). Indeed, a number of abiotic stresses are well known to cause DNA damage. The most obvious example is UV-B light (280–320 nm) that directly damages DNA by inducing the formation of CPDs. This results in DNA strand distortion, and hampers both transcription and DNA replication (Britt, 2004). Most CPDs are directly repaired by photolyases such as UVR2 (UV Response 2) in Arabidopsis (Willing et al., 2016), but tolerance of UV-B photodimers also requires TLS polymerases to allow DNA replication to proceed in spite of lesions (Curtis and Hays, 2007). When unrepaired, CPDs can activate the DDR. Indeed, exposure to UV-B light, like γ-irradiation, can induce PCD in root meristems, in a SOG1-dependent manner (Furukawa et al., 2010). PCD induction after γ-irradiation still occurs in *atm* and *atr* single mutants, although it is delayed, but not in double mutants, indicating that either kinase is sufficient to activate SOG1 (Furukawa et al., 2010). Likewise, zeocin-induced cell death was abolished in both *atm* and *atr* mutants in the root tip, while it seems to require only ATM in the inflorescence meristem, suggesting that DDR signaling components play partially specialized functions depending on cell types (Fulcher and Sablowski, 2009). In maize and Arabidopsis, histone acetylation has been associated to UV-B responses and damage repair (Campi et al., 2012; Fina et al., 2017). Interestingly, mutants deficient for histone acetyltransferases showed reduced growth inhibition after UV-B exposure, associated with altered expression of E2F transcription factors (Fina et al., 2017). Consistently, E2Fc know-down lines show less severe reduction of leaf growth in response to UV-B than the wild-type, suggesting that E2Fc could also play a role in the DDR activated by UV light (Gómez et al., 2019), as was previously suggested for the atypical E2Fe (Radziejwoski et al., 2011).

Another well documented example of abiotic stress activating the plant DDR is the exposure to heavy metals [for example cadmium (Cd), copper (Cu), lead (Pb) or mercury (Hg) (Küpper and Andresen, 2016; Lanier et al., 2019)], or other metallic ions such as aluminum (Al). These metallic ions can be divided into two categories: some, like copper or zinc are essential for plant growth but toxic at high doses, while others such as cadmium, mercury, and lead are not required for plant development. The toxic effects of these metals are varied, ranging from impairment of photosynthesis to inhibition of the uptake of other essential metal ions, but many of them cause DNA damage either directly, or through the induction of ROS production (Küpper and Andresen, 2016).

Among the metal elements that can affect plant growth, Al is probably one of the best studied, because it is very abundant, and because Al^{3+} ions that are predominant in acidic soils cause severe phytotoxicity, making this metal one of the primary growth limiting factors for agriculture. Exposure to Al^{3+} was shown to induce DNA damage in Arabidopsis (Nezames et al., 2012; Chen et al., 2019) but also in crops such as barley (Jaskowiak et al., 2018), and plant growth inhibition in response to this ion has been shown to require ATR and SOG1 (Rounds and Larsen, 2008; Sjogren et al., 2015; Zhang et al., 2018). Since Al causes DSBs, the improved root growth of *sog1* or *atr* mutants on Al containing medium may appear counter-intuitive. However, detailed genetic dissection of the response to low and high doses of Al allowed Chen and colleagues to propose a model according to which low levels of Al-induced DNA damage triggers ATR-dependent SOG1 activation leading to growth reduction and CYCB1/CDKB-dependent DNA repair. This pathway can be inactivated without compromising plant survival, suggesting that another pathway can allow activation of CYCB1-dependent DNA repair in the absence of ATR and SOG1. This alternative activation mechanisms could rely on RBR1 since *rbr1* mutants are hypersensitive to Al (Biedermann et al., 2017). By contrast, response to higher doses of Al and more severe DNA damage involves ATM-dependent SOG1 activation triggering the full activation of the DDR and leading to minimal growth, this pathway being indispensable for plant survival (Chen et al., 2019).

In addition to the well documented examples of UV-light or metal ions, there is accumulating evidence that a wide variety of stress conditions can induce DNA damage through unknown mechanisms that could involve ROS production. For example, prolonged chilling stress was found to induce DNA fragmentation in tobacco BY-2 cells (Koukalova et al., 1997) or maize root tip cells (Ning et al., 2002). Although one cannot rule out that some of the DNA damage observed in plants after exposure to stress is a consequence of the onset of PCD rather than actual stress-induced DNA damage, cold

stress has been shown to increase oxidative DNA damage in roots of *Cardamine pratensis* (Białkowski and Oliński, 1999). Consistently, Hong et al. (2017) recently reported that DDR activation in the root tip was essential to meristem survival after chilling stress. According to their model, cold stress induces DNA damage in the root tip, leading to selective PCD onset in the columella stem cell daughters. This response requires the canonical DDR players ATM, ATR, SOG1, and WEE1 and allows maintenance of the local auxin maximum in the root tip, thereby protecting meristem organization and allowing recovery after stress (Hong et al., 2017). Whether similar processes are activated in response to other kinds of stresses such as excess light, heat or drought remains to be fully explored, but a few studies support this notion. Indeed, the ANAC044 and ANAC085 transcription factors were found to promote cell cycle arrest in response to heat stress. Although this response is independent of SOG1, this finding demonstrates that some DDR components can be recruited in response to other types of abiotic stresses to induce cell cycle arrest (Takahashi et al., 2019). Furthermore, ozone induces DNA damage in wheat, particularly under water limiting conditions and heat or high light severely enhance DNA damage accumulation in rice mutants deficient for RNase H2 (Qiu et al., 2019). Activation of DNA repair also likely plays a key role during dehydration and rehydration in resurrection plant (Liu et al., 2018). Consistently, expression of a number of cell cycle inhibitors is induced in response to abiotic stresses, and *SMR5* and *7*, that are direct SOG1 targets have been shown to promote early exit of the cell cycle in response to chloroplastic stress (Hudik et al., 2014). Interestingly, *SMR5* is induced in response to heat, drought or high-light (Yi et al., 2014), and the same study revealed that SOG1 is phosphorylated in response to H_2O_2 accumulation, suggesting that generally, stress-induced ROS accumulation could trigger DDR activation. In agreement with this hypothesis, loss of the ROS detoxifying enzymes Ascorbate Peroxidase and Catalase 2 results in the activation of a WEE1-dependent cell cycle checkpoint (Vanderauwera et al., 2011) resulting in growth inhibition. Although this study was conducted in mutants in which ROS detoxification is severely compromised, it suggests that a similar response could be activated in wild-type plants exposed to stress. Together, these observations support the notion that many, if not all abiotic stresses, can activate the DDR, which could contribute to the plant growth reduction that is a common for all stress responses (Claeys et al., 2013). In this context, a better understanding of the plant DDR would open possible opportunities to counter environmentally induced yield-loss.

Finally, the plant DDR is clearly instrumental for seed viability and seedling vigor (Ventura et al., 2012; Waterworth et al., 2015). Indeed, both seed dehydration and germination, which are accompanied by a burst of ROS production, are highly damaging for DNA, and up-regulation of DNA repair genes during germination is well documented in Arabidopsis (Waterworth et al., 2010), *Medicago truncatula* (Balestrazzi et al., 2011) and *Phaseolus vulgaris* (Parreira et al., 2018). Consistently, *atm* mutants fail to delay germination in aged seeds, and show extensive chromosomal abnormalities (Waterworth et al., 2016), and HR-deficient or DDR mutants are hypersensitive to ABA during germination and at the seedling stage (Roy and Das, 2017). Thus, the probable contribution of the plant DDR to abiotic stress tolerance is supported by its essential role during germination, a particularly stressful step of the plant life cycle.

Maintenance of genome integrity is well known to be essential for meristem function, as illustrated by numerous examples of mutants affected in DNA Damage repair in which meristem organization is perturbed or its function is lost [e.g., (Wenig et al., 2013; Li et al., 2017; Han et al., 2018)], and it would thus not be surprising to find that DDR activation is a key factor for plant survival under abiotic stress conditions. In line with this hypothesis, DDR has been shown to shape directly or indirectly plant development in response to stress. In the root meristem, replacement of damaged stem cells relies on the reactivation of the ERF115 transcription factor to promote cell division (Heyman et al., 2013), and its transcriptional up-regulation occurs in cells that are in direct contact with damaged cells (Heyman et al., 2016). In the context of DNA-damage induced PCD, *ERF115* induction was shown to depend partially on SOG1 activity (Johnson et al., 2018). DDR was also shown to impact lateral root formation by modulating cytokinin signaling (Davis et al., 2016), and to account for the reduction of hypocotyl growth triggered by UV (Biever et al., 2014), suggesting that its activation could contribute to the well-known plasticity of plant development according to external conditions.

ROLE OF THE PLANT DDR IN BIOTIC STRESS RESPONSE

A similar connection can be drawn between the plant DDR and response to biotic stresses. It has long been known that pathogen infection or treatment with the defense hormone Salicylic Acid (SA) stimulates HR, suggesting that the DDR is activated by biotic stress (Lucht et al., 2002; Kovalchuk et al., 2003). Consistently, Song and colleagues reported that a variety of pathogenic, and even non-pathogenic micro-organism induce DNA damage in plant cells (Song and Bent, 2014). However, this accumulation of DNA damage does not depend on pathogen-induced ROS production, and the underlying mechanisms thus remain unknown. SA treatment has been shown to induce DNA damage (Yan et al., 2013) but this effect is debated, since in another study pre-treatment with SA was found to reduce DNA damage accumulation in response to infection, and SA alone failed to induce DNA damage (Song and Bent, 2014).

Thus the mechanisms leading to DNA damage accumulation during infection remain unclear, although some of these DNA lesions could simply reflect the induction of PCD as a defense mechanism. Nevertheless, there is accumulating evidence that DDR activation is relevant to plant immunity. First a number of DNA repair mutants have been reported to show enhanced susceptibility to *Pseudomonas syringae*. This is the case for plants lacking PARP2 (Poly ADP-ribose polymerase) that plays an important role for DNA repair (Song et al., 2015), and

mutants affected in DSB repair by HR such as *rad51* or *brca2* (Durrant et al., 2007; Wang et al., 2010; Song et al., 2011). Second, DDR signaling mutants such as *atm*, *atr*, or *rad17* have also been reported to be more susceptible to *P. syringae* (Yan et al., 2013; Song and Bent, 2014). One possible explanation for these observations would be that an efficient DDR activation and DNA repair is required for plant cell survival in response to biotic stress, possibly to avoid cell death due to the accumulation of DNA lesions; or on the contrary to contribute to PCD induction to limit pathogen growth. However, there is evidence that the DDR could enhance plant defense activation. Indeed, the above-mentioned *SNI1* gene was initially isolated as a negative regulator of systemic acquired resistance in a suppressor screen of the *npr1* (Non-expressor of PR genes) mutant (Li et al., 1999). As previously stated, *SNI1* was found to encode a sub-unit of the SMC5/6 complex (Yan et al., 2013) that plays a crucial role in DNA repair, notably in the removal of post-replicative damage (Diaz and Pecinka, 2018). *Sni1* mutants constitutively accumulate DNA damage, and show enhanced tolerance to pathogens, suggesting that DDR activation could stimulate biotic stress responses (Yan et al., 2013). Consistently, the recent genome-wide identification of SOG1 target genes revealed that a number of defense-related genes are SOG1 targets, providing a direct link between biotic stress and DDR (Bourbousse et al., 2018; Ogita et al., 2018).

In addition, DNA repair proteins have been proposed to play a direct role in the control of immune responses: activation of defense-related genes by SA in the *npr1 sni1* double mutant was largely dependent on BRCA2 (Wang et al., 2010). Furthermore, RAD51 and BRCA2 appear to directly bind the promoter of the *PR1* and *PR2* defense genes (Wang et al., 2010). These results thus led to a model according to which BRCA2 and RAD51 would directly control the transcription of immunity-related genes. However, the primary defect of the *sni1* mutant likely is in DNA repair since *sni1* is a sub-unit of the SMC5/6 complex (Yan et al., 2013). The accumulation of DNA damage in the *sni1* mutant is largely alleviated in the *atr* background (Yan et al., 2013), suggesting that the DNA lesions accumulate because ATR signaling triggers repair mechanisms that cannot be fully completed, possibly due to the absence of SMC5/6. In the absence of ATR activation, alternative pathways must be activated leading to a reduction of DNA damage accumulation. Under such a scenario, the activation of defense genes in *sni1* could be an indirect effect of DDR activation, possibly through the activation of SOG1. In that case, one could hypothesize that BRCA2 and other DNA repair proteins could contribute to the accumulation of repair intermediates that trigger the DDR. Loss of these proteins, including ATR, could reduce DNA damage accumulation by allowing alternative repair mechanisms to function, and thus DDR signaling through SOG1. In line with this hypothesis, 163 out of the 265 BRCA2-dependent defense genes identified by Wang et al. (2010) are differentially expressed in response to γ-irradiation according to Bourbousse et al. (2018). Thus, to fully ascertain the direct role of BRCA2/RAD51 complexes in immunity, the effect of the *brca2* or *rad51* mutations on defense gene expression should be analyzed in a wild-type background and a genome-wide analysis of BRCA2/RAD51 target genes during biotic stress response should be performed. Whether DNA repair proteins directly control the expression of several defense genes or not, there is converging evidence for a role of the plant DDR during immunity, which could, as we proposed in the case of abiotic stress, contribute to the growth inhibition induced by pathogens.

CONCLUDING REMARKS

The plant DDR is emerging as a key process shaping plant growth and development in response to environmental cues. Now that the main actors of this signaling pathway have been characterized, future work should elucidate the molecular connections between DDR and plant response to stress, thereby opening new prospects for crop improvement. Another promising line of research will be to decipher the connections between the DDR and chromatin dynamics. Indeed, replicative stress has been shown to affect the maintenance of gene silencing through DNA replication in yeast (Sarkies et al., 2010), a mechanism that most likely applies to plants, as evidenced by the large number of DNA replication proteins isolated in genetic screens for suppressors of silencing (Kapoor et al., 2005; Yin et al., 2009; Liu et al., 2010; Hyun et al., 2013). Furthermore, DNA repair processes require extensive chromatin remodeling to allow access of the repair machinery to DNA (Nair et al., 2017). Thus DNA damage represents a challenge for chromatin maintenance. Reciprocally, defects in chromatin dynamics can lead to genome instability and DNA damage accumulation (Ma et al., 2018). Mechanisms allowing chromatin reconstruction after DNA repair or connecting chromatin dynamics with genome stability have been little explored, particularly in plants, and will likely receive increasing attention in the future.

AUTHOR CONTRIBUTIONS

M-UN wrote the paragraph on DDR signaling. YH wrote the paragraph on DDR and abiotic stress. MB and CR conceived the review organization and figures. CR wrote the rest of the text and supervised the writing.

FUNDING

M-UN is supported by a grant from the Fondation pour la Recherche Médicale (ECO201806006824). YH is supported by a grant from the Chinese Science Council (201806690005).

ACKNOWLEDGMENTS

We thank J. Ramirez-Prado for critical reading of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 31 May 2019
doi: 10.3389/fpls.2019.00706

Seeds and the Art of Genome Maintenance

Wanda M. Waterworth[1], Clifford M. Bray[2] and Christopher E. West[1]*

[1] University of Leeds, Leeds, United Kingdom, [2] The University of Manchester, Manchester, United Kingdom

Successful germination represents a crucial developmental transition in the plant lifecycle and is important both for crop yields and plant survival in natural ecosystems. However, germination potential decreases during storage and seed longevity is a key determinant of crop production. Decline in germination vigor is initially manifest as an increasing delay to radicle emergence and the completion of germination and eventually culminating in loss of seed viability. The molecular mechanisms that determine seed germination vigor and viability remain obscure, although deterioration in seed quality is associated with the accumulation of damage to cellular structures and macromolecules including lipids, protein, and nucleic acids. In desiccation tolerant seeds, desiccation/rehydration cycles and prolonged periods in the dry quiescent state are associated with remarkable levels of stress to the embryo genome which can result in mutagenesis of the genetic material, inhibition of transcription and replication and delayed growth and development. An increasing number of studies are revealing DNA damage accumulated in the embryo genome, and the repair capacity of the seed to reverse this damage, as major factors that determine seed vigor and viability. Recent findings are now establishing important roles for the DNA damage response in regulating germination, imposing a delay to germination in aged seed to minimize the deleterious consequences of DNA damage accumulated in the dry quiescent state. Understanding the mechanistic basis of seed longevity will underpin the directed improvement of crop varieties and support preservation of plant genetic resources in seed banks.

Keywords: DNA repair, seeds, germination, priming, aging

OPEN ACCESS

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****Correspondence:***
Wanda M. Waterworth
w.m.waterworth@leeds.ac.uk

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *07 March 2019*
Accepted: *13 May 2019*
Published: *31 May 2019*

Citation:
Waterworth WM, Bray CM and West CE (2019) Seeds and the Art of Genome Maintenance. Front. Plant Sci. 10:706. doi: 10.3389/fpls.2019.00706

BACKGROUND

Successful germination is a key developmental transition that is critical for plant propagation and is essential for both agriculture and the plant lifecycle. Modern farming requires high quality seed lots, with robust germination and seedling establishment that is tolerant of environmental stresses. In addition, programs for the *ex situ* conservation of plant genetic resources in seed banks are reliant on seeds and their properties, providing a lifeline to future generations. Both agriculture and plant conservation requires the maintenance of seed germination vigor and viability during storage. Recent work has shed light on the molecular aspects of seed longevity, revealing DNA

Abbreviations: AP, apurinic; ATM, ataxia telangiectasia mutated; ATR, ATM and RAD3-related; BER, base excision repair; DDR, DNA damage response; DSB, double-strand break; HR, homologous recombination; NHEJ, non-homologous end-joining; PARP, poly (ADP-ribose) polymerase; PCD, programmed cell death; QTL, quantitative trait locus; RAM, root apical meristem; ROS, reactive oxygen species; SMR, Siamese related; SSB, single-strand break; 8-oxoG, 8-oxoguanine.

repair mechanisms and the DNA damage response (DDR) as key factors which control germination and dictate the germination potential of a seed.

Seed Germination

Seeds are propagules containing embryos in which growth is suspended. In this quiescent state, desiccation tolerant seeds, which represent the majority of plant species, exhibit a low moisture content (<15%) and repression of metabolic processes until rehydration occurs upon seed imbibition. Seeds that survive such low moisture contents are termed "orthodox" seeds, in contrast to those species incapable of withstanding such water loss which are termed "recalcitrant." Orthodox seeds can remain viable in this dehydrated state for long periods of time, before being stimulated to germinate upon rehydration under favorable conditions for growth. Seeds exhibit considerable interspecific and intraspecific variation in longevity, and in many species can retain viability for decades. Remarkably, date palm seeds excavated from the archeological site of King Herod's palace in Israel, were able to germinate after 2000 years (Sallon et al., 2008). Upon desiccation the cytoplasm transitions from a fluid to a glassy state which minimizes mobility of molecules and stabilizes cellular structures (Buitink and Leprince, 2008). The residual water in the desiccated seed is associated with biological molecules which provide resistance to freezing and formation of ice crystals. Seed germination is initiated by the imbibition of water by the seed and ends with the start of elongation of the embryonic axis and emergence of the radicle (Bewley and Black, 1994). Given an adequate supply of water, imbibition by the mature "dry" orthodox seed exhibits a triphasic pattern (Bewley, 1997). Phase I consists of water uptake that is largely a consequence of matric forces. In the mature seed, metabolism is reduced to very low levels, although all the components of a fully functional protein synthesizing system, including mRNA synthesized during the late stages of seed maturation are present in the quiescent embryo of a viable seed (Blowers et al., 1980). Within minutes of taking up water, imbibing seeds display rapid activation of respiratory and synthetic processes, *de novo* synthesis of protein and both ribosomal and messenger RNA along with mitochondrial ATP synthesis. Imbibition is followed by a lag phase (Phase II) in which water potential of the seed is in balance with its surroundings and there is no net water uptake. Phase III occurs as a consequence of radicle elongation and emergence that drives an increase in fresh weight. Both viable and non-viable seeds will exhibit phases I and II of water uptake but only viable seed are capable of entering phase III, which marks the completion of germination.

The Importance of Seed Longevity

Seeds deteriorate with time and seed aging is exacerbated under suboptimal environmental and poor storage conditions such as high relative humidity and temperatures. In agriculture, high seed vigor, defined as rapid, uniform germination, and robust seedling establishment tolerant of adverse environmental conditions, is a major determinant of crop yields (Rajjou et al., 2012; Finch-Savage and Bassel, 2016). Low quality seed negatively impacts on final yield through reduced emergence, poor seedling establishment and reduced harvesting efficiency arising from non-uniformity of crop growth. Low vigor seeds germinate and establish poorly under stresses including low temperature, drought and anoxic waterlogged soils. Yield losses resulting from using low vigor seeds are further exacerbated as young seedlings are particularly vulnerable to environmental stresses such as drought, predation, pathogen attack, and weed competition (Finch-Savage and Bassel, 2016). The strong link between seed vigor and successful seedling establishment highlights the great potential for increasing crop yields through improved seed germination performance in the field (Powell and Matthews, 2012). Seed longevity is determined by the interplay of complex genetic and environmental factors (Clerkx et al., 2004; Joosen et al., 2012), and despite its economic, agronomic and ecological importance our current understanding of the molecular basis of seed longevity remains incomplete to date. Desiccation and rehydration cycles in combination with prolonged periods in a dry quiescent state are accompanied by reduced cellular maintenance activities and the progressive accumulation of damage to cellular ultrastructure and biological macromolecules including DNA, RNA, proteins and lipids (Powell and Matthews, 2012). Reactive oxygen species (ROS) produced during desiccation, storage and imbibition are an important causative factor of seed aging although significantly ROS also play critical roles as signaling factors that promote germination (Kranner et al., 2010). Consequently, desiccation tolerant seeds have evolved powerful protection and repair systems to minimize damage to cellular structures and biological molecules. Upon seed imbibition, cellular repair activities facilitate recovery from damage incurred during quiescence, and the speed and capacity for repair are closely linked to germination performance and the successful establishment of the young seedling (Powell and Matthews, 2012). The molecular factors which influence seed longevity have been recently reviewed (Sano et al., 2015). However, an expanding body of studies is defining the important link between repair mechanisms, germination and seed longevity, in particular the role of genome maintenance mechanisms, and will form the focus of this review.

Factors Affecting Seed Vigor and Viability

The low metabolism of the quiescent embryo provides a barrier to repair activities, leading to the accumulation of macromolecular damage and seed aging. Suboptimal conditions during the late stages of seed development or during quiescence accelerate the deterioration of cellular components (Sattler et al., 2004). The increased requirement for repair leads to a delay to radicle emergence and reduced germination performance, ultimately resulting in failure to germinate and loss of seed viability. Seed aging not only delays radicle emergence but in many species leads to abnormal or weak seedlings (Powell and Matthews, 2012). Repair mechanisms reverse damage to cellular components, restoring cellular function prior to the initiation of growth post-germination. Genetic studies have identified the importance of pathways for cellular repair in maintaining the viability of the quiescent seed, as recently reviewed (Rajjou et al., 2012; Sano et al., 2015; Waterworth et al., 2015). The activity of these pathways influence seed longevity and there

is evidence that plants are able to adapt to environmental changes to promote seed viability over a relatively short timescale (Mondoni et al., 2014).

DNA Damage in Seeds

DNA, and the information it encodes, is irreplaceable if lost or degraded. DNA damage has immediate impacts on cellular function as DNA provides the template both for transcription and DNA replication. As meristems within the embryonic plant give rise to the mature plant, including the reproductive tissues, mutations incurred in seeds have the potential to be transmitted on to progeny (Ries et al., 2000). Accordingly, genome maintenance mechanisms in seeds are important not only for growth and development, but also in preserving the longer term stability of plant germplasm at the level of populations and species. Thus, DNA damage must be repaired early in imbibition prior to initiation of cell division, to maintain germination potential and minimize mutagenesis in subsequent seedling development (Waterworth et al., 2016). The requirement for extended repair of accumulated damage underlies the delay to germination characteristic of low vigor seed (Waterworth et al., 2010). In particular, seed aging is associated with progressive accumulation of DNA damage in the embryo, including increased levels of base loss, generating abasic sites, base modification, single strand DNA breaks (SSBs) and DNA double strand breaks (DSBs) (Cheah and Osborne, 1978; Dourado and Roberts, 1984a; Córdoba-Cañero et al., 2014). For example, naturally aged rye seeds accumulated DNA breaks as seeds deteriorated, leading to prolonged DNA repair synthesis prior to the onset of DNA replication in aged seed and germination coincident with delayed radicle emergence (Cheah and Osborne, 1978; Elder et al., 1987). The lowered moisture content of the desiccated orthodox seed reduces the rate of genome damage but in the absence of repair, lesions accumulate over time (Walters et al., 2006). Desiccated maize seed incurred 6-fold less base loss after dry storage at 20°C for 2 years than DNA in aqueous solution. Apurinic (abasic) sites were detected at a frequency of 3.8×10^{-5} per nucleotide in the quiescent embryo, and levels further increased 4-fold upon imbibition (Dandoy et al., 1987). DSBs are a particularly cytotoxic form of DNA damage. Cytological studies demonstrated extensive chromosome fragmentation and rearrangements upon seed aging and that even high vigor seeds display a background level of DSBs (Dourado and Roberts, 1984a). An early study published by Navashin in 1933 reported that the incidence of chromosome abnormalities in a seed lot stored for a number of years "*…strikingly resembled one obtained from soaked seed which had been treated by X-rays*" (Navashin, 1933), with extensive chromosomal defects in the majority of cells. The aberrant mitotic figures represent mis-joined chromosomes resulting from extensive induction of DNA double strand breaks in the desiccated quiescent seed (Waterworth et al., 2016). Desiccation as a strategy to survive extreme environments is termed anhydrobiosis, and is found in a broad range of organisms including bacteria, tardigrades, fungi, algae and mosses (Franca et al., 2007). Desiccation tolerance requires protection adaptions, for example the production of late embryogenesis abundant (LEA) proteins which were initially identified in plants but found in diverse phyla. However, while protective mechanisms enable organisms to withstand the physical effects of dehydration and rehydration alone, they are insufficient or unable to counter the accumulation of DNA damage during quiescence. Several organisms adapted for anhydrobiosis have evolved powerful DNA repair mechanisms to reverse genome damage incurred during quiescence. Examples include tardigrades and the desert dwelling bacterium *Deinococcus radiodurans*, organisms that exhibit extreme resistance to high energy irradiation (X-rays and gamma rays) due to their enhanced DNA repair capacity (Zahradka et al., 2006; Gladyshev and Meselson, 2008).

Genome Maintenance Mechanisms

The combination of endogenous factors and environmental stresses, including UV, background irradiation, soil and air pollutants, result in a wide spectrum of DNA damage. Furthermore, DNA modification by metabolic by-products (in particular ROS) and errors during DNA replication and transcription represent major sources of genome damage. Eukaryotes have evolved powerful DNA repair pathways specific for particular types of lesion (**Figure 1**) and sensitive DNA damage sensing mechanisms coupled to checkpoints that delay cell cycle progression in the presence of DNA damage (Sancar et al., 2004). Cellular survival depends on the concerted action of powerful repair pathways for base damage and single strand breaks (base excision repair or BER), broad specificity repair of damage to one strand of the duplex (nucleotide excision repair or NER) and repair of DNA double strand breaks (non-homologous end joining or NHEJ, homologous repair or HR, alternative NEJ pathways or alt-NHEJ). These pathways are highly conserved across eukaryotes, and well-characterized in plants, in particular in *Arabidopsis* and rice (Britt, 1999; Bray and West, 2005).

Excision Repair Pathways

Excision repair operates on one of the two strands of the DNA duplex by excising the damaged region followed by repair synthesis using the intact template strand as a guide. Damaged bases are typically removed by the Base Excision

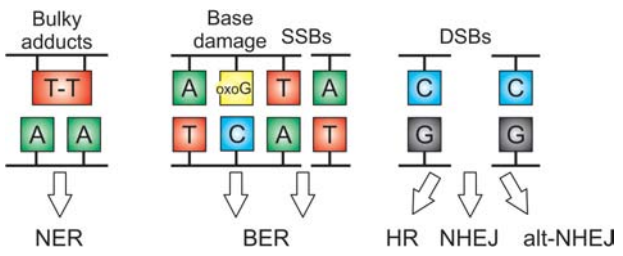

FIGURE 1 | DNA damage lesions and their DNA repair pathways in seeds. Nucleotide excision repair (NER) repairs damage on a single strand of the duplex, with specificity for bulky adducts and forms of damage that block RNA polymerase. Base excision repair (BER) removes damaged bases and repairs single strand breaks (SSBs). DNA double strand breaks (DSBs) are repaired by homologous recombination (HR), non-homologous end joining (NHEJ), or alternative NHEJ. Oxo G is 8-oxoguanine.

Repair pathway, initiated by DNA glycosylase enzymes that are specific to particular damage products, generating an abasic site which is followed by removal of the abasic site and DNA synthesis to fill the resulting gap. The most prevalent form of base damage is the oxidation product 8-oxoguanine (8-oxoG) and levels increase in seed subject to accelerated aging (Chen et al., 2012). Removal of 8-oxoG is mediated by either the 8-oxoguanine DNA glycosylase/lyase (OGG1) or formamidopyrimidine-DNA glycosylase (FPG) (Córdoba-Cañero et al., 2014). Both *OGG1* and *FPG* display increased expression during *Medicago truncatula* seed imbibition (Macovei et al., 2011) and levels of 8-oxoG base damage were significantly reduced in *Arabidopsis* seeds overexpressing *OGG1* (Chen et al., 2012). These lines also displayed enhanced resilience to seed aging under abiotic stress conditions, with improved seed viability when germinated at elevated temperatures or in the presence of salt stress (NaCl), relative to wild type (Chen et al., 2012). More bulky forms of DNA damage, representing steric changes in DNA duplex structure including base dimers, are repaired by nucleotide excision repair (NER), in which an oligonucleotide of ∼30 bases is excised and DNA polymerase fills in the single stranded region. This pathway can also use stalled RNA polymerase to identify polymerase blocking lesions which are then fed into the NER pathway. Mutation in xeroderma pigmentosum group B protein (XPB1), which mediates DNA helicase activity in NER, resulted in reduced germination relative to WT seeds after treatment with hypochlorite, which induces oxidative DNA damage. This suggests that NER is active in imbibing seeds and is required for maintenance of seed viability (Costa et al., 2001). NER gene expression increased toward the end of *Phaseolus vulgaris* L seed development, consistent with NER activity in imbibing seeds (Parreira et al., 2018). To-date there are no reports that core NER components are required to repair aging-induced genome damage, although recently co-expression network analysis in *Medicago* and *Arabidopsis* identified DNA repair factors such as *DNA LIGASE I* (*LIG1*) as genes associated with seed longevity (Righetti et al., 2015). Genes in this cluster were also expressed in response to pathogens, light and auxin, raising the possibility that seed longevity may have evolved through co-opting pathways which control defense against pathogens (Righetti et al., 2015). Interestingly the DDR signaling network is common to a broader range of stresses and has been implicated in the response to pathogen attack (Ogita et al., 2018).

Repair of DNA Double Strand Breaks

Double-strand breaks are highly cytotoxic DNA damage products which occur spontaneously in the cell, especially during DNA replication and under oxidative stress. DSBs are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR), characterized by random-end-joining or homology mediated repair of broken chromosomes, respectively. NHEJ is the predominant mechanism in vegetative tissues of vascular plants, as indicated by the extreme hypersensitivity of NHEJ mutants to X-rays and radiomimetics (West et al., 2002; Friesner and Britt, 2003). Recombination-mediated repair of DSBs is essential for cell viability and maintenance of genomic integrity in response to genotoxic stresses (Charbonnel et al., 2011). The elevated frequencies of chromosomal abnormalities in aged seeds (Abdalla and Roberts, 1968) arise from chromosomal fusions formed through errors in re-joining of DNA DSBs by the cell's recombination pathways. Even high vigor seeds display a background level of chromosomal aberrations, indicative of higher levels of genome stress in germination relative to other stages of plant development (Waterworth et al., 2016). In *Arabidopsis* seeds, the presence of chromosomal breaks is sufficient to slow or block germination and failure to repair this damage prior to germination results in genome instability and low vigor seedlings (Waterworth et al., 2010). Analysis of DNA ligase mutants, deficient in NHEJ repair of DSBs, established the genetic link between DNA repair and seed longevity. DNA LIGASE 4 (LIG4) and DNA LIGASE 6 (LIG6), respectively, function in the canonical and back-up (alt-NHEJ) pathways, and mutant seed are hypersensitive to accelerated aging (Charbonnel et al., 2011). The additive phenotype of the *lig4 lig6* mutant indicates distinct roles for each pathway in maintenance of germination potential. Interestingly, a genome wide analysis of genetic determinants of seed longevity identified a QTL in *Arabidopsis* that coincided with the chromosomal location of *LIG4* (Nguyen et al., 2012). HR-mediated repair of DSBs is also important in seeds, identified by analysis of gamma irradiated maize *rad51* mutants which displayed delayed germination and high seedling mortality relative to wild type lines (Li et al., 2007). The hypersensitivity to aging of seeds deficient in DSB repair implicates the importance of chromosome break repair in maintaining high seed vigor (Waterworth et al., 2010). Conversely, increased DNA repair capacity results in enhanced seed longevity and resistance to aging (Chen et al., 2012) and seeds that are maintained in a hydrated state and which have not undergone maturation drying do not display such levels of genome stress, with reduced chromosomal abnormalities (Villiers, 1974). During the later stages of seed development in *Phaseolus vulgaris*, in which maturation drying reduces seed water content, seeds display upregulation of DSB repair associated genes, which may reflect the stress induced during the drying phase and may prime seeds with repair factors required in early imbibition (Parreira et al., 2018). These results establish a strong link between DNA damage incurred during seed aging with decreased seed quality and weak seedlings that establish poorly on soil.

DNA Damage Signaling

DNA damage sensing mechanisms are coupled to control of cell cycle progression to limit the potentially highly mutagenic effects of DNA replication or chromatid segregation in the presence of DNA damage (Sancar et al., 2004). In order to minimize the cellular consequences of genotoxic stresses, the DDR orchestrates a coordinated network of responses including activation of cell cycle checkpoints, DNA repair factors, programmed cell death (PCD) and endoreduplication (Fulcher and Sablowski, 2009; Adachi et al., 2011). Plant growth and development requires cellular responses to genotoxic stress, which are type-specific and dependent on damage levels (Fulcher and Sablowski, 2009). The protein kinases ATAXIA TELANGIECTASIA MUTATED

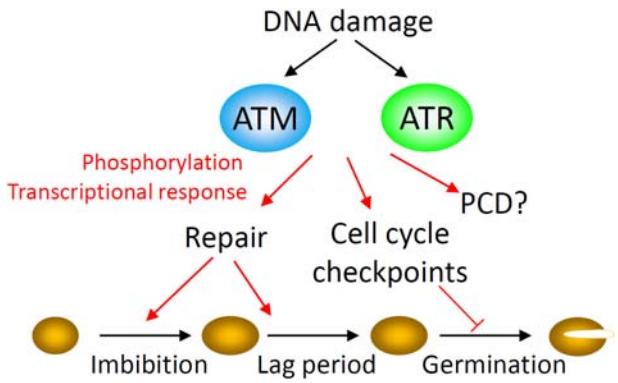

FIGURE 2 | The DNA damage response (DDR) in seeds. The DNA damage response executes a coordinated network of responses in order to minimize the consequences of genome damage to the cell, including activation of cell cycle checkpoints, DNA repair factors, and programmed cell death (PCD). The master kinases ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) control the cellular response to DNA damage in eukaryotes through activation of downstream responses at the transcriptional and post-transcriptional levels. ATM controls advancement of germination in aged seeds, in part through transcriptional control of the cell cycle inhibitor *SIAMESE RELATED 5* (*SMR5*). Both ATM and ATR influence seed viability but the molecular mechanism is unknown. In plants the transcriptional DDR encompasses hundreds of genes encoding proteins involved in DNA repair, chromatin remodeling and DNA metabolism. In the early stages of imbibition, seeds exhibit a large and rapid ATM-dependent transcriptional DNA damage response early in imbibition. DNA repair synthesis is detectable from the earliest stages of imbibition. As seed aging progresses and radicle emergence is delayed, this lag phase to germination is accompanied by an ATM-mediated delay of cell cycle activation in the root apical meristem (RAM) and extension of DNA repair activities.

(ATM) and ATM AND RAD3-RELATED (ATR) function as master controllers of the cellular response to DNA damage in eukaryotes and cell cycle arrest is activated, in part, by the transcriptional upregulation of CYCLIN DEPENDENT KINASE (CDK) inhibitors (**Figure 2**) (Yi et al., 2014; Hu et al., 2016). In plants the transcriptional DDR encompasses hundreds of genes encoding proteins involved in DNA repair, chromatin remodeling and DNA metabolism (Culligan et al., 2006). In the early stages of imbibition, seeds exhibit a large and rapid ATM-dependent transcriptional DDR, indicative of high levels of genotoxic stress (Waterworth et al., 2010). However, the DDR is negligible in mature barley seeds which have not undergone desiccation, storage and rehydration, indicating that ATM activation during imbibition of the desiccated seed is a direct response to high levels of DNA damage incurred during or after maturation drying (Waterworth et al., 2016). Recent studies identified that aged mutant *atr* and *atm* seeds display higher germination rates than wild type control seed, indicating deficiencies in the regulation of germination in response to damage in these lines (Waterworth et al., 2016). In ATM-deficient seeds, germination of aged seeds coincides with extensive chromosomal abnormalities and the resulting seedlings establish poorly on soil (Waterworth et al., 2016). Similarly, natural loss of seed vigor is associated with increased frequencies of non-viable seedlings carrying cytogenetic defects and leads to reduced crop yields in agricultural species (Dourado and Roberts, 1984b; Finch-Savage and Bassel, 2016). These recent findings collectively implicate DNA damage checkpoints as important determinants of vigor and viability of both seeds and seedlings, highlighting the importance of DNA damage signaling in germination to promote robust seedling growth.

Cell Cycle Activity in Germination

An increasing body of studies is linking control of cell cycle in germination with seed vigor. Cell cycle progression is linked to genome integrity through the activity of cell cycle checkpoints which control cell cycle advancement. Checkpoints are activated at critical phases of the cell cycle including DNA replication (the G1/S transition and intra-S phase), and before partitioning of sister chromatids into daughter cells during mitosis (the G2/M checkpoint) (Hu et al., 2016). Advancement through the plant cell cycle is driven by CDKs (cyclin dependent kinases) and their regulatory cyclin partners and is stimulated by CDK activating kinases (CAKs). Negative regulators integrate environmental and developmental signaling to control cell cycle activity. These include the WEE1 kinase, involved in the S-phase checkpoint, and two families of small inhibitory proteins: cyclin-dependent kinase inhibitors (CKIs, also known as kip-related proteins (KRPs) and the SIAMESE/SIAMESE RELATED (SIM/SMR) family proteins (Hu et al., 2016). In most mature desiccated seeds the majority of cells are in the resting or G1 stage of the plant cell cycle (Velappan et al., 2017). Cell expansion drives embryo growth in *Arabidopsis* and the cell cycle is initiated in the cells of the root apical meristem (RAM) around the time of radicle emergence from the seed coat (Vázquez-Ramos and de la Paz Sánchez, 2007). Recent studies showed that phytohormones including gibberellin and auxin promote cell cycle activation prior to germination (Lara-Núñez et al., 2008; Resentini et al., 2015; Godínez-Palma et al., 2017), whereas activation of cell division in the cotyledons and shoot apical meristem (SAM) occurs largely post-germination, several hours later than the root meristem cells (Barroco et al., 2005; Masubelele et al., 2005). Cell cycle activity in the RAM is required for high vigor and there is evidence for regulatory roles of cyclins, KRPs, and SMR proteins in seed germination. Mutants lacking the D-type cyclins, CYCD1:1 and CYCD1:4 exhibited delayed radicle emergence (Barroco et al., 2005; Masubelele et al., 2005), while CYCD-CDK kinase activity in imbibing maize seeds is stimulated by auxin (Lara-Núñez et al., 2008).

Endocycles, whereby cells replicate DNA and increase ploidy without mitotic division, is associated with cell expansion and in seeds is implicated in stimulating germination (Finch-Savage and Bassel, 2016). For example, the *Arabidopsis* CDK inhibitor ICK3/KRP5 is expressed in the transition zone between the root and the hypocotyl. Mutants display delayed germination, consistent with a role for KRP5 in the induction of endocycles promoting radicle emergence (Wen et al., 2013). KRP6 is also suggested to promote germination through endocycles. However, KRP6 has additional inhibitory roles, counteracting the gibberellin-mediated activation of mitotic cell cycle activity, leading *krp6* mutants to germinate faster than wild type lines

(Resentini et al., 2015). Recently, the second family of small inhibitory proteins (SIM/SMR) was also shown to have roles in seeds. In response to DNA damage, *Arabidopsis* ATM induces expression of *SMR5* and *SMR7* which results in cell cycle arrest (Yi et al., 2014) and *SMR5* and *SMR7* induction is also observed during imbibition (Waterworth et al., 2016). This is indicative of a mechanism whereby DNA damage slows germination through the ATM-dependent cell cycle regulation mediated by SMR factors. Recent studies identified that in aged seeds of *atm* mutant lines, S-phase is advanced relative to wild type seed, consistent with ATM-mediated control of a G1/S checkpoint and extending the lag period to completion of germination. This reveals DNA damage signaling as a major factor which controls germination in aged seed, integrating germination progression with surveillance of genome integrity and imposing the lag period to germination as vigor declines in response to aging-related DNA damage.

Cell Death in Aged Seeds

Rapid and sensitive responses to genotoxic stresses are crucial to safeguard the fidelity of genetic information, in particular in meristematic tissues of plant embryos where actively dividing cell populations are the progenitors of all cells in the future plant. The genome integrity of the meristem cells, and especially the stem cell initials and the quiescent centre (QC), is therefore crucially important. The slow division rate of QC cells allows greater time for repair of genome damage and may underlie the greater tolerance of these cells to DNA damage (Heyman et al., 2014). This contrasts with the rapidly dividing stem cell initials which display hypersensitivity to genotoxins, leading to high levels of PCD in these tissues, in a pathway dependent on DNA damage signaling by the ATM and ATR kinases (Fulcher and Sablowski, 2009; Furukawa et al., 2010). Both kinases act through a transcription factor SOG1 which is proposed to have key roles in the resumption of embryo growth in germination subsequent to genome damage (Johnson et al., 2017). In seeds, cells remain in G1 prior to germination and the role of PCD and its contribution to seed vigor and subsequent seedling growth is unclear. However, hallmarks of cell death are observed as seed deterioration progresses and damage to cellular components exceeds repair capacity (Kranner et al., 2010). The appearance of DNA laddering, a characteristic hallmark of programmed nuclease activity in PCD, was detectable in both sunflower and pea seeds after aging, increasing in incidence as seed deterioration progressed (El-Maarouf-Bouteau et al., 2011; Chen et al., 2013). Transcriptomic analyses of aging pea seeds were consistent with a switch from PCD to senescence associated gene expression as seed viability is lost in pea. PCD in aged seeds may contribute to loss of viability, in addition to cell death arising from senescence of cells suffering irreversible damage, leading to "exhaustion," which is likely to underlie the loss of germination potential in aged seeds (Kranner et al., 2010).

Chromatin Remodeling

DNA repair, DNA replication and transcription all take place in the context of chromatin in which DNA is packaged with histone proteins into nucleoprotein complexes. Accessibility of proteins, including the transcription and repair machinery, is achieved through chromatin remodeling enzymes and post-translational modification of histones (Donà and Mittelsten Scheid, 2015). This provides a powerful mechanism for transcriptional control during development and in response to the environment, in addition to protecting DNA from cellular factors. The mechanisms which function to stabilize and protect the genome in the dry quiescent state, and in transitions in nuclear architecture between the hydrated and desiccated state, are unclear (Neumann et al., 2009). In *Arabidopsis* seeds, a programmed decrease in nuclear size and chromatin compaction are associated with the dormant and the desiccated state and persist until germination is completed (van Zanten et al., 2011). Chromatin remodeling plays important roles in the modulation of dormancy, which represents a block to germination even under favorable conditions, and recent studies are also revealing key roles in germinating seeds. Treatment of *Arabidopsis* seeds with histone deacetylase inhibitors stimulates germination (Wang et al., 2016), while *Arabidopsis* mutants in the histone deacetylases HDA6, HDA9 and HDA19 displayed reduced dormancy (Zanten et al., 2014; Nelson et al., 2017). HDA19 functions in a complex with SWI-INDEPENDENT3 (SIN3)-LIKE1 (SNL1) and SNL2 during seed maturation, and establishes seed dormancy through reducing expression of genes involved in ABA turnover, thereby promoting ABA accumulation (Wang et al., 2013). Upon imbibition, SNL1 and SNL2 expression is reduced, which results in increased histone acetylation in target genes and leads to auxin signaling, increased expression of CYCD1;1 and CYCD4;1 and promotion of germination (Wang et al., 2016). Deacetylation inhibitors, used at higher concentrations than those that increased *Arabidopsis* seed vigor, inhibited *Medicago* seed germination and resulted in increased DNA strand breaks around the time of radicle protrusion (Pagano et al., 2018). This DNA damage was coincident with upregulation of transcripts encoding antioxidant genes and the DNA repair factors OGG1 (BER) and LIG4 (NHEJ) (Pagano et al., 2018). Understanding how nuclear compaction is mediated with local changes in chromatin structure, and the impact of these modifications on germination and maintenance of genome integrity in the desiccated state, will provide important new insight into the mechanisms underlying seed longevity.

Germination Enhancement Treatments: Seed Osmopriming

Deterioration in seed vigor is manifest as decreasing rapidity and synchronicity of germination and this increased delay to radicle emergence is accompanied by an extended period of genome repair. Several crop species, including high value vegetable seeds and sugar beet are routinely improved by priming, a pre-germinative seed treatment in which controlled hydration increases the speed of germination and enhances field emergence (Heydecker et al., 1973). Controlled hydration is thought to allow cellular repair processes to proceed without completion of germination (Heydecker et al., 1973; McDonald, 1999). Priming evidently reverses the lag period to germination

exhibited as seed lose vigor and promotes uniformity and stress tolerance in emerging seedlings. Seedling field emergence for many commercial species, typically >70% in the case of sugar beet, can be increased 5–10% by priming. The advantages of priming treatments are reductions in both the spread of germination and mean time to germination in low vigor seed lots. However, priming can result in a significant reduction in seed longevity (accelerated loss of viability over time) resulting in substantial economic losses in crop species (Tarquis and Bradford, 1992; Dekkers et al., 2015). The molecular basis for this loss of storability remains unknown, although over-priming, where germination is allow to progress to the initiation of DNA replication, was associated with reduced viability in tomato (van Pijlen et al., 1996).

Biochemistry of Osmopriming

Our understanding of the molecular basis of priming remains limited, although storage protein mobilization, endosperm weakening and DNA repair synthesis have been identified in a number of priming studies (Capron et al., 2007; Waterworth et al., 2015). Restoration of genome integrity by repair processes is common to priming in a range of species, including a correlation of DNA repair synthesis with improved germination after priming leek (*Allium porrum* L.) seeds (Ashraf and Bray, 1993; van Pijlen et al., 1996). Both repair of nuclear DNA and replication of mitochondrial DNA were observed during the priming period in leek embryos, whereas nuclear replicative DNA synthesis and cell cycle progression occurred post priming. Repair of mitochondria is likely to be of critical importance, as ATP is virtually absent in the quiescent embryo and mitochondrial oxidative phosphorylation is a major source of ATP from the start of imbibition. Loss of vigor has been shown to be reflected in reduced levels of nucleoside triphosphates and nucleotide sugars needed for nucleic acid synthesis and repair along with cell wall synthesis during cell expansion and division in the embryo of the germinating seed (Standard et al., 1983). Nuclear DNA replication is not observed during priming of leek seeds (Gray et al., 1990b). However, cell cycle progression during osmopriming treatments is species dependent and seeds of some species contain immature embryos which need to increase appreciably in size before the radicle tip emerges through the seed coat at germination. Such immature embryos of both carrot and celery seeds show a 3–4 fold increase in cell number and cell volume before they are able to germinate (Gray et al., 1990a; Karssen et al., 1990). During priming cells of the root tip of tomato embryos progress from the G1 phase of the cell cycle into G2 via a round of replicative DNA synthesis but do not undertake cell division (Bino et al., 1992; Dawidowicz Grzegorzewska, 1997; de Castro et al., 2000), consistent with cell cycle activity contributing to the advancement of germination conferred by seed priming.

Genome Maintenance in the Hydrated Seed

In the natural environment, seeds can persist in the soil seedbank undergoing dormancy cycling for many years, experiencing transitions between wet-dry states dependent on soil hydration levels (Footitt et al., 2011). Seed-bearing plants are thought to have evolved dormancy and desiccation tolerance as distinct adaptive strategies which facilitate survival and propagation in varying environments, with many species exhibiting interspecific in addition to intraspecific adaptation to different climatic conditions (Nguyen et al., 2012; He et al., 2014). In the dormant hydrated state, genome maintenance activities reverse cellular damage accumulated in the desiccated state (Elder and Osborne, 1993), potentially reducing the acute requirement for DNA repair during germination observed in imbibing seeds. The negative correlation between seed dormancy and longevity indicated that repair capacity may be linked to the ecological niche that a species is adapted to Nguyen et al. (2012). Thus, seeds from dry environments may have lower dormancy but a greater requirement for cellular repair, resulting in enhanced longevity, whilst wetter environments support continuous background levels of cellular repair, but require greater control in the timing of germination. DNA repair synthesis is observed in hydrated, dormant wild oat seeds (*Avena fatua*) which initiate DNA replication only after transfer of seeds to temperatures permissive of germination (Elder and Osborne, 1993). Recent studies identified that *Arabidopsis* seeds display significant upregulation of mRNA transcripts of genome maintenance factors, including *LIG6*, *SMR5* and *ATM*, during prolonged hydration in the dormant state, consistent with repair activity in the soil seed bank (Waterworth et al., 2016). Notably, dormant, hydrated lettuce seed sustained less chromosomal damage and retained germination vigor for extended time periods in comparison to their dry stored counterparts (Villiers, 1974). DNA repair activities in desiccation-rehydration cycles has also been identified which functions to help maintain Artemesia seed viability in harsh desert conditions. These seeds contain a water-absorbing proteinaceous surface pellicle and the partial hydration of this pellicle by night-time desert dews was correlated with significant DNA repair activity serving to maintain the integrity of the embryo genome (Yang et al., 2011). Genome maintenance is required to minimize the mutational load as seeds deteriorate and germination vigor is lost (Waterworth et al., 2015). The spectrum of mutations incurred upon seed aging can be transmitted to future generations, with the potential to influence plant genome stability at the population level (Ries et al., 2000; Jiang et al., 2014). Seeds of wild populations are particularly sensitive to environmental perturbation (Cochrane et al., 2011) and stresses experienced at this stage of the plant life cycle may have significant impact on genome stability.

Homeostasis of Reactive Oxygen Species in Seeds

Oxidative stress is a major cause of DNA damage, although oxidation of macromolecules is associated with both promotion of germination through ROS-mediated signaling in addition to the accumulation of oxidative damage as seeds deteriorate (Kranner et al., 2010). Oxidative stress activates components of the plant DDRs through ATM kinase signaling (Yi et al., 2014).

In other eukaryotes ATM acts as a direct sensor of oxidative stress, although the mechanism of ATM activation is not reported in plants. Levels of ROS in seeds are controlled by non-enzymatic ROS scavenging systems and antioxidant enzymes such as peroxidases (catalase, peroxiredoxins), superoxide dismutase, and enzymes of the glutathione and ascorbate cycles (Bailly, 2004; Kranner et al., 2010; Sano et al., 2015). In wheat seeds, the peroxidase 1-cys peroxiredoxin (PER1) forms part of a nuclear-localized redox system (Pulido et al., 2009). Recently, ectopic expression of PER1 from sacred lotus, a species with extreme seed longevity, was shown to confer resistance to *Arabidopsis* seed aging, accompanied by reduced levels of ROS and lower lipid peroxidation (Chen et al., 2016). Lotus PER1 reduces Fe^{3+} mediated cleavage of plasmid DNA *in vitro*, and this activity together with nuclear localisation of this redox factor, provides a potential mechanisms for the protection of the seed genome.

Combinatorial Consequences of Seed Deterioration

All the components of a fully functional protein synthesizing system, including messenger RNA, are present in the dry embryo of seeds. Viable embryos require only the imbibition of water for activation of metabolism and *de novo* protein synthesis is detectable within minutes of imbibing water in viable embryo (Bewley and Black, 1994). Germination is associated with massive transcriptional reprogramming as stored transcripts associated with seed maturation and quiescence are degraded in early imbibition and replaced by *de novo* synthesis of mRNA species required for seedling growth (Rajjou et al., 2004). DNA repair synthesis is initiated very early upon seed imbibition with the first burst of metabolic activity (Elder and Osborne, 1993). This is suggestive that at least some DNA repair factors may be either stored in the quiescent seed and become activated upon imbibition or produced by *de novo* synthesis upon resumption of transcription/translation (Elder and Osborne, 1993). During seed deterioration, damage to DNA, RNA, and protein progressively accumulates, increasingly impacting on the efficiency of transcription and translation processes in germination and early seedling growth. An important consequence of the requirement for *de novo* protein synthesis in germination is that seeds must preserve the translation machinery, as if it inactivated the capacity for production of replacement proteins becomes limiting (Rajjou et al., 2008; Dirk and Downie, 2018). Protein oxidation and mis-folding will also impact on efficiency of enzyme activities, including those of DNA repair factors such as DNA ligase and DNA polymerase, which decline in activity as seeds near the viability threshold (Elder et al., 1987; Gutiérrez et al., 1993; Coello and Vázquez-Ramos, 1996). However, the temporal progression of DNA damage signaling and repair processes in germination and how these are affected during seed aging largely remains to be determined.

Future Questions

Recent studies have implicated important roles for DNA damage signaling in control of germination in the aging seed. However, how DNA repair processes and the DNA damage signaling networks are integrated with other key regulatory factors which control germination, dormancy and seed longevity remains to be established. Additionally, the genome maintenance mechanisms operative in dormancy and priming remain to be defined at the molecular level. The plant DDR is a complex signaling network with hundreds of downstream targets which orchestrates the cellular response to DNA damage (Culligan et al., 2006). Although ATM controls progression of germination in part through control of cell cycle activation in the RAM (Waterworth et al., 2016), further targets of DNA damage signaling and their functions remain to be determined. Furthermore, the contribution of DNA damage and roles of the DDR in loss of seed viability remains unknown. Future work will uncover these signaling pathways and provide an understanding of how germination is linked to genome integrity, with the identification of specific regulatory mechanisms and the cells and tissues of the plant embryo in which they operate. This will include analysis of agronomically important species, enabling the prediction and improvement of germination under stress conditions, through marker assisted breeding and utilization of intraspecific variation. Germination potential is also important to natural ecosystems, and defining the repair activities in seeds undergoing wet- dry cycling cycles in the soil seed bank will provide new insight into how genome integrity is preserved during environmental stresses. While repair factors are important, understanding the roles of chromatin remodeling, antioxidant systems and cellular protective factors in maintenance of germination potential will also help both understand and improve seed longevity.

CONCLUSION AND OUTLOOK

The use of seeds for crop production was central to the development of human civilisation, underpinning agriculture and food production from Neolithic times until the present day. The increased demand for food with growth of the global population is leading to escalation in the value of the commercial seed market, projected to reach $92 billion by 2025 (Anon, 2019). Additional pressures on global agriculture result from the reduction in arable land, changing climate and the rising demand for biofuels. These factors necessitate the development of improved crop varieties that are tolerant of suboptimal environmental conditions and reduced losses arising from poor germination and field establishment. The escalating global population places enormous pressure on the environment, threatening many species with extinction. This has led to programs for plant germplasm conservation in seed banks, reliant on the storage properties of seeds. Both agriculture and plant conservation requires the maintenance of seed viability during storage, and recent work is shedding light on the molecular aspects of seed longevity, including key factors that dictate the germination potential of a seed. The seed stage of the plant lifecycle is associated with particularly high levels of genotoxic stress which need to be countered by powerful DNA repair and response mechanisms. These mechanisms maintain germination potential but also play a vital role in preservation of the genetic material transmitted between generations within

the embryo genome. As such, DNA repair and response factors represent promising targets for the genetic improvement of crop germination performance in the field, in particular under stress conditions. Quantification of DNA damage levels or repair factors which are highly conserved across plant species, could also provide early and sensitive predictive markers for the evaluation of seed lot deterioration. Understanding genome maintenance mechanisms in seeds will be fundamental for the prediction and improvement of germination to help us meet major global challenges on the road ahead.

AUTHOR CONTRIBUTIONS

WW, CB, and CW conceived and wrote the review.

FUNDING

This work was financially supported by the EU FP7 grant 311840 (EcoSeed) and the Biological Sciences Research Council grant BB/S002081/1 to WW and CW.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 June 2019
doi: 10.3389/fpls.2019.00761

The *dmc1* Mutant Allows an Insight Into the DNA Double-Strand Break Repair During Meiosis in Barley (*Hordeum vulgare* L.)

Miriam Szurman-Zubrzycka[1†], Brygida Baran[1†], Magdalena Stolarek-Januszkiewicz[1], Jolanta Kwaśniewska[2], Iwona Szarejko[1] and Damian Gruszka[1]*

[1] Department of Genetics, Faculty of Biology and Environment Protection, The University of Silesia in Katowice, Katowice, Poland, [2] Department of Plant Anatomy and Cytology, Faculty of Biology and Environment Protection, The University of Silesia in Katowice, Katowice, Poland

OPEN ACCESS

Edited by:
Ayako N. Sakamoto, National Institutes for Quantum and Radiological Science and Technology, Japan

Reviewed by:
Charles I. White, Centre National de la Recherche Scientifique (CNRS), France
Shuhei Nasuda, Kyoto University, Japan

***Correspondence:**
Damian Gruszka
damian.gruszka@us.edu.pl

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *06 March 2019*
Accepted: *24 May 2019*
Published: *11 June 2019*

Citation:
Szurman-Zubrzycka M, Baran B, Stolarek-Januszkiewicz M, Kwaśniewska J, Szarejko I and Gruszka D (2019) The dmc1 Mutant Allows an Insight Into the DNA Double-Strand Break Repair During Meiosis in Barley (Hordeum vulgare L.). Front. Plant Sci. 10:761. doi: 10.3389/fpls.2019.00761

Meiosis is a process of essential importance for sexual reproduction, as it leads to production of gametes. The recombination event (crossing-over) generates genetic variation by introducing new combination of alleles. The first step of crossing-over is introduction of a targeted double-strand break (DSB) in DNA. DMC1 (Disrupted Meiotic cDNA1) is a recombinase that is specific only for cells undergoing meiosis and takes part in repair of such DSBs by searching and invading homologous sequences that are subsequently used as a template for the repair process. Although role of the *DMC1* gene has been validated in *Arabidopsis thaliana*, a functional analysis of its homolog in barley, a crop species of significant importance in agriculture, has never been performed. Here, we describe the identification of barley mutants carrying substitutions in the *HvDMC1* gene. We performed mutational screening using TILLING (Targeting Induced Local Lesions IN Genomes) strategy and the barley TILLING population, *Hor*TILLUS, developed after double-treatment of spring barley cultivar 'Sebastian' with sodium azide and *N*-methyl-*N*-nitrosourea. One of the identified alleles, *dmc1.c*, was found independently in two different M_2 plants. The G2571A mutation identified in this allele leads to a substitution of the highly conserved amino acid (arginine-183 to lysine) in the DMC1 protein sequence. Two mutant lines carrying the same *dmc1.c* allele show similar disturbances during meiosis. The chromosomal aberrations included anaphase bridges and chromosome fragments in anaphase/telophase I and anaphase/telophase II, as well as micronuclei in tetrads. Moreover, atypical tetrads containing three or five cells were observed. A highly increased frequency of all chromosome aberrations during meiosis have been observed in the *dmc1.c* mutants compared to parental variety. The results indicated that DMC1 is required for the DSB repair, crossing-over and proper chromosome disjunction during meiosis in barley.

Keywords: barley, chromosome aberrations, crossing-over, DMC1, meiosis, TILLING

INTRODUCTION

Meiosis is a process of essential significance for sexual reproduction. During meiosis distribution of genetic material to gametes is associated with recombination which is achieved through crossing over and chromosome segregation. The recombination event (crossing-over) takes place during the first meiotic prophase between non-sister chromatids of homologous chromosomes. It leads to establishing physical links between homologous chromosomes, called chiasmata. Meiotic crossing-over shuffles genetic information, creates new combinations of alleles, and therefore generates genetic variations and drives evolution. The process of recombination during meiosis starts with a programmed DNA double-strand break (DSB). Meiotic DSBs are introduced through the catalytic action of the evolutionarily-conserved SPO11 (Sporulation Protein 11) protein complex which is an enzyme related to type II DNA topoisomerases (Keeney et al., 1997; Robert et al., 2016; Vrielynck et al., 2016). In general, DSBs can be repaired through two major pathways: homologous recombination (HR) and/or non-homologous end joining (NHEJ) (Ohnishi et al., 2009). Programmed DSBs during meiosis are eliminated by HR in the DSBR pathway (Double Strand Break Repair model). The model of DSB repair was first proposed by Szostak and coworkers in the 1980s (Szostak et al., 1983). The extensive studies of this process in *Saccharomyces cerevisiae* implemented only several alterations to the original model (reviewed in Andersen and Sekelsky, 2010). After introduction of DSB, the DNA ends are resected and long (about 1 kbp) 3′single-stranded overhangs are created, called 3′ssDNA tails (Ohnishi et al., 2009). RAD51 (Radiation sensitive 51) and DMC1 (Disrupted Meiotic cDNA1) recombinases attach to these tails and form nucleoprotein filaments that search for and invade homologous sequences either on a sister chromatid or on a homologous chromosome (Bishop et al., 1992; Shinohara et al., 1992). The latter case may lead to genetic recombination. After invasion on homologous sequence, the next step in the DSBR pathway is establishing the D-loop structure followed by formation of a double Holliday Junction (dHJ) intermediate. Then, the two strands at each HJ are nicked by specific enzymes and ligated. The resolution of dHJ can result in both, crossover and non-crossover repair products (COs and NCOs, respectively) (Andersen and Sekelsky, 2010). DMC1 and RAD51 belong to the same protein family of recombinases, involved in DNA repair through HR, which are related to the bacterial RecA (Bianco et al., 1998). They catalyze the process of pairing and invasion of 3′ssDNA tails formed at the DSB sites into homologous double-stranded DNA. Both of these proteins take part in the meiotic recombination events, however, DMC1 is specific only for cells undergoing meiosis, while RAD51 is ubiquitous and acts also in DSB repair in somatic cells. It is suggested that DMC1 promotes only the CO recombination with the homologous chromosome, which is unique to meiosis, and RAD51 plays its role mainly in sister chromatid exchange or the NCO recombination (Shinohara and Shinohara, 2004; Neale and Keeney, 2006). However, a recent work has shown that in the case of absence of the RAD51-mediated strand exchange activity, the DMC1 activity is sufficient to repair all DSBs during meiosis into both CO and NCO products and it does not affect meiotic crossing-over rates or patterns (Cloud et al., 2012; Da Ines et al., 2013; Singh et al., 2017).

In the plant kingdom, meiosis has been studied to the greatest degree in *Arabidopsis thaliana* (for review see Mercier et al., 2015). Cereals with large genomes and large chromosomes, such as barley (*Hordeum vulgare*), are characterized by highly skewed distribution of meiotic crossovers. Consequently, the large sub-centromeric regions, representing substantial proportions of the physical map, are seldom recombined (Higgins et al., 2012; Ramsay et al., 2014). Therefore, the molecular mechanisms underlying meiotic events may be distinct for model Arabidopsis with genome size of ∼135 Mbp (The Arabidopsis Genome Initiative [AGI], 2000) contained within five chromosomes and for barley with genome size of ∼5.3 Gbp contained within seven chromosomes (International Barley Genome Sequencing Consortium Mayer et al., 2012; Mascher et al., 2017). Our knowledge on the DMC1 function in plants comes mainly from studies performed in Arabidopsis. Moreover, its detailed function in DSB repair during meiosis is still extensively discussed. For example, some contradictory reports have appeared in rice (*Oryza sativa* L.): one, showing that OsDMC1 is required for homologous pairing (Deng and Wang, 2007), and the other, reporting that it is dispensable in this process (Wang et al., 2016), which is different from the role of DMC1 described in other species. These results imply that the function of DMC1 may be distinct in diverse organisms and a direct transfer of knowledge from related species may not be feasible. The recent findings in rice have been obtained studying rice insertion mutants (Wang et al., 2016). Although some *in silico* studies of DMC1 have been performed in monocot crops, including barley (Barakate et al., 2014), only very recently role of the barley homolog was analyzed in a spontaneous mutant (Colas et al., 2019).

Barley (*Hordeum vulgare* L.), ranking fourth in production and acreage, belongs to the most important cereal crops worldwide. Here, we present the identification of barley mutants in the *DMC1* gene isolated using TILLING strategy in the *Hor*TILLUS population derived from chemical mutagenesis of spring cultivar 'Sebastian'. Cytological analysis of male meiocytes in the identified *dmc1* mutants revealed various abnormalities during meiosis, in anaphase/telophase I and anaphase/telophase II, as well in tetrads. Our results indicate that DMC1 is involved in the DSB repair, crossing-over and chromosome disjunction during meiosis process in barley.

MATERIALS AND METHODS

Plant Material

The *Hor*TILLUS (*Hordeum vulgare* – TILLING – University of Silesia) population has been used for mutation detection in the *HvDMC1* gene through TILLING approach. This population was developed after double treatment of spring barley cultivar 'Sebastian' with sodium azide and *N*-methyl-*N*-nitrosourea (Szurman-Zubrzycka et al., 2018). Each M_2 plant of the *Hor*TILLUS population originated from a different M_1 plant. Eight-fold DNA pools from M_2 *Hor*TILLUS plants served as templates for mutational screening. The homozygous lines of the

isolated *dmc1* mutants were backcrossed with their parent variety and homozygous mutants selected from the F_2 populations have been used for cytological analyses of meiosis. Barley cv. 'Sebastian' has been used as a wild type in this study.

Mutational Screening in *HvDMC1* Using the TILLING Strategy

The sequence of the *DMC1* gene in barley was identified and published by Klimyuk et al. (2000) in the NCBI database (Acc. no. AF234170.1). Its genomic and coding sequences consist of 5654 bp and 1035 bp, respectively. The *HvDMC1* gene is composed of 14 exons and encodes a protein which is 344 amino acid in length (**Figure 1**). Our bioinformatics analysis revealed that *HvDMC1* gene has no paralogs in barley genome (**Supplementary Materials 1**, **2**). The DMC1 sequence is strongly conserved among various species representing the plant and animal kingdom. The bioinformatics tools: ClustalOmega[1] and CODDLE (Codons Optimized to Discover Deleterious Lesions) were used to select fragment of the *HvDMC1* gene for mutational screening. This *in silico* analysis enabled selection of the gene fragment which is highly conserved among homologous sequences from different plant species (**Figure 2**). Sequence encoding the Rad51 functional domain, which is characteristic for proteins involved in the DNA repair, was mapped in the *HvDMC1* gene with the use of Pfam tool[2]. Based on these bioinformatics analyses, the 811 bp long fragment of the *HvDMC1* gene containing exons 7 to 11, encoding a part of the Rad51 domain, was chosen as an amplicon for the TILLING screening. PCR reaction was optimized for specific primers labeled with IRDye-700 (forward) and IRDye-800 (reverse) (**Supplementary Material 3**). TILLING was performed on DNA of 5,376 M_2 plants of the *Hor*TILLUS population. The method of mutational screening applied in this study was performed according to the protocol described elsewhere (Szurman-Zubrzycka et al., 2017; Jost et al., 2019). Briefly, the eight-fold pools were used for PCR reaction with IRDye-700 and IRDye-800 labeled and unlabeled primers (**Supplementary Material 3**). The next step, formation of heteroduplexes, was performed at 95°C for 3 min for initial denaturation, and then at 70°C for 20 sec (×70 cycles, −0.1°C per cycle) for slow renaturation. Heteroduplexes appeared only in pools with mutations within the analyzed amplicon. After heteroduplex formation the samples were treated with 20 µl of 0.1× Celery Juice Extract (CJE) containing Cel I enzyme that specifically recognizes and cuts DNA mismatches. The enzymatic cleavage was performed at 45°C for 15 min. The products of cleavage were purified with 96% ethanol with 1% sodium acetate and then washed with 70% ethanol. After centrifugation the pellets were dried and dissolved in 3 µl of STOP buffer (containing 5% bromophenol blue-xylene, 40% formamide and 1% EDTA). Before loading on polyacrylamide gel the samples were denatured. The electrophoresis was carried out in LI-COR sequencers in denaturing 6% polyacrylamide gels in 1xTBE (Tris – Boric Acid – EDTA) running buffer at the following settings: 3000 V, 30 mA and 30 W. The lanes with additional bands indicating putative mutations in the analyzed bulks were selected for further analysis (**Supplementary Material 4**). For identification of single plants carrying the mutations, each sample from the selected bulk was then analyzed by mixing its DNA individually with DNA of the parent variety following the same method described for the eight-fold pools. The analyzed fragments from the identified plants were sequenced in order to confirm the presence of mutations.

[1]www.ebi.ac.uk/Tools/msa/clustalo/

[2]http://pfam.sanger.ac.uk/

Preparation of Material for Microscopic Analyses of Meiosis

Plants of the *dmc1* mutants as well as their parental cultivar 'Sebastian' were grown in a greenhouse at 22/20°C (day/night, respectively), under a photoperiod of 16 h/8 h and a light intensity of 400 $\mu E/m^2s$ for approximately 4 months, until their spikes reached length of 2.5–5 cm. Immature ears were harvested and immediately fixed in the methanol:acetic acid (3:1, v/v) overnight at room temperature.

To investigate the involvement of HvDMC1 in the DSB repair during meiosis, anthers from immature florets were used for preparation of male meiocyte spreads. Only cells in meiotic phases after crossing over were analyzed. Particular focus was given on cells in anaphase I/II and telophase I/II as well as tetrads, in which it was possible to observe micronuclei.

Cytogenetic slides were prepared using the Feulgen's squash technique. Three anthers were isolated to prepare one slide. Cytological analyses were performed for each genotype in three repetitions with 15 slides per replica. The frequencies of anaphase/telophase I and anaphase/telophase II cells with chromosome aberrations were analyzed, on average, in 158 and 165 cells per slide, respectively. The frequencies of cells in tetrad stage with the micronuclei were estimated, based on analysis of, on average, 170 cells per slide. Preparations were examined with the Nikon ECLIPSE Ni bright field microscope. Images were captured by the Nikon DS.- Fi1c camera under 40× magnification.

RESULTS

Mutation Identification and Characterization

After screening of 5,376 M_2 plants of the *Hor*TILLUS population, six independent mutations in the *HvDMC1* gene were identified (**Table 1**). All identified mutations were confirmed by sequencing and all of them are G/C to A/T transitions. Based on the number of the identified mutations in the *HvDMC1* gene (6), the length of amplicon (811 bp) and the number of M_2 plants screened (5,376), the calculated mutation density in this gene was 1 mutation per 729 kbp.

Six mutations identified in the *HvDMC1* gene gave five new alleles (*dmc1.a* – *dmc1.e*) (**Table 1** and **Figure 1**). The same mutation G2571A (the *dmc1.c* allele) was induced and identified independently in two different M_2 plants which originated from different M_1 individuals: plant no. 3041/001 and plant

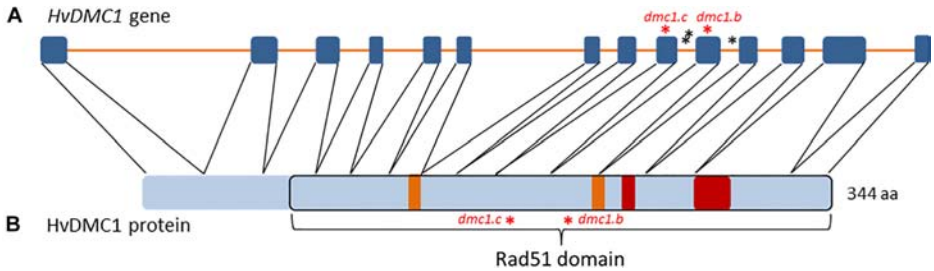

FIGURE 1 | (A) The structure of the *HvDMC1* gene. Blue boxes symbolize exons and orange lines introns. The identified mutations are indicated by asterisks (red – missense mutations, black – mutations in non-coding regions). **(B)** The structure of the HvDMC1 protein with localization of the Rad51 domain. Orange boxes symbolize the Walker A and Walker B functional motifs that are responsible for ATP binding, red boxes symbolize ssDNA-binding loops 1 and 2. The positions of amino-acid residues mutated in the *dmc1.c* and *dmc1.b* alleles are indicated by red asterisks.

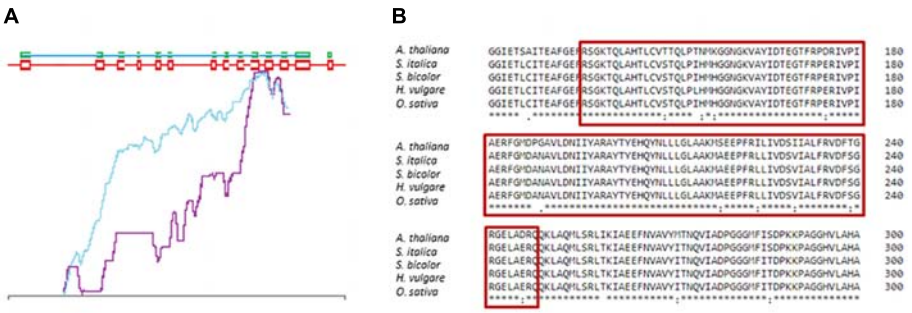

FIGURE 2 | Selection of the *HvDMC1* gene fragment for TILLING analysis **(A)** CODDLE analysis showing the most conserved fragment of the gene (blue line – score missense changes and truncations; violet – the PSSM difference). **(B)** Comparison of the DMC1 protein sequences from various plant species. The red frame shows conserved fragment of the protein which is encoded by the analyzed fragment of the *HvDMC1* gene.

TABLE 1 | Characteristics of mutations identified in the *HvDMC1* gene.

Allele	No of M_2 plant	Mutation position in genomic sequence	Type of mutation	Alteration in protein sequence	SIFT score	Mutation state in M_2 plant
dmc1.a	1519/001	C2515T	NC	–		Homozygous
dmc1.b	1694/001	G2742A	MS (exon 10)	G212S	0,43	Homozygous
dmc1.c	3041/001	G2571A	MS (exon 9)	R183K	0,03	Heterozygous
dmc1.c	3223/001	G2571A	MS (exon 9)	R183K	0,03	Heterozygous
dmc1.d	3896/002	C2495T	NC	–		Homozygous
dmc1.e	3976/001	C2837T	NC	–		Heterozygous

NC, mutation in non-coding sequence; MS, missense mutation.

no. 3223/001. To distinguish the origin of the mutated allele, it is hereafter named as *dmc1-3041* or *dmc1-3223* depending on the mutated line. Three mutations – *dmc1.a*, *dmc1.d* and *dmc1.e* occurred in non-coding, intron regions of the *HvDMC1* gene. They were analyzed *in silico* and the positions of these mutations are neither in donor/acceptor sites of introns nor in polypyrimidine tracts or branch points, so they are probably not essential for splicing and do not have any impact on the encoded protein. Homozygous plants carrying these intronic mutations did not show any visible morphological changes when compared to 'Sebastian'. Three other mutations – *dmc1.b*, *dmc1.c-3041* and *dmc1.c-3223*, occurred in coding sequence (*dmc1.b* in exon 10, *dmc1.c* in exon 9) and they cause amino acids alterations at the protein level. The *dmc1.b* mutation changes glycine-212 to serine (G212S) and the *dmc1.c* mutation changes arginine-183 to lysine (R183K). Potentially, both of them can be used for functional analysis of the *DMC1* gene in barley. The SIFT (Sorting Intolerant From Tolerant) tool was used to analyze *in silico* the influence of the identified mutations on protein activity and functioning. If the SIFT score is less than 0.05 the mutation is considered as deleterious for protein activity (Ng and Henikoff, 2003; Kumar et al., 2009). According to this bioinformatics analysis, the *dmc1.b* mutation is functionally neutral (SIFT score = 0.43), whereas the *dmc1.c* mutation is deleterious (SIFT score = 0.03). The multiple alignment of the DMC1 proteins from various species showed that the amino acid substituted in the *dmc1.b* mutant (glycine-212) is conserved among plant species, while the amino


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HsDMC1      GGIESMAITEAFGEFRTGKTQLSHTLCVTAQLPGAGGYPGGKIIFIDTENTFRPDRLRDI   173
ZmDMC1      GGIETLCITEAFGEFRSGKTQLAHTLCVSTQVPIHMHGGNGKVAYIDTEGTFRPERIVPI   180
HvDMC1      GGIETLCITEAFGEFRSGKTQLAHTLCVSTQLPLHMHGGNGKVAYIDTEGTFRPERIVPI   180
TaDMC1      GGIETLCITEAFGEFRSGKTQLAHTLCVSTQLPLHMHGGNGKVAYIGTEGTFRPERIVPI   180
OsDMC1      GGIETLCITEAFGEFRSGKTQLAHTLCVSTQLPIHMHGGNGKVAYIDTEGTFRPERIVPI   180
AtDMC1      GGIETSAITEAFGEFRSGKTQLAHTLCVTTQLPTNMKGGNGKVAYIDTEGTFRPDRIVPI   180
NtDMC1      GGIETSAITEAFGEFRSGKTQLAHTLCVSTQLPTSMKGGNGKVAYIDTEGTFRPDRLVPI   177
            ****: .*********:*****:*****::*:*      .**: :*.**.****:*:  *

HsDMC1      ADRFNVDHDAVLDNVLYARAYTSEHQMELLDYVAAKFHEEAGIFKLLIIDSIMALFRVDF   233
ZmDMC1      AERFGMDANAVLDNIIYARAYTYEHQYNLLLGLAAKMAEE--PFKLLIVDSVIALFRVDF   238
HvDMC1      AERFGMDANAVLDNIIYARAYTYEHQYNLLLGLAAKMAEE--PFRLLIVDSVIALFRVDF   238
TaDMC1      AERFGMDANAVLDNIIYARAYTYEHQYNLLLGLVAKMAEE--PFRLLIVDSVIALFRVDF   238
OsDMC1      AERFGMDANAVLDNIIYARAYTYEHQYNLLLGLAAKMAEE--PFRLLIVDSVIALFRVDF   238
AtDMC1      AERFGMDPGAVLDNIIYARAYTYEHQYNLLLGLAAKMSEE--PFRILIVDSIIALFRVDF   238
NtDMC1      AERFGMDAGAVLDNIIYARAYTHEHQYSLLLGLAAKMAEE--PFRLLIVDSVIALFRVDF   235
            *.**.:* .*****::****** *** .**  .**: **   *::**:**::*******

HsDMC1      SGRGELAERQQKLAQMLSRLQKISEEYNVAVFVTNQMTADPGATMTFQADPKKPIGGHIL   293
ZmDMC1      SGRGELAERQQKLAQMLSRLTKIAEEFNVAVYITNQVIADPGGGM-FITDPKKPAGGHVL   297
HvDMC1      SGRGELAERQQKLAQMLSRLTKIAEEFNVAVYITNQVIADPGGGM-FITDPKKPAGGHVL   297
TaDMC1      SGRGELAERQQKLAQMLSRLTKIAEEFNVAVYITNQVIADPGGGM-FITDPKKPAGGHVL   297
OsDMC1      SGRGELAERQQKLAQMLSRLTKIAEEFNVAVYITNQVIADPGGGM-FITDPKKPAGGHVL   297
AtDMC1      TGRGELADRQQKLAQMLSRLIKIAEEFNVAVYMTNQVIADPGGGM-FISDPKKPAGGHVL   297
NtDMC1      TGRGELAERQQKLAQMLSRLIKIAEEFNVAVYMTNQVIADPGGGV-FISDPKKPAGGHVL   294
            :******:*********** **:**:****::***: ****. : * :***** ***:*
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FIGURE 3 | Multiple alignment of fragment of the DMC1 protein sequences from various species with positions of substituted amino acids in the *dmc1.b* and *dmc1.c* mutants indicated with red frames. *Hs – Homo sapiens, Zm – Zea mays, Hv – Hordeum vulgare, Ta – Triticum aestivum, Os – Oryza sativa, At – Arabidopsis thaliana, Nt – Nicotiana tabacum*.

FIGURE 4 | Spikes of cv. 'Sebastian' and the *dmc1.b, dmc1.c-3041,* and *dmc1.c-3223* mutants displaying partial sterility. Red arrows indicate examples of places where no grains were developed.

acid changed in the *dmc1.c* mutant (arginine-183) is conserved not only among plants, but also in *Homo sapiens* (**Figure 3**). This also suggests that the *dmc1.c* mutation may have more significant impact on the protein function, nevertheless for further investigation we have used all three mutant lines carrying missense mutations – *dmc1.b*, *dmc1.c-3041* and *dmc1.c-3223*. We have developed homozygous mutant lines and used plants of the M_4/M_5 generation for backcross with their parent variety 'Sebastian' in order to reduce the number of putative background mutations. We selected homozygous mutant plants form the

BC (backcross) F_2 generations and used them for cytological analysis of meiosis.

Cytological Observations of Meiosis in the *hvdmc1.b*, *hvdmc1.c-3041* and *hvdmc1.c-3223* Mutants

The first observed phenotypic feature of all the identified *dmc1* mutants selected for the cytological analysis was partial sterility of their spikes, which indicated some fertility disorders (**Figure 4**). Apart from that, the mutants did not show any evident morphological changes when compared to the wild type 'Sebastian'. In all three *dmc1* mutants we observed chromosome aberrations such as chromosomal bridges and chromosome fragments during anaphase and telophase I and II and micronuclei in tetrads (**Figure 5**). In the *dmc1.c-3041* mutant an abnormal tetrads consisting of three or five haploid cells were observed (**Figure 6**). Additionally, we observed that both *dmc1.c* mutants showed disturbances in the formation of bivalents plate in metaphase I (**Figure 7**). However, this observation needs further investigations.

In all analyzed meiosis phases, the *dmc1.c-3041* and *dmc1.c-3223* mutants displayed statistically significant differences in chromosomal aberration frequency compared to the wild type variety 'Sebastian' (**Figures 8**, **9**). In anaphase/telophase I, the parent variety exhibited the chromosome aberration frequency at the level of 3%, whereas in the *dmc1.c* mutants the chromosome aberration frequency was significantly higher: 17% and 26% in *dmc1.c-3041* and *dmc1.c-3223*, respectively (**Figure 8A**). In anaphase/telophase II, the frequency of cells with chromosome aberrations in the mutants was 19 and 40% (in the *dmc1.c-3041* and *dmc1.c-3223*, respectively; **Figure 8B**) compared to 4% in 'Sebastian'. The frequency of tetrads with micronuclei was also significantly higher in the mutants – 14 and 33% (in *dmc1.c-3041* and *dmc1.c-3223*, respectively) than in the wild type, where it reached 1% (**Figure 9**).

The *dmc1.b* mutant also showed abnormalities during meiosis, but with much lower frequencies than the *dmc1.c* mutants. In anaphase and telophase I, the frequency of chromosomal aberrations in the *dmc1.b* mutant was 5.5%, about two times higher than in the wild type 'Sebastian'. In anaphase and telophase II, the frequency of chromosome aberration (5%) observed in *dmc1.b* did not differ statistically from the parent variety 'Sebastian' (4%) (**Figure 8**). Analysis of micronuclei in tetrads has also shown no significant differences between this mutant and its parent variety (**Figure 9**).

DISCUSSION

We have performed the analysis of meiosis in the *dmc1.b* mutant carrying missense mutation leading to the G212S substitution and both the *dmc1.c* lines (*dmc1.c-3041* and *dmc1.c-3223*) carrying missense mutation causing the R183K change. Observation of meiosis in the mutants revealed that the *dmc1.c* lines showed differences in chromosomal aberrations frequency

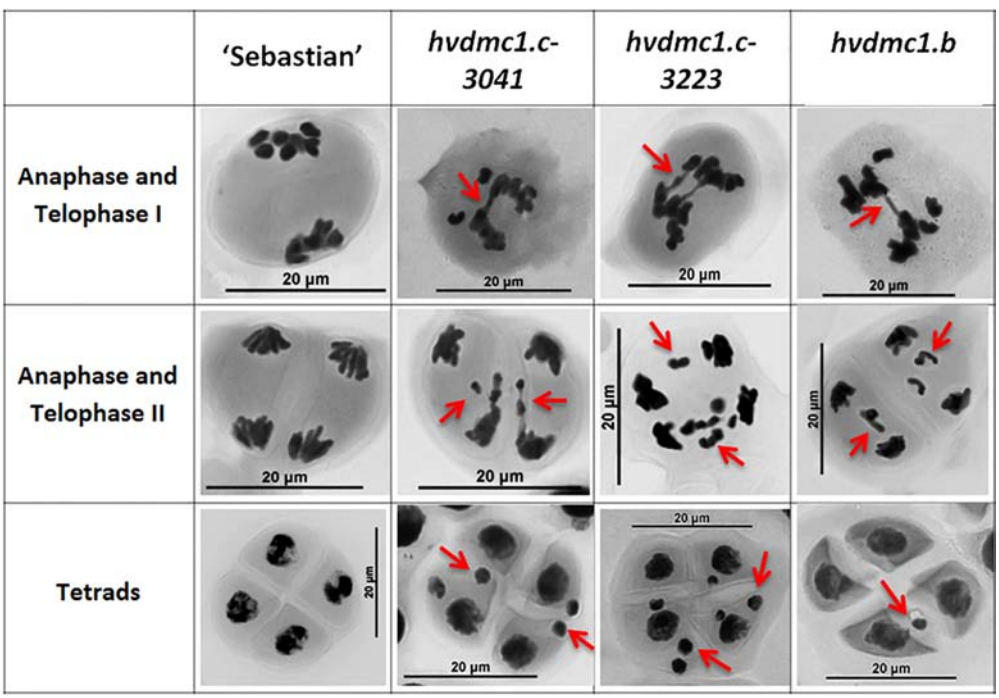

FIGURE 5 | The summary panel with the examples of meiotic cells in different stages: anaphase/telophase I, anaphase/telophase II and in tetrads in 'Sebastian' and the *dmc1.c-3041, dmc1.c-3223 and dmc1.b* mutants. In the anaphase and telophase I the chromosome bridges, in the anaphase and telophase II the chromosome fragments, whereas in tetrad stage the micronuclei are indicated by red arrows.

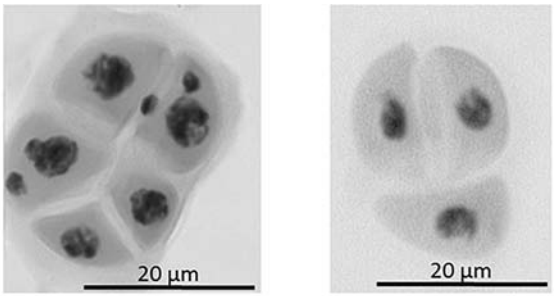

FIGURE 6 | Example of abnormal tetrad formation in the *dmc1.c-3041* mutant. On the left: tetrad composed of five cells, two of the cells show the presence of two micronuclei. On the right: tetrad composed of three cells, no micronuclei are present.

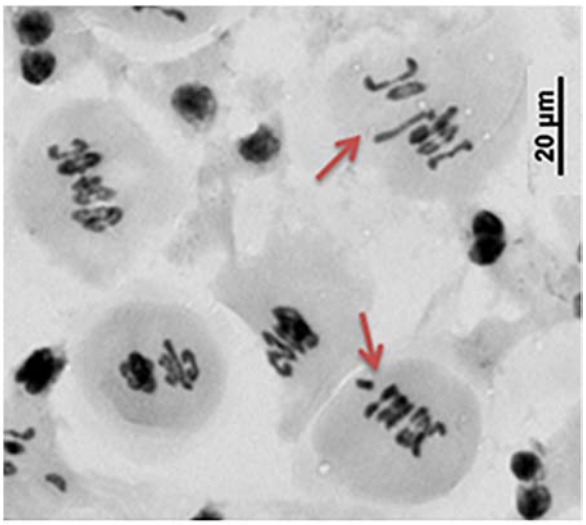

FIGURE 7 | The abnormal chromosome assembling during metaphase I in the *dmc1.c-3223* mutant.

when compared to the wild type, whereas the *dmc1.b* mutant did not show significant disorders. This results are consistent with the *in silico* analysis of conservation of the substituted amino acid positions and with the SIFT values, which have shown that the *dmc1.c* mutation should have a more significant impact on protein function than *dmc1.b*.

Our *dmc1.c* mutants exhibited very high chromosome aberration frequencies in anaphase/telophase I/II and also very high number of tetrads with micronuclei. We suggest that these changes are the result of defects in DSB repair and anomaly in crossing-over, what strongly confirms that the *DMC1* gene is involved in the DSB repair, recombination and chromosome disjunction during meiosis. Both *dmc1.c* mutants showed disturbances in the chromosome assembling during the metaphase I. Such abnormalities have previously been observed in other species, both in plants and animals. The *DMC1*-knock-out mice displayed aberrant chromosomal pairing or non-homologous chromosome pairing in spermatocytes (Habu et al., 1996). Our findings are also consistent with the observations of the *atdmc1* mutants which exhibited abnormalities in the formation of bivalents and chiasmata (Da Ines et al., 2013). Moreover, in meiotic cells of the rice *osdmc1a osdmc1b* insertional double mutant, univalents and abnormal number of chromosomes in the metaphase plate during the second meiotic division were observed (Wang et al., 2016). In our study, one mutated line, *dmc1.c-3041,* formed irregular tetrads containing three or five haploid cells. Similar anomalies were observed in the rice *osdmc1a osdmc1b* double mutant (Wang et al., 2016). This type of anomaly may be the result of abnormal, uneven segregation of chromosomes to the opposite poles of the cell during meiotic divisions. The *dmc1.b* as well as both *dmc1.c* mutant lines show partial sterility of the spikes. Previously reported *dmc1* mutants in different species showed sterility or partial sterility. For example, rice *OsDMC1*-RNAi lines, as well as the insertional *osdmc1a osdmc1b* double mutant grow normally during their vegetative phase, but they are characterized by total sterility (Deng and Wang, 2007; Wang et al., 2016). The insertional Arabidopsis mutant, *atdmc1*, produces viable seeds at very low ratio (1.5%) (Da Ines et al., 2013). The *DMC1* knock-out mice displayed total sterility (Habu et al., 1996). This suggests that fertility disorders are a common feature of individuals lacking the *DMC1* gene in different species. Our *dmc1* mutants are not a knock-out type, they carry missense mutations in the analyzed gene and, as it was predicted in our analysis (**Figure 1**), the substituted amino-acid residues are located in the Rad51 domain, however outside the functional motifs of the DMC1 protein (such as the Walker A and B motifs, and loops 1 and 2), therefore the effect on sterility is not that strong as in other species.

In our study the mutational screening of 5,376 M_2 plants from the *Hor*TILLUS population revealed six independent G/C to A/T mutations within the *HvDMC1* gene. Most mutations (88%) found to date in the *Hor*TILLUS population represented this type of transition (calculated based on data of 32 genes TILLed; Szurman-Zubrzycka et al., 2018). Both mutagens used for creation of our TILLING population (MNU and NaN_3) cause such DNA lesions. *N*-methyl-*N*-nitrosourea belongs to alkylating agents that are known to alkylate guanine and create O^6-metG – the lesion with strong mutagenic property (Kleibl, 2002). O^6-metG mispairs with thymine, which leads to its replacement by adenine in the subsequent replication cycle. If this methylation is induced in a non-transcribed (sense) DNA strand, it leads to G to A transition, whereas if it occurs in transcribed (antisense) DNA strand it results in C to T transition. TILLING populations which were developed after treatment with MNU for *Glycine max* and *Oryza sativa* showed 89.4 and 91.7% G/C to A/T transitions, respectively (Cooper et al., 2008; Suzuki et al., 2008). Sodium azide, the other mutagen used for establishing the *Hor*TILLUS population, is mutagenic only for some plant species, among them barley and rice (reviewed in Gruszka et al., 2012). It was used as the only mutagen in other barley TILLING population - TILLMore developed for cultivar 'Morex', where it caused mainly G/C to A/T transitions (95.5%, Talamè et al., 2008; Sparla et al., 2014).

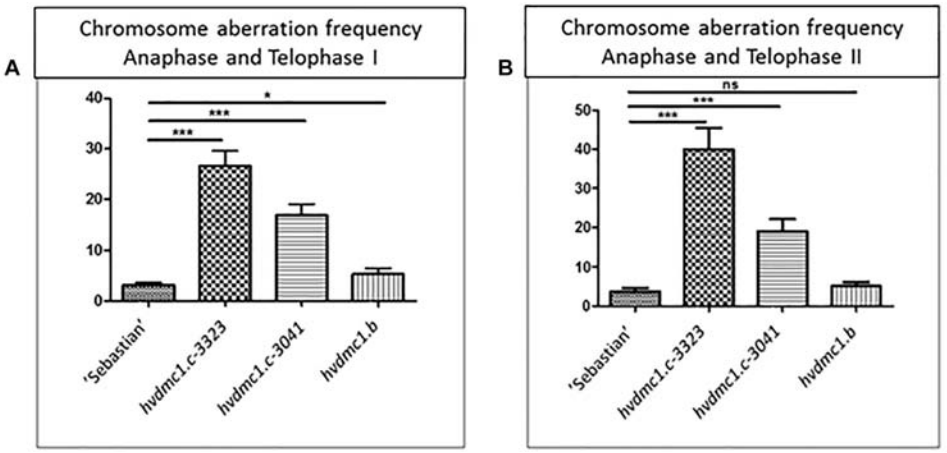

FIGURE 8 | (A) Comparison of the chromosome aberration frequencies in anaphase and telophase I in 'Sebastian' (wt) and three *dmc1* mutants. **(B)** Comparison of the chromosome aberration frequencies in anaphase and telophase II in 'Sebastian' and three *dmc1* mutants. Stars indicate statistical significant differences (ANOVA; $p < 0,05$) between 'Sebastian' and the three *dmc1* mutants, ns – differences between compared genotypes were not statistically significant.

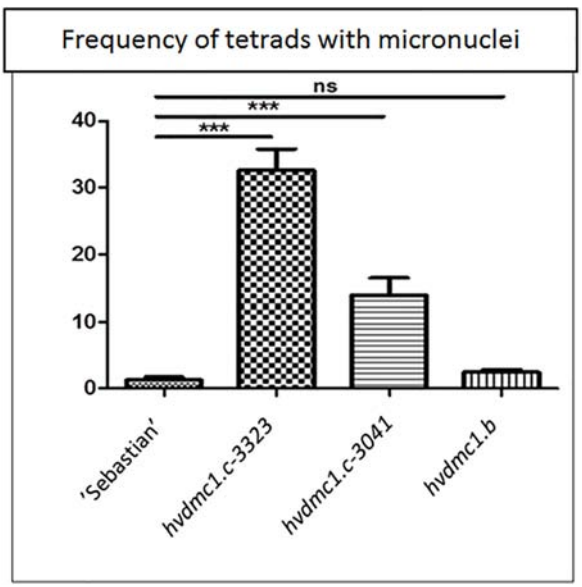

FIGURE 9 | Comparison of the frequency of tetrads with micronuclei in 'Sebastian' (wt) and three *dmc1* mutants. Stars indicate statistically significant differences (ANOVA; $p < 0,05$) between 'Sebastian' and the three *dmc1* mutants, ns – differences between compared genotypes were not statistically significant.

The mutation density calculated based on mutations found in the *HvDMC1* amplicon is 1 per 729 kbp. The average mutation density in the *Hor*TILLUS population is 1 per 477 kbp, however it varies between gene fragments (Szurman-Zubrzycka et al., 2018). The value obtained for *HvDMC1* is slightly lower, what could be caused by amplicon base content (G/C – 42%; A/T – 58%).

One very important aspect in terms of functional genetics is the presence of paralogs within the genome that can take over functions of gene of interest (functional redundancy). If there are two or more closely related genes, usually it is necessary to produce individuals with mutations in both or all of paralogs to perform functional analysis. Barley genome is one of the largest diploid genomes sequenced with a haploid genome size of more than 5 Gbp in seven large chromosomes (International Barley Genome Sequencing Consortium Mayer et al., 2012; Mascher et al., 2017). In order to check if there are any paralogs of *HvDMC1* in barley genome we have screened its 2nd version that has been recently released (Mascher et al., 2017) with the use of the EnsemblPlants[3] and the IPK Barley BLAST[4] servers. Our analysis indicated that the *HvDMC1* gene (HORVU5Hr1G040730) is located on chromosome 5 and has no paralogs in the genome (**Supplementary Materials 1**, **2**), so our *dmc1.b* and *dmc1.c* mutants are good tools to study the function of this gene, because the risk of gene redundancy is very low.

An issue which is sometimes raised considering TILLING mutants is that observed phenotype may be caused by other, than analyzed, mutations in the genome (so called background mutations). Taking into consideration the size of barley genome and the overall mutation density found in the *Hor*TILLUS population (ca. 1/500 kbp; Szurman-Zubrzycka et al., 2018), we can assume that each M_2 plant carries more than 10,000 mutations. However, vast majority of them occur in non-coding regions, since genes (annotated coding sequences) make up 1.3% of barley genome (65.3 Mbp; Mascher et al., 2017). So, statistically, the number of mutations in genes equals to ca. 130 and, probably, most of them are silent and/or do not affect the protein function. Therefore, the probability of the presence of other deleterious mutation in a gene related to the same process of interest is very low. Nevertheless, in order to further decrease this probability, we performed backcrosses of

[3]http://plants.ensembl.org/index.html

[4]http://webblast.ipk-gatersleben.de/barley_ibsc/viroblast.php

the identified mutants with their parent variety 'Sebastian', which reduced the number of (putative) background mutations by half. The homozygous mutants selected from the F_2 populations were phenotyped in this study. What is more, we observed that two different *dmc1.c* mutant lines (*dmc1.c-3041* and *dmc1.c-3223*), that originated from different M_1 plants and possess different mutational background, show similar defects during meiotic divisions. These two lines share only the mutation leading to the R183K substitution in DMC1, whereas any putative background mutations differ between them, which strongly suggests that the identified mutation is responsible for this phenotype.

CONCLUSION

The role of *DMC1* has been validated mostly in model plant species, such as *Arabidopsis thaliana* and rice. Here, we described functional analysis of *HvDMC1* in barley, which belongs to the most important cereal species worldwide. Our barley TILLING population, *Hor*TILLUS, has been used for mutational screening in the *HvDMC1* gene. We have identified and characterized a new allele, named *dmc1.c,* responsible for abnormalities during meiosis. Two mutated lines, from different M_1 plants carrying the same mutation (G2571A that causes the R183K substitution), showed similar defects in this process, which strongly suggests that *HvDMC1* is involved in the proper course of meiosis in barley. We conclude that DMC1 is required for DSB repair during meiosis, the process which has yet to be fully elucidated.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

IS and DG conceived the project and designed the experiments. DG supervised the project. MS-Z, BB, MS-J, and JK conducted the research. MS-Z, IS, and DG wrote the manuscript.

FUNDING

The authors gratefully acknowledge financial support of the research by the Food and Agriculture Organization (FAO) and International Atomic Energy Agency (IAEA) (Grant No. 15657 R0-R3) and by the Polish Ministry of Science and Higher Education (Grant Nos. 687/W-IAEA/2010/0, 773/W-IAEA/2010/0, 2214/FAO/IAEA/2011/0, and 2725/FAO/IAEA/2013/0).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00761/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 July 2019
doi: 10.3389/fpls.2019.00990

Characterization of DNA Repair Foci in Root Cells of *Arabidopsis* in Response to DNA Damage

Takeshi Hirakawa† *and Sachihiro Matsunaga**

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Chiba, Japan

As a sessile organism, plants are constantly challenged by diverse environmental stresses that threaten genome integrity by way of induction of DNA damage. In plants, each tissue is composed of differentiated cell types, and the response to DNA damage differs among each cell type. However, limited information is available on the subnuclear dynamics of different cell types in response to DNA damage in plants. A chromatin remodeling factor RAD54, which plays an important role in the exchange reaction and alteration of chromatin structure during homologous recombination, specifically accumulates at damaged sites, forming DNA repair foci (termed RAD54 foci) in nuclei after γ-irradiation. In this study, we performed a time-course analysis of the appearance of RAD54 foci in root cells of *Arabidopsis* after γ-irradiation to characterize the subnuclear dynamics in each cell type. A short time after γ-irradiation, no significant difference in detection frequency of RAD54 foci was observed among epidermal, cortical, and endodermal cells in the meristematic zone of roots. Interestingly, cells showing RAD54 foci persisted in roots at long time after γ-irradiation, and RAD54 foci in these cells localized to nuclear periphery with high frequency. These observations suggest that the nuclear envelope plays a role in the maintenance of genome stability in response to DNA damage in *Arabidopsis* roots.

Keywords: DNA damage response, DNA repair, homologous recombination, RAD54, nuclear envelope

OPEN ACCESS

Edited by:
Ayako N. Sakamoto, National Institutes for Quantum and Radiological Science and Technology, Japan

Reviewed by:
Keishi Osakabe, Tokushima University, Japan
Thomas Eekhout, VIB-UGent Center for Plant Systems Biology, Belgium

***Correspondence:**
Sachihiro Matsunaga
sachi@rs.tus.ac.jp

†***Present address:***
Takeshi Hirakawa, Biological Science, Nara Institute of Science and Technology, Takayama, Japan

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *06 March 2019*
Accepted: *15 July 2019*
Published: *30 July 2019*

Citation:
Hirakawa T and Matsunaga S (2019) Characterization of DNA Repair Foci in Root Cells of Arabidopsis in Response to DNA Damage. Front. Plant Sci. 10:990. doi: 10.3389/fpls.2019.00990

INTRODUCTION

Genome integrity is constantly threatened by exogenous (e.g., ionizing radiation, ultraviolet light, and chemical components) and endogenous stresses (e.g., stalled DNA replication forks and reactive oxygen species) that induce DNA damage in organisms. In plants, DNA damage is also caused by diverse environmental stresses, such as stress-mediated reactive oxygen species, pathogen infection, high boron concentration, and aluminum ions (Rounds and Larsen, 2008; Sakamoto et al., 2011; Baxter et al., 2014; Song and Bent, 2014). Signaling of DNA damage is rapidly coordinated with several mediators to maintain genome stability in plants. In response to DNA damage in plants, ataxia telangiectasia mutated (ATM) and ATM/rad3-related kinases, which are sensor proteins for DNA double-strand breaks (DSBs) and single-strand DNA, respectively, activate the SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) transcription factor through phosphorylation (Culligan et al., 2004; Yoshiyama et al., 2013a). The active form of SOG1 directly regulates expression of genes participating in DNA repair, cell cycle progression, pathogen response, and phytohormone signaling (Ogita et al., 2018).

After the induction of DSBs, programmed cell death (PCD) is induced specifically in stem cells of the root meristematic zone and the central zone of shoot apical meristems in *Arabidopsis* (Fulcher and Sablowski, 2009). In contrast, the quiescent center (QC) cells, which maintain the homeostasis of stem cells, do not show PCD or morphological alterations in roots with DSBs. In the epidermis and cortex of roots, DSBs induce both arrest of the cell cycle and endoreduplication. Endoreduplication is triggered by inhibiting G2/M progression and specialized cell cycle where DNA replication is repeated without mitosis and cytokinesis, following expansion of the cell volume (Adachi et al., 2011). These findings suggest that the cellular response to DNA damage differs among each cell type in roots following DNA damage. However, little is known about the subnuclear dynamics in each cell type during the response to DNA damage.

In response to DNA damage, DNA repair foci, which are the subnuclear foci formed by DNA repair factors that accumulate specifically at damaged sites, are detected as distinct spots in nuclei (Rothkamm et al., 2015). A phosphorylated histone variant H2AX, termed γH2AX, which is detected around damaged sites and functions as a marker recruiting other DNA repair factors, forms several subnuclear foci upon DNA damage (Rogakou et al., 1999). In plants, the phosphorylation of H2AX is downstream of the activation of ATM by DSBs, and the detection frequency of γH2AX foci increases in a dose-dependent manner following induction of DSBs (Friesner et al., 2005). Thus, γH2AX foci are used as tools to measure DNA repair activity in plant cells upon DNA damage. However, γH2AX foci are undetectable in living cells because immunostaining using a specific antibody is involved. Several studies have shown that certain DNA repair factors form DNA repair foci in living cells of *Arabidopsis* in response to DNA damage (Lang et al., 2012; Jia et al., 2016; Biedermann et al., 2017; Horvath et al., 2017; Liu et al., 2017). Previously, we observed that the chromatin-remodeling factor RAD54, which regulates the spatiotemporal arrangement of homologous loci with DSBs, accumulates specifically at damaged sites, resulting in formation of DNA repair foci termed RAD54 foci (Hirakawa et al., 2015, 2017). RAD54 plays an important role in strand exchange and the alteration of chromatin structure during homologous recombination (HR) repair in eukaryotes (Heyer et al., 2010). *In vitro* analysis showed that yeast RAD54 has an activity of unwinding duplex DNA to promote the exchange reaction in HR (Mazin et al., 2000). In addition, human RAD54 slides nucleosomes along chromatin in an ATP-dependent manner to promote the homology search during HR *in vitro* (Zhang et al., 2007). The *Arabidopsis rad54* mutant shows low HR repair activity and high sensitivity to several genotoxic stresses (Osakabe et al., 2006), and as a result, RAD54 foci contribute to the progression of HR repair. In the present study, we monitored the formation of RAD54 foci in each cell type in *Arabidopsis* roots after the induction of DSBs to characterize the subnuclear dynamics following DNA damage of these cells.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plants used in this study were *Arabidopsis thaliana* ecotype Col-0. Transgenic plants expressing RAD54-EYFP with the *rad54-1* background were constructed in our previous study (Hirakawa et al., 2017). The double-mutant *crwn1/4* was used in a previous study (Sakamoto and Takagi, 2013). Sterilized seeds were sown on half-strength Murashige and Skoog (1/2 MS) medium plates (supplemented with 1% sucrose and 1% agar). After incubation at 4°C for 24 h, the plates were placed in an incubator maintained at 22°C with a 16/8 h (light/dark) photoperiod.

γ-Irradiation and Microscopy

Five-day-old seedlings were exposed to 100 Gy γ-irradiation using a ^{137}Cs source at a dose rate of 0.762 Gy/min at the Research Institute for Biomedical Science, Tokyo University of Science. After γ-irradiation, the roots were observed with a FV1200 confocal microscope equipped with a GaAsP detector (Olympus). To stain the cell walls, seedlings were immersed in 10 μg/ml propidium iodide/D.W. (Sigma-Aldrich) for 2 min. The detection frequency was obtained by dividing the cells showing RAD54 foci by RAD54 positive cells.

EdU and DAPI Staining

Detection of 5-ethynyl-2′-deoxyuridine (EdU) was performed with the Click-iT® Plus EdU Alexa Fluor® 594 Imaging Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Five-day-old seedlings were exposed to 100 Gy γ-irradiation. After 24 h, the seedlings were incubated in liquid 1/2 MS medium containing 10 μM EdU for 20 min to specifically label cells during S phase at that time. The seedlings were fixed with 4% (w/v) paraformaldehyde/PBS for 40 min, washed in PBS, and then incubated in 0.5% (w/v) Triton X-100/PBS for 20 min. The samples were washed in PBS twice and incubated in the Click-iT reaction cocktail for 30 min in the dark. The Click-iT reaction cocktail was removed, and the samples were washed in PBS three times. The samples were washed in PEMT (50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, 0.5% Triron X-100) buffer three times for 5 min each, and then washed in PBS. The samples were incubated in a mixture of DNA-staining solution (Sysmex)/PBS (3:1, v/v) for 3 min and then washed in PBS three times for 5 min each. The samples were mounted under a cover glass with 25% (v/v) 2,2′-thiodiethanol/PBS. Samples were observed with a FV1200 confocal microscope equipped with a GaAsP detector.

Immunostaining

Immunostaining was performed as previously described (Hirakawa et al., 2017). Root tips of 5-day-old seedlings sampled 8 h after γ-irradiation (100 Gy) were analyzed. Rabbit anti-γH2AX (Hirakawa et al., 2017) was used as the primary antibody and diluted 1:100. Anti-rabbit Alexa Fluor

488 (Thermo Fisher Scientific) was used as the secondary antibody and diluted 1:1,000. The specimens were observed with a FV1200 confocal microscope equipped with a GaAsP detector (Olympus).

Shoot Growth Analysis Following MMS Treatment

Sterilized seeds were incubated at 4°C for 24 h. The seeds were sown on 1/2 MS medium plates (1% sucrose and 0.8% agar) containing 0.05% MMS (Sigma-Aldrich). Shoot fresh weight was recorded after 14 days.

RESULTS

Appearance of RAD54 Foci in Each Cell Type of Roots With DNA Double-Strand Breaks

To investigate the DNA repair activity in each cell type in response to DNA damage, we observed the formation of RAD54 foci in root cells after γ-irradiation, which induces DSBs in DNA. RAD54 foci are subnuclear foci where HR repair might occur in chromatin, thus RAD54 foci can be used to monitor the activity of HR repair in living cells (Hirakawa et al., 2017). In the epidermis, cortex, and endodermis of the meristematic zone of roots, the number of cells showing RAD54 foci peaked at 4 h after 100 Gy γ-irradiation, and thereafter decreased from 8 to 24 h after γ-irradiation (**Figures 1A,B**). The detection frequency of cells with RAD54 foci did not differ in these cell types at each time point after γ-irradiation (**Figure 1B**). Thus, the HR repair activity was similar in the epidermis, cortex, and endodermis of roots with DSBs. Next, we monitored the formation of RAD54 foci in stem cells and QC cells in the meristematic zone of roots after γ-irradiation. At 10 min after γ-irradiation, stem cells showing RAD54 foci were detected in the stem cell niche, and the number of these cells increased until 8 h after γ-irradiation (**Figure 1C**). Stem cells with RAD54 foci were rarely observed in stem cell niches containing a greater number of dead cells at 24 h after γ-irradiation. In contrast, RAD54 foci were never detected in QC cells after γ-irradiation, which indicated that HR repair activity in QC cells differed from that in stem cells with DSBs.

RAD54 Foci Are Detected With High Frequency During G1 or G2 Phase Cells at Long Time After Induction of DNA Double-Strand Breaks

The detection frequency of RAD54 foci decreased from 8 to 24 h after γ-irradiation; however, RAD54 foci were detected in each cell type except QC cells in the root at 24 h after γ-irradiation (**Figures 1A–C**). Thus, we characterized the RAD54 foci persisting in root cells at long time after the induction of DSBs. At 24 h after γ-irradiation, the number of RAD54 foci differed substantially among nuclei of the root epidermal cells (**Figure 2A**). In a previous study, we showed that most RAD54 foci were detected at high frequency in epidermal cells in the S to G2 phases of the cell cycle a short time (10 min) after γ-irradiation (Hirakawa et al., 2017). The DNA content, which increases with progression from the S phase to the G2 phase of the cell cycle, is correlated with nucleus size (Jovtchev et al., 2006). Therefore, we measured the nucleus size of cells that showed RAD54 foci to investigate the relationship between the formation of RAD54 foci and the cell cycle. At 24 h after γ-irradiation, most RAD54 foci were detected in nuclei of a wide range of sizes (4–12 μm^2) in the epidermal cells of roots. The correlation coefficient between the number of RAD54 foci and nucleus size was low ($R^2 = 0.28$) (**Figure 2B**). To further analyze the effect of cell cycle on the formation of RAD54 foci, γ-irradiated seedlings were incubated in liquid 1/2 MS medium containing EdU, which is incorporated into cells during the S phase and enables distinction between G1–G2 phase cells and S phase cells (Hayashi et al., 2013). We classified the cells showing RAD54 foci into EdU-labeled cells and non-labeled cells. In the epidermis of roots, the detection frequency of non-labeled cells was higher than that of EdU-labeled cells at 24 h after γ-irradiation (**Figures 2C,D**). This result might suggest that RAD54 foci formed or remained with high frequency in G1 or G2 phase cell.

Nuclear Envelope Is Involved in Formation of RAD54 Foci With DNA Double-Strand Breaks

Subnuclear architecture and chromatin structure affect the efficiency of DNA repair in eukaryotes, including plants (Waterworth et al., 2011; Donà and Scheid, 2015). In cultured animal cells, the rate of DNA repair in heterochromatic regions is slower than that of euchromatic regions after γ-irradiation (Goodarzi et al., 2008). Thus, we investigated whether RAD54 foci were detected in heterochromatic regions with high frequency at long time after γ-irradiation. To visualize heterochromatic regions, we generated transgenic plants expressing RAD54-EYFP and CENH3-tdTomato, which is a centromere-specific histone H3 variant co-localized to a repetitive sequence of 180 bp present in all centromeres (Talbert et al., 2002). We classified the cells into three classes on the basis of the number of RAD54 foci (n = 1–3, 4–8, and 9≤; **Figure 3A**). In the epidermis of the meristematic zone of roots, RAD54 foci, which were merged with or attached to CENH3 signals, were rarely detected in nuclei at 24 h after γ-irradiation (**Figure 3B**). In cultured animal cells, the condensation of chromatin prevents the induction of DSBs from ionizing radiation (Takata et al., 2013). To check whether DSBs were induced at heterochromatic regions, we observed the formation of the histone variant γH2AX, which is the phosphorylated histone variant H2AX detected specifically at damaged sites, at chromocenters where chromatins are condensed in nuclei (Friesner et al., 2005). At 8 h after γ-irradiation, the frequency of the interaction

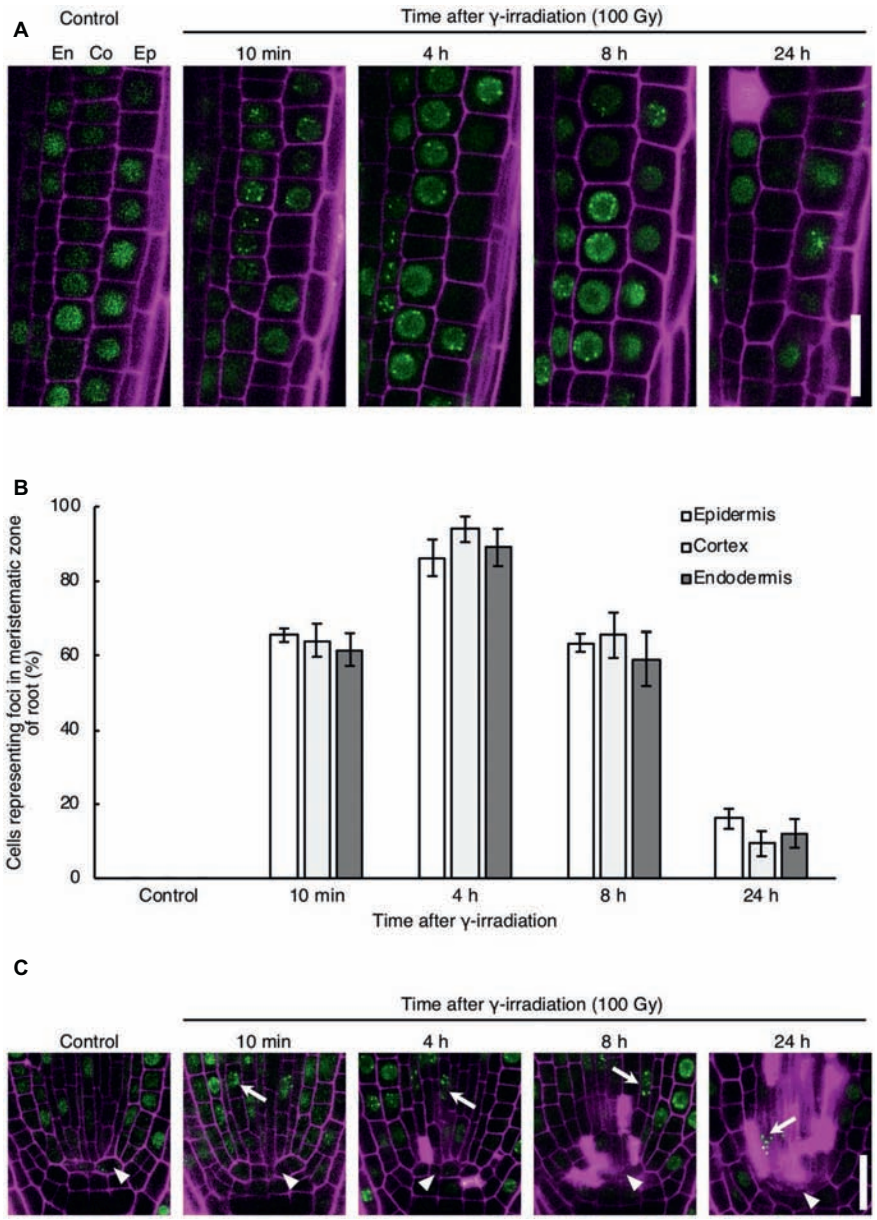

FIGURE 1 | Dynamics of the formation of RAD54 foci in *Arabidopsis* root cells with DNA double-strand breaks. **(A)** Epidermal, cortical, and endodermal cells in the root meristematic zone of plants expressing RAD54-EYFP after γ-irradiation (100 Gy). Green: RAD54-EYFP. Magenta: propidium iodide. Scale bar: 20 μm. **(B)** Detection frequency of cells showing RAD54 foci in the epidermis, cortex, and endodermis in the root meristematic zone at 10 min, 4 h, 8 h, and 24 h after γ-irradiation (100 Gy). Error bars indicate the standard error. At least five roots were counted for each group. **(C)** Stem cells and quiescent center (QC) cells in the root meristematic zone of plants expressing RAD54-EYFP after γ-irradiation (100 Gy). White arrows and arrowheads indicate stem cells and QC cells, respectively. Green: RAD54-EYFP. Magenta: cell wall. Scale bar: 20 μm.

between γH2AX foci and chromocenters was low in nuclei of the root meristematic zone (**Supplementary Figure S1**). These results suggested that the condensation of chromatin presented a barrier for the induction of DSBs in *Arabidopsis*. The nuclear envelope (NE) performs an important function in repairing persistent DSBs in chromatin of mammals and yeast (Gerhold et al., 2015; Amaral et al., 2017). To investigate whether the NE was involved in the formation of RAD54 foci, we generated transgenic plants expressing RAD54-EYFP and SUN1-TagRFP, which is an inner nuclear membrane (INM) protein localized to the nuclear periphery (Oda and Fukuda, 2011). The cells were classified into three classes on the basis of the number of RAD54 foci in nuclei (n = 1–3, 4–8, and 9≤; **Figure 3C**). At 24 h after γ-irradiation, more than 50% of the RAD54 foci were attached to the NE in the epidermal cells of the meristematic zone of roots (**Figure 3D**). To further analyze the relationship between the NE and RAD54 foci, we observed the nuclear dynamics of

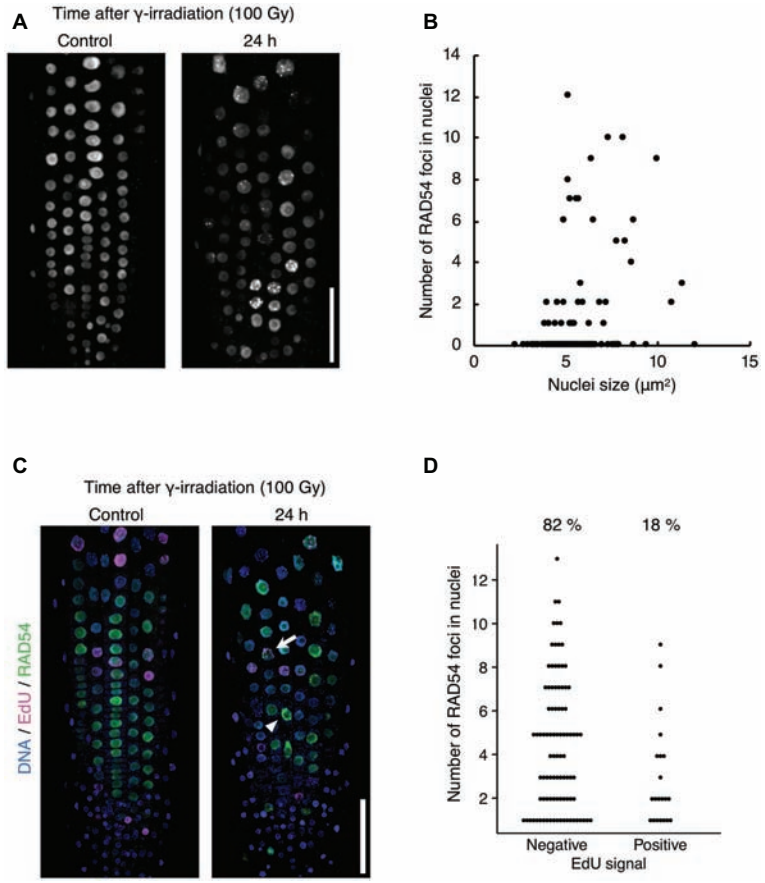

FIGURE 2 | RAD54 foci are detected with high frequency during G1 or G2 phase cells at long time after γ-irradiation. **(A)** Epidermis of the root meristematic zone of plants expressing RAD54-EYFP at 24 h after γ-irradiation. Scale bar: 50 μm. **(B)** Relationship between the number of RAD54 foci in nuclei and nucleus size. The correlation coefficient is 0.28 ($p < 0.01$, $n = 189$). **(C)** Epidermis of the root meristematic zone in plants expressing RAD54-EYFP stained with DAPI and EdU at 24 h after γ-irradiation. Blue: DNA. Magenta: EdU. Green: RAD54-EYFP. White arrows and arrowheads indicate RAD54 foci positive cells labeled with EdU and RAD54 foci positive cells not labeled with EdU, respectively. Scale bar: 50 μm. **(D)** Number of RAD54 foci in cells negative and positive for EdU signals at 24 h after γ-irradiation (100 Gy). Seven roots were counted for each group. Cells lacking RAD54 foci were not counted. Upper percentages are the detection frequency of EdU-labeled cells and non-labeled cells showing RAD54 foci at 24 h after γ-irradiation. EdU negative: $n = 93$; EdU positive: $n = 21$.

RAD54 in a double-mutant of the CROWDED NUCLEI (CRWN) family after γ-irradiation. The CRWN family, which are plant-specific INM proteins, function in the regulation of nuclear morphology and the arrangement of heterochromatic regions in nuclei (Sakamoto and Takagi, 2013; Wang et al., 2013). A recent study showed that a mutation in members of the CRWN family causes high sensitivity to the genotoxic agent methyl methanesulfonate (MMS) and accumulation of DNA damage following MMS treatment which suggests that the CRWN family contributes to DNA repair in response to DNA damage (Wang et al., 2019). At 24 h after γ-irradiation, the number of RAD54 foci attached to the NE in the *crwn1/4* mutant was lower than that in the wild type (**Figure 3E**). In addition, the *crwn1/4* mutant showed the high sensitivity to MMS relative to the WT control during shoot development (**Supplementary Figure S2**). These results suggested that the NE was involved in HR repair, and that CRWN1 and CRWN4 play roles in the repair of DSBs at long time after γ-irradiation in *Arabidopsis*.

DISCUSSION

In this study, we monitored the temporal change in appearance of RAD54 foci in *Arabidopsis* roots after γ-irradiation, to evaluate the DNA repair activity in each cell type of the root. Previous studies have reported that each cell type in roots shows a specific response to DSBs. In the epidermis and cortex, endoreduplication accompanied with an increase in cell volume is induced by zeocin, which is a DSB-inducing agent in plants (Adachi et al., 2011). In contrast, PCD was observed specifically in the stem cells of root tips in response to zeocin treatment (Fulcher and Sablowski, 2009). The present microscopic analysis showed that the detection frequency of cells with RAD54 foci was not significantly different in the epidermis and cortex at each time point of observation after γ-irradiation (**Figures 1A,B**). In addition, the pattern of stem cells with RAD54 foci detected after γ-irradiation was similar to that in the epidermis and cortex (**Figure 1C**). These results suggested that RAD54-dependent HR repair occurred at the same frequency in the

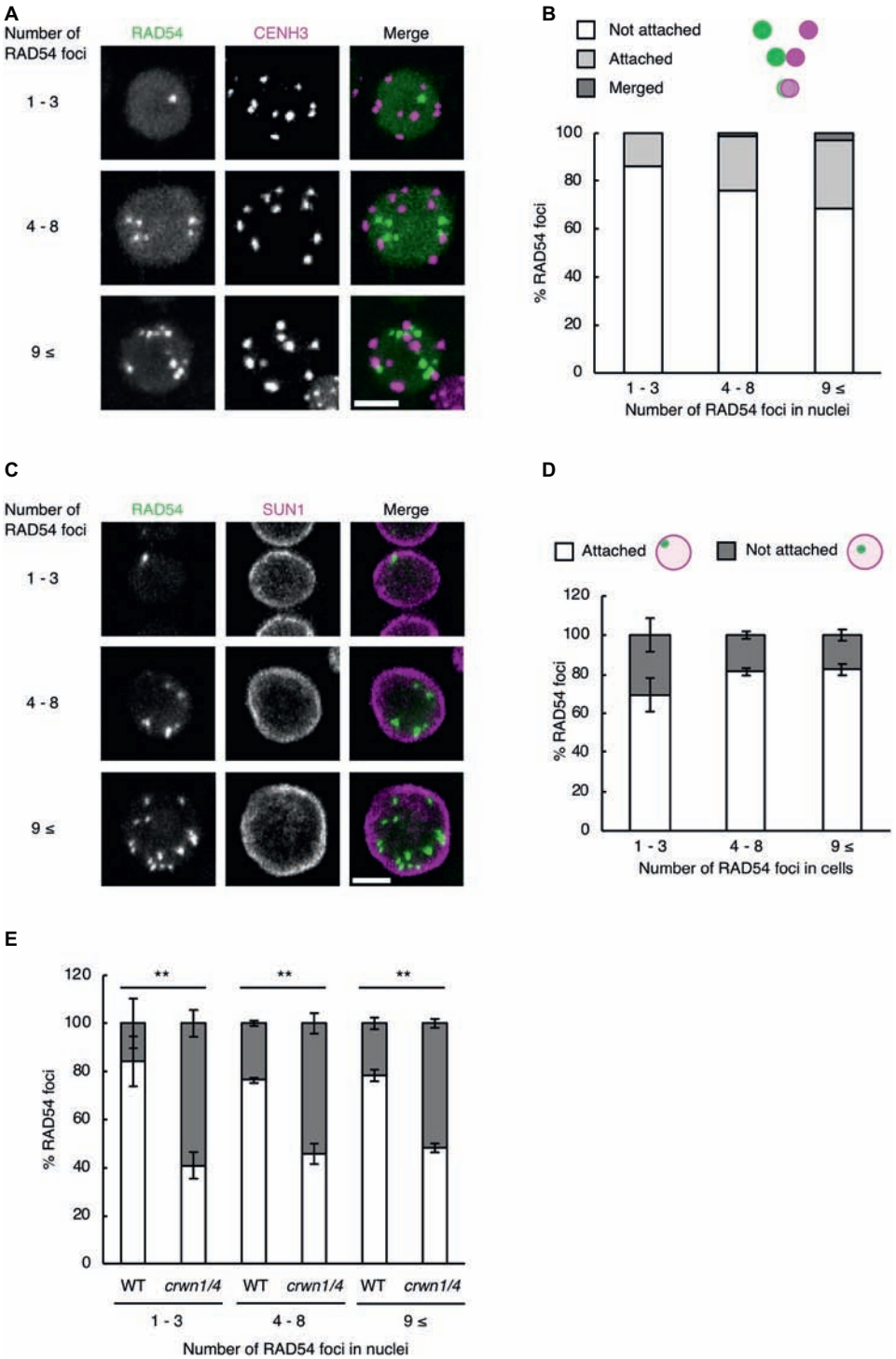

FIGURE 3 | RAD54 foci attached to the nuclear periphery are detected with high frequency at long time after γ-irradiation. **(A)** Nucleus of cells in the root meristematic zone in plants expressing RAD54-EYFP and CENH3-tdTomato at 24 h after γ-irradiation. Green: RAD54-EYFP. Magenta: CENH3-tdTomato. Scale bar: 5 μm. **(B)** Detection frequency of RAD54 foci interacted with CENH3 at 24 h after γ-irradiation. The interaction pattern between RAD54 foci and CENH3 were categorized in three classes (merged with CENH3, attached to CENH3, and not attached to CENH3; $n = 74$). **(C)** Nuclei of cells in the root meristematic zone in plants expressing RAD54-EYFP and SUN1-TagRFP at 24 h after γ-irradiation. Green: RAD54-EYFP. Magenta: SUN1-TagRFP. Scale bar: 5 μm. **(D)** Detection frequency of RAD54 foci attached and not attached to the nuclear envelope (NE) at 24 h after γ-irradiation. Error bars indicate the standard error. Three roots were counted for each group. **(E)** Detection frequency of RAD54 foci attached and not attached to NE in the wild type and the *crwn1/4* double-mutant at 24 h after γ-irradiation. Error bars indicate the standard error. At least three roots were counted for each group. $^{**}p < 0.01$ (Fisher's exact test). The perimeter of nuclei stained with DAPI was defined as the nuclear envelope in this experiment.

epidermis, cortex, and stem cells, whereas these cells showed different responses to DSBs. In stem cell niches, cells showing RAD54 foci and cells undergoing PCD were detected after γ-irradiation (**Figure 1C**). Given that the PCD pathway is closely associated with the signaling pathways in response to DNA damage, PCD might affect the formation of RAD54 foci (Nowsheen and Yang, 2012). Although the signaling pathways activated following DNA damage in plants have been studied in detail, the mechanism controlling PCD in response to DNA damage is still unclear (Yoshiyama et al., 2013b). A number of nucleases and proteases, such as *BIFUNCTIONAL NUCLEASE 1* and *CYSTEINE ENDOPEPTIDASE 1*, could be used to visualize the PCD process in plants (Farage-Barhom et al., 2008; Zhang et al., 2014). Thus, it might be possible to reveal the relationship between the formation of RAD54 foci and PCD by dual fluorescence imaging of RAD54 and these markers of PCD. Interestingly, RAD54 foci were not detected in QC cells at each time point after γ-irradiation (**Figure 1C**). This result is consistent with the observation that progression of the cell cycle in QC cells is arrested at the G1 phase when HR repair activity is low owing to the absence of sister chromatids (Forzani et al., 2014). There are findings about the mechanisms to maintain genome stability in QC cells of animals. In hematopoietic stem cells of mice, non-homologous end-joining mediated repair but not HR repair is preferentially used for repair of DNA damage during the quiescence phase (Mohrin et al., 2010). The detection frequency of γH2AX foci induced by heat stress in quiescent human endometrial mesenchymal cells (MSCs) is considerably lower than that in proliferating MSCs (Alekseenko et al., 2018). Thus, it is suggested that the mechanism of DNA repair in QC cells also differs substantially from that in differentiated cells and stem cells in plants.

We found that RAD54 foci were detected with high frequency during G1 or G2 phase cells in roots at 24 h after γ-irradiation (**Figure 2D**). This result indicates the possibility that RAD54 formed or remained in these cells at long time after the induction of DSBs. To address this question, it might be effective to monitor the appearance and disappearance of RAD54 foci in nuclei after γ-irradiation by time-lapse imaging of RAD54. Additionally, the visualization of G1 and G2 phase cells could definitely reveal the close relationship between these phases and RAD54 foci at long time after γ-irradiation. We also observed that cells showing RAD54 foci persisted in roots and that RAD54 foci attached to the NE were detected with high frequency in these cells at 24 h after γ-irradiation (**Figures 3C,D**). In *Drosophila*, DSBs in heterochromatic regions move to the nuclear periphery to complete HR repair, and the defect of anchoring DSBs at the nuclear periphery reduces tolerance to γ-irradiation and induces ectopic recombination, which might occur between repetitive sequences in heterochromatic regions (Chiolo et al., 2011; Ryu et al., 2015). Persistent DSBs induced by the budding yeast HO-endonuclease system are relocalized to the nuclear periphery, where the DSBs directly bind to the Nup84 nuclear pore complex (Nup84, Nup120, and Nup133) and the INM protein Mps3 (Nagai et al., 2008; Kalocsay et al., 2009). In addition, the budding yeast mutants of Nup120 and Mps3 show high sensitivity to MMS and unequal exchange of sister chromatids (Horigome et al., 2014). The present *Arabidopsis* mutant analyses showed that the plant-specific INM proteins CRWN1 and CRWN4 are required for attachment of RAD54 foci to the nuclear periphery at a long time after γ-irradiation (**Figure 3E**). Thus, we suggest that the NE contributes to the progression of HR repair in eukaryotes, and that CRWN1 and CRWN4 are involved in NE-mediated HR repair and maintenance of genome stability in response to DSBs in plants.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because our manuscript does not include sequence data sets, for example, RNA-seq and ChIP-seq. Requests to access the datasets should be directed to sachi@rs.tus.ac.jp.

AUTHOR CONTRIBUTIONS

TH and SM designed this research and wrote the manuscript. TH performed all experiments.

FUNDING

This research was supported by a CREST grant from the Japan Science and Technology Agency to SM and MEXT/JSPS KAKENHI grants to TH (19J00658, 16J06389) and SM (15H05955, 15H05962).

ACKNOWLEDGMENTS

We thank Dr. Yuki Sakamoto (Osaka University, Japan) for providing the seeds of *crwn1/4* and the plasmid of SUN1-TagRFP, and Dr. Takuya Sakamoto (Tokyo University of Science, Japan) for providing the plasmid of CENH3-tdTomato.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00990/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | γH2AX foci are not detected at chromocenters after γ-irradiation. **(A)** Nucleus showing γH2AX foci in the root meristematic zone at 8 h after γ-irradiation. Green: γH2AX. Magenta: DNA. Scale bar: 5 μm. **(B)** Detection frequency of γH2AX foci interacted with chromocenters at 8 h after γ-irradiation. The interaction pattern between γH2AX foci and chromocenters were categorized in three classes (merged with chromocenters, attached to chromocenters, and not attached to chromocenters; $n = 49$).

SUPPLEMENTARY FIGURE S2 | *crwn1/4* mutants show high sensitivity to MMS during shoot development. **(A)** Shoot development of wild type and *crwn1/4* plants treated with and without 0.05% MMS. Scale bar: 0.5 cm. **(B)** Shoot fresh weight of WT and *crwn1/4* plants treated with and without 0.05% MMS ($n = 20$). $p < 0.01$ (Student's *t*-test).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors SM.

frontiers in Plant Science

REVIEW
published: 30 August 2019
doi: 10.3389/fpls.2019.01055

DNA Base Excision Repair in Plants: An Unfolding Story With Familiar and Novel Characters

Teresa Roldán-Arjona[1,2,3], *Rafael R. Ariza*[1,2,3] *and Dolores Córdoba-Cañero*[1,2,3]*

[1] Maimónides Biomedical Research Institute of Córdoba (IMIBIC), Córdoba, Spain, [2] Department of Genetics, University of Córdoba, Córdoba, Spain, [3] Reina Sofia University Hospital, Córdoba, Spain

Base excision repair (BER) is a critical genome defense pathway that deals with a broad range of non-voluminous DNA lesions induced by endogenous or exogenous genotoxic agents. BER is a complex process initiated by the excision of the damaged base, proceeds through a sequence of reactions that generate various DNA intermediates, and culminates with restoration of the original DNA structure. BER has been extensively studied in microbial and animal systems, but knowledge in plants has lagged behind until recently. Results obtained so far indicate that plants share many BER factors with other organisms, but also possess some unique features and combinations. Plant BER plays an important role in preserving genome integrity through removal of damaged bases. However, it performs additional important functions, such as the replacement of the naturally modified base 5-methylcytosine with cytosine in a plant-specific pathway for active DNA demethylation.

Keywords: DNA repair, DNA damage, DNA glycosylase, AP endonuclease, *Arabidopsis*

OPEN ACCESS

Edited by:
Alma Balestrazzi, University of Pavia, Italy

Reviewed by:
Seiichi Toki, National Institute of Agrobiological Sciences, Japan
Ales Pecinka, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**
Dolores Córdoba-Cañero
b72cocad@uco.es

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *05 April 2019*
Accepted: *30 July 2019*
Published: *30 August 2019*

Citation:
Roldán-Arjona T, Ariza RR and Córdoba-Cañero D (2019) DNA Base Excision Repair in Plants: An Unfolding Story With Familiar and Novel Characters. Front. Plant Sci. 10:1055. doi: 10.3389/fpls.2019.01055

INTRODUCTION

The genomes of all organisms are susceptible to a variety of DNA lesions arising from endogenous and exogenous sources (Lindahl, 1993). Such threats to genome integrity are counteracted by diverse DNA repair pathways that are best understood in bacteria, yeast, and mammals. The base excision repair (BER) pathway is a critical DNA repair mechanism for removal of damaged bases arising from oxidation, alkylation, or deamination (Krokan and Bjoras, 2013). BER is initiated by DNA glycosylases that excise the damaged base and completed by additional proteins that remove the remaining sugar–phosphate moiety, fill the subsequent gap, and perform ligation. Knowledge about the BER pathway in plants has greatly advanced in the last two decades, mainly through studies in the model organism *Arabidopsis thaliana*, although additional progress has been made in other species. Results obtained so far indicate that plants have orthologs of most BER genes previously identified in other organisms. However, they also possess some plant-specific BER proteins, as well as distinctive enzyme combinations not found in other kingdoms. In the following sections, we first present a brief overview of the major stages in the BER pathway and then focus on the plant enzymes involved in every step, discussing their similarities and differences with BER factors from bacteria, yeast, and mammals.

OVERVIEW OF BASE EXCISION REPAIR

BER is a complex mechanism that occurs in several steps: i) excision of the damaged DNA base, ii) cleavage of the sugar–phosphate backbone at the generated abasic (apurinic/apyrimidinic, AP) site, iii) clean-up of the resulting DNA ends, iv) gap filling through DNA synthesis, and v) DNA ligation (**Figure 1**). Repair factors involved in these stages have been identified primarily through studies in bacterial and mammalian systems.

The first BER step involves the excision of a modified or incorrect base through the action of a DNA glycosylase that cleaves the N-glycosidic bond, thus releasing the target base and leaving an AP site with the sugar–phosphate backbone intact. There are multiple DNA glycosylases with different substrates specificities (Friedberg et al., 2006; Jacobs and Schar, 2012).

Subsequent AP site processing can be achieved either by an AP lyase activity, usually associated with a subset of DNA glycosylases, or by AP endonucleases. Based on their catalytic activities, DNA glycosylases are classified into monofunctional and bifunctional. Monofunctional DNA glycosylases only remove the target base, thus generating an AP site, whereas bifunctional glycosylases possess an associated AP lyase activity that, after base excision, catalyzes 3′ incision to the AP site by β-elimination, generating 3′-α, β unsaturated aldehyde (3′-PUA), and 5′-hydroxyl (OH) termini. Some bifunctional DNA glycosylases perform a later δ-elimination reaction converting the 3′-PUA end in a 3′-phosphate (3′-P) terminus. The AP site generated by monofunctional DNA glycosylases is usually processed by an AP endonuclease, which cleaves the DNA backbone 5′ to the abasic site, thus generating 3′-OH and 5′-deoxyribose-5-phosphate (5′-dRP) termini (Levin and Demple, 1990; Dianov et al., 1992).

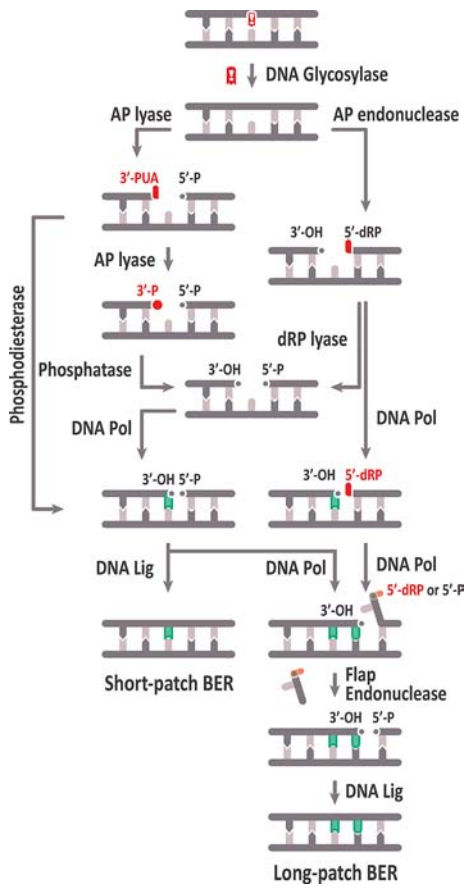

FIGURE 1 | Schematic representation of the base excision repair (BER) pathway. See text for details.

Unconventional ends generated by AP lyases (3′-PUA or 3′-P) and AP endonucleases (5′-dRP) need to be processed to conventional 3′-OH and 5′-P termini, respectively, to allow DNA polymerization and ligation. Cleaning of 3′-PUA ends is performed by the 3′-phosphodiesterase activity of AP endonucleases, whereas the 3′-P termini are processed by a DNA 3′-phosphatase, which in mammalian cells is polynucleotide kinase phosphatase (PNKP) (Pascucci et al., 2002; Wiederhold et al., 2004). The 5′-dRP end must be processed to a 5′-P end by a deoxyribosephosphate (dRP) lyase activity that, in mammalian cells, is associated to DNA polymerase β (Srivastava et al., 1998).

Once the blocked termini have been processed to 5′-P and 3′-OH ends, gap filling may proceed either by insertion of one nucleotide (short-patch or "single-nucleotide BER," SP-BER) or 2–13 nucleotides (long-patch, LP-BER). In mammals, DNA polymerase β is involved in nucleotide insertion during SP-BER (Srivastava et al., 1998), and the resulting nick is ligated by a complex of XRCC1 and LigIIIα (Nash et al., 1997). In contrast, LP-BER requires replicative DNA polymerases (Pol δ and Pol ε, in mammals), which displace the strand containing the 5′-dRP terminus, generating a flap structure that is processed by a flap endonuclease (FEN1), and finally, the generated nick is sealed by LIG1 (Levin et al., 1997).

Plants possess homologs of most BER proteins identified in other organisms (Britt, 2002; Hays, 2002; Roldan-Arjona and Ariza, 2009b) (**Table 1**), and the complete BER pathway was reproduced *in vitro* using *Arabidopsis* cell extracts (Cordoba-Cañero et al., 2009). However, some factors are absent in plants, such as Pol β (Garcia-Diaz and Bebenek, 2007; Roy et al., 2008), others are encoded by multiple gene copies in plant genomes, such as PCNA and FEN1 (Kimura et al., 2003; Strzalka and Ziemienowicz, 2011), and additionally some BER proteins appear to be restricted to plants (Choi et al., 2002; Gong et al., 2002). Such differences suggest that plant-specific characteristics arose during BER evolution. In the following sections, we review plant factors involved in the main BER stages.

BASE REMOVAL

BER is initiated by DNA glycosylases that recognize and excise the modified or damaged bases by hydrolytic cleavage of the N-glycosidic bond between the C1′ of the 2′-deoxyribose and the N atom at the target base. Most DNA glycosylases studied to date remove the target base through a base-flipping mechanism that involves DNA bending and distortion to facilitate base extrusion.

TABLE 1 | Proteins involved in BER in bacteria, yeast, humans, and *Arabidopsis*.

BER enzyme	*E. coli*	*S. cerevisiae*	*H. sapiens*	*Arabidopsis*		
				Name	Gene ID	Reference
DNA glycosylases						
Uracil DNA glycosylases superfamily	Ung	Ung1p	UNG	AtUNG	AT3G18630	(Cordoba-Cañero et al., 2010)
	Mug		TDG			
			Smug1			
AAG			MPG	AthAAG	AT3G12040	(Santerre and Britt, 1994)
H2TH superfamily	MutM			AtFPG	AT1G52500	(Ohtsubo et al., 1998)
	Nei		NEIL1			
			NEIL2			
			NEIL3			
HhH-GPD superfamily	Nth	Ntg1p	NTHL1	AtNTH1	AT2G31450	(Roldan-Arjona et al., 2000)
		Ntg2p		AtNTH2	AT1G05900	(Gutman and Niyogi, 2009)
		Ogg1p	OGG1	AtOGG1	AT1G21710	(Dany and Tissier, 2001; Garcia-Ortiz et al., 2001)
	MutY		MYH	AtMUTY	AT4G12740	
	AlkA	Mag1p		AtAlkA	Two putative homologs	
	Tag			AtTag	Nine putative homologs	
			MBD4	AtMBD4L	AT3G07930	(Ramiro-Merina et al., 2013)
DML family				ROS1	AT2G36490	(Gong et al., 2002)
				DME	AT5G04560	(Morales-Ruiz et al., 2006)
				DML2	AT3G10010	(Ortega-Galisteo et al., 2008)
				DML3	AT4G34060	(Ortega-Galisteo et al., 2008)
AP endonucleases						
Xth family	Xth	Apn2p	APE1	ARP	AT2G41460	(Cordoba-Cañero et al., 2011)
				AtAPE1L	AT3G48425	(Li et al., 2015)
			APE2	AtAPE2	AT4G36050	(Li et al., 2015)
Nfo family	Nfo	Apn1p				
3′ DNA phosphatases						
		Tpp1p	PNKP	ZDP	AT3G14890	(Petrucco et al., 2002; Martinez-Macias et al., 2012)
DNA polymerases						
Family A	Pol I	Pol γ	Pol γ			
				AtPolIA	AT1G50840	(Trasvina-Arenas et al., 2018)
				AtPolIB	AT3G20540	(Trasvina-Arenas et al., 2018)
			Pol θ	AtPol θ	AT4G32700	(Inagaki et al., 2006)
Family B		Pol α	Pol α	AtPol α	AT1G67630	
		Pol δ	Pol δ	AtPol δ	AT2G42120	
		Pol ε	Pol ε	AtPol ε	AT1G08260	
Family X			Pol β			
		PolIV	Pol λ	AtPolλ	AT1G10520	(Amoroso et al., 2011; Roy et al., 2011)
Flap endonucleases		Rad27p	FEN1	AtFEN1	AT5G26680	(Zhang et al., 2016b)
DNA ligases						
NAD^+-dependent	LigA					
ATP-dependent		Cdc9p	LIG1	AtLig1	AT1G08130	(Cordoba-Cañero et al., 2011)
			Lig3			
			LIG4	AtLIG4	AT5G57160	(Waterworth et al., 2010)
				LIG6	AT1G66730	(Waterworth et al., 2010)

Then, the damaged base is inserted into a hydrophobic pocket so that catalytic residues can access the N-glycosidic bond, and an amino acid (the *base flipper* residue) fills in the vacant space left behind in the double helix. In some cases, the intercalated residue and/or other enzyme residues make specific interactions with the orphan opposite base in the complementary strand (Huffman et al., 2005; Dalhus et al., 2009). Monofunctional DNA glycosylases cleave the N-glycosidic bond using an activated water molecule as nucleophile to attack the C1′ of the target nucleotide, whereas bifunctional DNA glycosylases use as nucleophile the amine moiety of a residue from the active site, thereby forming a Schiff base intermediate.

There are different types of DNA glycosylases, each specialized for a particular type of chemical damage or a range of structurally related lesions. Five structural superfamilies of DNA glycosylases have been identified: uracil DNA glycosylase (UDG), alkyladenine DNA glycosylase (AAG), helix-hairpin–helix (HhH-GPD), helix–two-turn–helix (H2TH), and HEAT-like repeat (HLR) (Dalhus et al., 2009). Despite their different structures, it seems clear that all DNA glycosylase families, except the HLR family (Mullins et al., 2015), use a base-flipping strategy to recognize and excise their substrates. Since HLR-like DNA glycosylases are mostly prokaryotic and not present in plants, in the following sections, we will concentrate on the remaining four superfamilies.

UDG Superfamily

Uracil DNA glycosylases (UDG) are monofunctional glycosylases that remove uracil from DNA. In addition to spontaneous deamination of cytosine to uracil, which contributes significantly to the accumulation of mutagenic U:G mispairs, dUMP can be misincorporated during replication in U:A pairs (Kavli et al., 2007). UDG activity has been partially purified in some plant species, such as carrot, wheat, onion, or maize (Blaisdell and Warner, 1983; Maldonado et al., 1985; Bensen and Warner, 1987; Talpaert-Borle, 1987; Bones, 1993).

All members of the UDG superfamily are proteins with a single domain comprising four-stranded parallel twisted β-sheet flanked by α-helices (Mol et al., 1995). On the basis of substrate specificity, UDGs are classified into six families distributed across eubacteria, archaea, yeast, animals, and plants (Schormann et al., 2014). Family 1 of UDG, represented by *Escherichia coli* Ung and human UNG, is the most extensively studied and the most widely distributed, present in most species examined, with some remarkable exceptions such as *Drosophila melanogaster* and Archaea (Aravind and Koonin, 2000).

A member of the Family-1 UDG from *Arabidopsis*, AtUNG (AT3G18630), has been purified and characterized (Cordoba-Cañero et al., 2010). The AtUNG protein sequence conserves the active site motifs A and B present in the five UDG families and the critical residues implicated in base recognition and catalysis in Family-1 enzymes (Cordoba-Cañero et al., 2010). In human cells, two isoforms of UNG, with different cellular localizations, are generated by alternative splicing: UNG1 in the mitochondria and UNG2 in the nucleus (Nilsen et al., 1997). The N-terminal sequence of AtUNG contains a putative PCNA-binding motif and shows higher degrees of similarity to human UNG2 than to UNG1 (Cordoba-Cañero et al., 2010). So far, no evidence of AtUNG multiple targeting has been found in *Arabidopsis*, although UDG activity has been detected in mitochondrial extracts and an AtUNG-eGFP fusion protein, transiently expressed in *N. benthamiana* leaves, colocalized with mitochondria in protoplasts generated from the agro-infiltrated tissues (Boesch et al., 2009). Therefore, the possibility that plant UNG is targeted to mitochondria and/or chloroplasts cannot be ruled out.

E. coli and human UNG excise uracil but no other 5-substituted pyrimidines, except for 5-fluorouracil (5-FU) (Mauro et al., 1993; Krokan et al., 2002), probably because uracil and 5-FU residues are small enough to fit the tight uracil-binding pocket compared to the larger chloro-, methyl-, bromo-, and iodo-substituted uracils (Liu et al., 2002). In contrast to bacterial and human enzymes, AtUNG lacks detectable activity on 5-FU (Cordoba-Cañero et al., 2010), suggesting that steric constraints imposing selectivity and specificity for uracil against other pyrimidines are more strict in the plant enzyme.

Available evidence suggests that AtUNG encodes the major UDG activity detected in *Arabidopsis* cell extracts, since such activity disappears in *atung* null mutants (Cordoba-Cañero et al., 2010). Similarly to other multicellular organisms, *atung* mutant plants show neither visible phenotypic alterations nor detectable increased levels of uracil in the genome, although neither UDG activity nor uracil BER is detected (Cordoba-Cañero et al., 2010). However, inactivation of the *AtUNG* gene protects plants against the cytotoxic effect of 5-FU, indicating that UDG activity is harmful for cells with high levels of dUTP/dTTP ratio (Cordoba-Cañero et al., 2010). The *Arabidopsis* genome contains another gene (AT2G10550) with partial sequence similarity to UNG, and it has been suggested that it is an inactive paralog interrupted by two transposon insertions, probably originated by a gene duplication process (Cordoba-Cañero et al., 2010). UDG Family 2 (exemplified by *E. coli* Mug and human TDG), Family 3 (typified by vertebrate SMUG1), and Families 4 and 5 (identified in thermophilic bacteria and archaea) are not represented in plants (Cordoba-Cañero et al., 2010).

AAG Superfamily

The members of the AAG superfamily, also known as alkylpurine-DNA glycosylases or N-methylpurine DNA glycosylases, are compact single-domain enzymes with a mixed α/β structure and a positively charged DNA-binding surface (Brooks et al., 2013). These enzymes, unrelated to other BER enzymes, are monofunctional glycosylases that remove alkylated purines and ethenopurines, and the best characterized is human AAG (hAAG). In land plants, a hAAG ortholog (AtAAG) was first isolated in *Arabidopsis* (Santerre and Britt, 1994). AtAGG complements the sensitive phenotype to methyl methanesulfonate (MMS) of an *E. coli* double mutant deficient in N3-methyladenine (N3-meA) glycosylases and excises N3-meA, but not N7-methylguanine (N7-meG) (Santerre and Britt, 1994; Malhotra and Sowdhamini, 2013). Expression of *AtAAG* seems to be higher in growing tissues, supporting the importance of maintaining genome integrity in dividing cells (Santerre and Britt, 1994; Shi et al., 1997). AAG genes have been also detected in other higher plants, including maize (Fu et al., 2010; Wang et al., 2015), wheat (Mak et al., 2006), grape (Tillett et al., 2012), and *Brachypodium distachyon* (Kim et al., 2012).

HhH-GPD Superfamily

The HhH-GPD superfamily is the most heterogenous DNA glycosylase superfamily, with widely different substrate specificities. Its characteristic HhH motif is a DNA-binding domain that is present in a number of proteins that bind DNA in a sequence-independent manner (Thayer et al., 1995; Doherty et al., 1996). This superfamily includes both monofunctional and bifunctional members, and their structures share two characteristic domains with the active site located at their junction. The core fold consists of four N-terminal and six to seven C-terminal α-helices, linked by a type-II β-hairpin (Doherty et al., 1996). The HhH motif is followed by a loop (GPD motif) containing glycine (G), proline (P), and an invariable aspartic acid (D) residue (Huffman et al., 2005). The conserved aspartic acid activates the nucleophile (a molecule of water or a lysine residue in monofunctional or bifunctional DNA glycosylases, respectively) for attack of the N-glycosidic bond (Huffman et al., 2005). These enzymes remove a broad

spectrum of lesions, including those generated by alkylation, oxidation, or hydrolytic damage.

Mammals do not appear to possess homologs of the 3-methyladenine DNA glycosylases belonging to this family (Tag and AlkA), and rather, they use AAG to remove alkylated purines (Dalhus et al., 2009). However, in addition to AtAAG *Arabidopsis* possesses 9 and 2 putative homologs of Tag and AlkA enzymes, respectively (Britt, 2002), none of which has been characterized so far.

Oxidatively damaged pyrimidines in *E. coli* are repaired by Nth, also known as Endonuclease III (EndoIII), a bifunctional glycosylase with AP lyase activity (Katcher and Wallace, 1983). *Arabidopsis* possesses two structural and functional homologs of Nth: AtNTH1 (AT2G31450) (Roldan-Arjona et al., 2000) and AtNTH2 (AT1G05900) (Gutman and Niyogi, 2009). AtNTH1 exhibits DNA glycosylase activity on urea and thymine glycol from double-stranded DNA and also possesses AP lyase activity (Roldan-Arjona et al., 2000). AtNTH2 has three splice variants described. Expressed AT1G05900.2 splice variant exhibited significant glycosylase/lyase activity on DNA containing thymine glycol (Gutman and Niyogi, 2009). AtNTH1 and AtNTH2 (AT1G05900.2 splice variant) fused to GFP seem to be targeted to chloroplast nucleoids (Gutman and Niyogi, 2009). An alternative AtNTH1 transcription initiation site would allow translation from a downstream ATG to generate a predicted protein with a putative nuclear localization signal and lacking chloroplast targeting (Roldan-Arjona et al., 2000; Gutman and Niyogi, 2009). A phylogenetic analysis of EndoIII homologs in bacteria, archaea, and eukaryotes reveals major phylogenetic relationships of AtNTH1 with eukaryotic proteins, being most similar to EndoIII from *Schizosaccharomyces pombe* (Roldan-Arjona et al., 2000). In *Saccharomyces cerevisiae*, there are also two functional homologs (Ntg1p and Ntg2p) of *E. coli* EndoIII, with Ntg1p localizing primarily to mitochondria and Ntg2p to the nucleus (You et al., 1999). In humans, however, the only functional homolog identified so far (hNTH1) contains a putative nuclear localization signal at the N-terminus (Aspinwall et al., 1997), although it has been located in both nucleus and mitochondria (Takao et al., 1998). The subcellular localization of other splice variants of AtNTH2 remains to be determined. Therefore, AtNTH1 and AtNTH2 could have a role in the removal of oxidative lesions in both nuclear and organellar genomes.

The major oxidation product of purines is 7-hydro-8-oxoguanine (8-oxoG), which is originated as a consequence of the oxidation of the hydroxyl radical of C8 of a guanine (Dizdaroglu, 1985). It is a highly mutagenic lesion due to its capacity to pair with both cytosine and adenine (Shibutani et al., 1991). Repair of 8-oxoG in eukaryotes is performed by 8-oxoguanine DNA glycosylases (OGG), bifunctional glycosylases belonging to the HhH-GPD superfamily, that catalyze the excision of 8-oxoG and cleave the generated AP site by a β-elimination mechanism (Girard and Boiteux, 1997). Ogg1 homologs are present in eukaryotes, including humans (Radicella et al., 1997; Roldan-Arjona et al., 1997), and in some archaea, but not in bacteria (Eisen and Hanawalt, 1999). *Arabidopsis* has an OGG1 homolog with more than 40% identity with yeast and human OGG1 proteins (Dany and Tissier, 2001; Garcia-Ortiz et al., 2001). In contrast with the mammalian *OGG1* gene that produces several splice variants with mitochondrial or nuclear localization (Nishioka et al., 1999), in *Arabidopsis*, only one isoform of this protein seems to be produced (Dany and Tissier, 2001). The *Arabidopsis* OGG1-predicted protein possesses a putative nuclear localization signal at the N-terminus, but lacks identifiable signal sequences for targeting to plastids or mitochondria (Dany and Tissier, 2001; Garcia-Ortiz et al., 2001). Although it has been suggested that there is a putative mitochondrial targeting sequence in MtOGG1 from *Medicago truncatula* (Macovei et al., 2011), the subcellular localization of OGG1 in plants remains to be determined.

Expression of AtOGG1 abolishes the mutator phenotype of an *E. coli mutM mutY* mutant strain, thus indicating its capacity to excise 8-oxoG *in vivo* (Dany and Tissier, 2001; Garcia-Ortiz et al., 2001). *Arabidopsis atogg1* mutants show no obvious phenotypic differences in comparison with wild-type plants (Murphy, 2005), but *in vitro* BER assays with *atogg1* mutant cell extracts show that AtOGG1 contributes to the excision of 8-oxoG and counteracts accumulation of oxidative DNA damage (Cordoba-Cañero et al., 2014). Biochemical characterization of AtOGG1 demonstrated its activity on DNA substrates containing 8-oxoG (Dany and Tissier, 2001; Garcia-Ortiz et al., 2001) and the imidazole ring-opened derivative 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (Morales-Ruiz et al., 2003). The enzyme preferentially excises 8-oxoG paired to guanine, in comparison with 8-oxoG:A pairs generated with high frequency during replication (Morales-Ruiz et al., 2003). In *E. coli*, the excision of A mispaired to 8-oxoG is catalyzed by MutY (Michaels et al., 1992). Homologs to bacterial *mutY* have been characterized in both eukaryotes and archaea (Eisen and Hanawalt, 1999). *Arabidopsis* possesses a putative MutY homolog (AT4G12740), which remains uncharacterized.

Spontaneous deamination of 5-methylcytosine (5-meC) to thymine leads to T:G mispairs targeted by thymine-DNA mismatch glycosylases, such as bacterial MIG and mammalian MBD4 (also known as MED1) (Horst and Fritz, 1996; Hendrich et al., 1999; Berti and McCann, 2006). MBD4, which possesses a methyl-CpG-binding domain (MBD) and a HhH-GPD DNA glycosylase domain, is a monofunctional DNA glycosylase that excises U or T mispaired to G, with a preference for mismatches at a CpG context (Nash et al., 1996; Hendrich and Bird, 1998; Bellacosa et al., 1999; Hendrich et al., 1999; Petronzelli et al., 2000a; Petronzelli et al., 2000b; Turner et al., 2006). A plant MBD4 homolog, termed MBD4-like (AtMBD4L, AT3G07930), has been identified in *Arabidopsis* (Ramiro-Merina et al., 2013). AtMBDL4 and other plant MBD4 homologs lack the MBD domain present at the N-terminus of metazoan MBD4 proteins, but share a C-terminal catalytic domain with critical residues specifically conserved in MBD4 glycosylases. AtMBD4L excises uracil and 5-substituted uracil derivatives, such as 5-BrU or 5-FU, with more efficiency than thymine (Ramiro-Merina et al., 2013). Since AtMBD4L shows a clear preference for a CpG sequence context, where the majority of plant DNA methylation takes place, it has been suggested that this enzyme plays a role in preventing the potential mutagenic effects of 5-meC deamination (Ramiro-Merina et al., 2013). Four alternative splice variants of AtMBD4L have been described, two of which (AtMBD4L3 and AtMBD4L4) are expressed in leaves and flowers, whereas another

one (AtMBD4L3) has been localized in the nucleus (Nota et al., 2015). Interestingly, plants overexpressing AtMBD4L3 show increased expression of AtLIG1 (Nota et al., 2015).

DML Family

The DEMETER-LIKE (DML) family is a plant-specific DNA glycosylase family belonging to the HhH-GPD superfamily. Its founding members are four *Arabidopsis* proteins: DME (DEMETER), ROS1 (REPRESSOR OF SILENCING 1), DME-like 2 (DML2), and DME-like 3 (DML3) (Choi et al., 2002; Gong et al., 2002; Ortega-Galisteo et al., 2008). All four enzymes are 5-meC DNA glycosylases/lyases involved in active DNA demethylation through a BER process. Proteins from the DML family appear to be unique to plants, with putative orthologs present in mosses (*Phycomitrella patens*) and unicellular green algae (*Ostreococcus*, for example), suggesting that active demethylation through excision of 5-meC may have appeared early during plant evolution (Roldan-Arjona and Ariza, 2009a).

All DML proteins possess an HhH-GPD motif with the invariant aspartate, a conserved lysine residue characteristic of bifunctional DNA glycosylases, and a [4Fe–4S] cluster. They are very large proteins, ranging from 1,100 to 2,000 amino acids, in comparison to other members of the HhH-GPD superfamily (200–400 amino acids). One of its distinctive characteristics is their discontinuous catalytic domain, comprised of two conserved regions separated by a predicted unstructured sequence whose length varies across family members (Ponferrada-Marin et al., 2011). They also contain a conserved carboxy-terminal domain, that is not related with any known protein family (Choi et al., 2002; Gong et al., 2002; Morales-Ruiz et al., 2006) but is required for catalytic activity (Ponferrada-Marin et al., 2010; Hong et al., 2014), and a short amino-terminal domain significantly rich in lysine that facilitates demethylation in long substrates (Ponferrada-Marin et al., 2010). In addition to 5-meC, ROS1, DME, and DML3 excise T mispaired with G and show a preference for CpG contexts (Morales-Ruiz et al., 2006; Ortega-Galisteo et al., 2008), thus supporting an additional DNA repair role similar to that of MBD4L in counteracting the mutagenic consequences of 5-meC deamination.

Members of the DML family are bifunctional DNA glycosylase/lyases that excise the target base and cleave the phosphodiester backbone by β- or β, δ-elimination, generating a single-nucleotide gap with the 3′-PUA or 3′-P termini, respectively (Agius et al., 2006; Gehring et al., 2006; Morales-Ruiz et al., 2006; Penterman et al., 2007; Ortega-Galisteo et al., 2008). Such 3′-blocked ends must be processed to the 3′-OH termini before a DNA polymerase and a DNA ligase may fill and seal the gap, respectively.

H2TH Superfamily

Proteins of the H2TH superfamily (also known as Fpg/Nei) are characterized by a common structure comprising of domains separated by a flexible linker sequence. The catalytic amino acid that acts as nucleophile is a conserved proline located at the N-terminal domain, whereas the C-terminal domain contains a zinc finger required for DNA binding (Sugahara et al., 2000). All of them are bifunctional DNA glycosylases that cleave the sugar–phosphate backbone by β, δ-elimination activity, and they are mostly involved in the repair of oxidative damage (Fromme and Verdine, 2004; Huffman et al., 2005). The two founding members of the H2TH superfamily are the *E. coli* proteins Formamidopyrimidine DNA glycosylase (Fpg, also known as MutM) and Endonuclease VIII (Nei). Fpg recognizes formamidopyrimidines, 8-oxoG, as well as its oxidation products guanidinohydantoin (Gh), and spiroiminodihydantoin (Sp), whereas Nei primarily acts on damaged pyrimidines (Kathe et al., 2009).

Phylogenetic analysis has confirmed that both Fpg and Nei homologs are widely distributed in prokaryotes. In eukaryotes, Fpg homologs are only found in plant and fungi clades, whereas Nei homologs are restricted to metazoans, although they have been lost in many non-vertebrate lineages (Kathe et al., 2009). Mammals possess three Nei-like proteins (NEIL1, NEIL2, and NEIL3) (Wallace, 2013).

Although plants have both Ogg and Fpg homologs (Ohtsubo et al., 1998; Dany and Tissier, 2001; Garcia-Ortiz et al., 2001; Scortecci et al., 2007; Macovei et al., 2011), the relative roles of these two types of enzymes in counteracting oxidative DNA damage are not well understood. Alternative splicing of *Arabidopsis* FPG leads to seven different isoforms, and two of them show variation in the expression levels depending on the analyzed tissue (Ohtsubo et al., 1998; Murphy and Gao, 2001). AtFPG1 is the only isoform characterized biochemically, and whereas its activity excising 8-oxoG was almost undetectable, it shows a potent AP lyase activity (Kathe et al., 2009). The inability of AtFPG1 to excise 8-oxoG has been attributed to the presence of a very short version of the a-F-b9/10 loop, which is involved in 8-oxoG recognition (Duclos et al., 2012).

T-DNA insertion mutant plants lacking both AtFPG and AtOGG proteins do not show any obvious phenotype distinguishable from the wild type (Murphy, 2005). However, there is evidence that both enzymes participate in 8-oxoG repair and contribute to counteract the oxidative DNA damage in *Arabidopsis* (Cordoba-Cañero et al., 2014). Interestingly, *atfpg atogg1* double mutants show increased levels of oxidative DNA damage not only in the nucleus but also in the mitochondria (Cordoba-Cañero et al., 2014).

AP SITE INCISION

AP sites are frequently found in DNA due to the spontaneous hydrolysis of the N-glycosylic bond. Additionally, they are also repair intermediates generated by monofunctional DNA glycosylases during BER (**Figure 1**). It has been estimated that more than 10,000 bases are lost spontaneously per day per mammalian cell, being purines much more susceptible to spontaneous loss than pyrimidines (Lindahl and Nyberg, 1972). AP sites are DNA lesions with cytotoxic effects due to their capacity to block DNA replication and transcription, but also have potential mutagenic consequences if they are bypassed by DNA polymerases (Loeb, 1985; Prakash et al., 2005). AP site repair is initiated by either AP endonucleases or AP

lyases, generating single-strand breaks (SSB) with either 5′- or 3′-blocked ends, respectively, that cannot be used as substrates by DNA polymerases or DNA ligases. Such SSBs can be converted into highly toxic double-strand breaks (DSB) if not processed before DNA replication (Caldecott, 2001).

AP Endonucleases

AP endonucleases recognize AP sites and perform hydrolysis at their 5′-side, yielding SSBs with 3′-OH and 5′-dRP ends (Levin and Demple, 1990) (**Figure 1**). Based on structural folding and amino acid sequence similarity to the major AP endonucleases of *E. coli*, these enzymes are classified under Endonuclease IV (EndoIV, also known as Nfo) and Exonuclease III (ExoIII) families. Under physiological conditions, ExoIII is responsible for the vast majority of AP endonuclease activity detected in *E. coli* (Weiss, 1976), whereas EndoIV is induced during oxidative stress (Chan and Weiss, 1987). Although EndoIV and ExoIII families have overlapping DNA substrate specificities, they are distinguished by their modes of DNA damage recognition (Redrejo-Rodriguez et al., 2016). Moreover, their tertiary structure and their divalent metal requirements are completely different; while ExoIII family proteins are Mg^{2+}-dependent, EndoIV family members are Zn^{2+}-dependent, indicating that they have evolved independently from different ancestors. Importantly, ExoIII family members are present in all kingdoms of life, while EndoIV members are absent in some groups, such as mammals and plants (Daley et al., 2010). An EndoIV homolog in *S. cerevisiae* (Apn1) has been identified as the main AP endonuclease activity in this species (Popoff et al., 1990). In *S. pombe*, an EndoIV homolog exists, too, but seems to play only a backup role in DNA repair (Ramotar et al., 1998).

Mammalian genomes encode two proteins, APE1 and APE2 (also known as APEX1 and APEX2), with sequence similarity to ExoIII. APE1 is the major AP endonuclease activity, performing more than 95% of total AP site incision (Demple and Sung, 2005), whereas the activity of APE2 is significantly lower (Hadi and Wilson, 2000). APE1 possesses a C-terminal region responsible for interaction with DNA and AP endonuclease activity (Fritz, 2000) and a unique N-terminal region, absent in ExoIII, required for a redox activity regulating the DNA-binding potential of several transcription factors (Georgiadis et al., 2008).

The *Arabidopsis* genome encodes three AP endonuclease homologs of ExoIII: APE1L, ARP, and AtAPE2. APE1L (AT3G48425) and ARP (AT2G41460) are similar to the major human AP endonuclease APE1, and AtAPE2 (AT4G36050) is similar to the human APE2 (Murphy et al., 2009). Homologous sequences have been identified also in sugarcane (Maira et al., 2014; Cabral Medeiros et al., 2019) and rice (Joldybayeva et al., 2014).

Like its human APE1 homolog, *Arabidopsis* ARP possesses a repair-independent redox activity able to regulate the DNA-binding capacity of some transcription factors (Babiychuk et al., 1994). On the other hand, its DNA incision activity is essential during uracil or AP site repair *in vitro* (Cordoba-Cañero et al., 2011). ARP also processes AP sites generated by AtFPG and/or AtOGG1 during 8-oxoG repair and performs an important role in repairing oxidative DNA damage accumulated during seed aging (Cordoba-Cañero et al., 2014). Several T-DNA insertion mutants in *ARP* show no phenotypic differences with wild-type plants (Gutman and Niyogi, 2009; Murphy et al., 2009; Cordoba-Cañero et al., 2011), despite the fact that ARP acts as a protective factor when levels of uracil in DNA are artificially increased by 5-FU treatment (Cordoba-Cañero et al., 2011). ARP fusion proteins to GFP are targeted to chloroplasts, and the capacity of chloroplast protein extracts to incise osmium tetroxide-treated DNA is reduced in *Arabidopsis arp* mutants (Gutman and Niyogi, 2009).

All three AP endonucleases from *Arabidopsis* have been biochemically characterized by several groups (Lee et al., 2014; Li et al., 2015; Li et al., 2018). AP endonuclease activity of ARP, APE1L, and AtAPE2 has been demonstrated, with AtAPE2 activity the weakest (Lee et al., 2014; Li et al., 2015). Unlike human APE1, ARP discriminates between AP sites generated by spontaneous base loss or by enzymatic excision. Thus, ARP cleaves AP sites generated by N7-meG excision but is unable to process AP sites originated due to spontaneous depurination of N7-meG, suggesting that these two types of AP sites possess different chemical or structural properties not yet identified (Barbado et al., 2018). In addition to AP endonuclease activity, AP endonucleases are endowed with phosphodiesterase and/or phosphatase activities involved in cleaning blocked DNA ends (see the section *Cleaning of DNA Termini*).

Whereas deletion of the *APE1* gene results in very early embryonic lethality in mice (Xanthoudakis et al., 1996), *Arabidopsis* T-DNA insertional mutants of APE1L, AtAPE2, or ARP display no phenotypic defects (Murphy et al., 2009). However, the simultaneous inactivation of APE1L and AtAPE2 leads to a seed abortion phenotype, whereas a joint deficiency with either APE1L or AtAPE2 does not cause any effect. These results indicate that APE1L and AtAPE2 are probably performing overlapping functions required for seed viability (Murphy et al., 2009), likely in repair of DNA damage generated during seed development and/or the 3′-blocked ends generated by DML DNA glycosylases during active DNA demethylation (see the section *DML Family*). Although ARP is dispensable for normal seed development, it performs a protective role against the adverse effects of seed aging (Cordoba-Cañero et al., 2014).

AP Lyases

Although it has been widely assumed that AP sites are mainly processed by AP endonucleases, accumulating evidence points to an additional important role for AP lyases. For example, in both *S. cerevisiae* and *S. pombe*, AP sites are first incised by the AP lyase activity of Nth1 homologs, which produce 3′-PUA blocked termini that are subsequently processed by AP endonucleases (Pascucci et al., 2002; Li et al., 2015). Evidence of an important role of AP lyases in the processing of abasic sites has also been reported recently in plants. In *Arabidopsis*, spontaneous depurination of MMS-induced N7-meG generates AP sites that are not recognized by ARP (see above) and are exclusively repaired through an AP endonuclease-independent route initiated by the AP lyase activity of AtFPG (Barbado et al., 2018). AtFPG is the major, possibly the only, AP lyase activity detectable

in *Arabidopsis* cell extracts (Barbado et al., 2018). AP site incision catalyzed by AtFPG generates a 3′-P end that is converted to 3′-OH by the DNA 3′-phosphatase ZDP (see the section *Blocked 3′-Termini*) before repair is completed (Barbado et al., 2018).

CLEANING OF DNA TERMINI

Blocked 3′-Termini

Blocked 3′-termini arise from the incision activity of bifunctional DNA glycosylases/AP lyases. Incisions performed by β-elimination generate 3′-PUA blocked ends, whereas those caused by β, δ–elimination produce 3′-P ends (**Figure 1**).

Human APE1 possesses 3′-phosphodiesterase activity to remove 3′-PUA blocked ends and also exhibits a weak 3′-phosphatase activity (Demple and Harrison, 1994; Suh et al., 1997). In contrast, human APE2 has weak AP endonuclease activity but potent 3′-phosphodiesterase and 3′→5′-exonuclease activities (Burkovics et al., 2006).

In *Arabidopsis*, APE1L is able to efficiently process the 3′-PUA ends *in vitro* (Lee et al., 2014; Li et al., 2015). Furthermore, APE1L has been demonstrated to function in the active DNA demethylation pathway by processing the 3′-PUA termini generated by the bifunctional 5-meC DNA glycosylases/lyases of the DML family (Li et al., 2015). It has been also shown that APE1L and APE2 possesses 3′-phosphatase activity *in vitro* (Li et al., 2015; Li et al., 2018). The wheat homolog of APE1L possesses a weak AP endonuclease activity, as compared to human APE1, but displays 3′-phosphodiesterase, 3′-phosphatase, and 3′→5′ exonuclease activities (Joldybayeva et al., 2014). It has been also demonstrated that *Arabidopsis* ARP exhibits NIR (Nucleotide Incision Repair) and 3′→5′ exonuclease activities (Akishev et al., 2016).

When BER is initiated by bifunctional DNA glycosylases that perform β, δ-elimination, a gap flanked by phosphates is generated (**Figure 1**). The 3′-P blocked end is not a substrate for DNA polymerases, and AP endonucleases seem not to be efficient 3′-phosphatases. In mammalian BER, this problem is solved using polynucleotide kinase/3′-phosphatase (PNKP) for 3′-P removal (Jilani et al., 1999). Mammalian PNK functions in AP endonuclease-independent BER of oxidative DNA damage (Wiederhold et al., 2004) as well as in SSBs and DSBs repair (Whitehouse et al., 2001; Chappell et al., 2002).

In plants, proteins with 3′-DNA phosphatase activity have been described in maize (ZmDP2) and *Arabidopsis* (ZDP, zinc finger DNA 3′-phosphoesterase, AT3G14890). They show partial sequence similarity to mammalian PNKP, but lack the associated 5′-kinase activity, suggesting that, unlike PNKP, they are unable to phosphorylate the 5′-hydroxyl termini at SSBs (Betti et al., 2001; Petrucco et al., 2002; Martinez-Macias et al., 2012).

ZDP, which apparently is the only enzyme responsible for the DNA 3′-phosphatase activity detectable in *Arabidopsis* cell extracts, participates in the processing of the 3′-P ends generated by AtFPG and AtOGG1 during 8-oxoG repair, as well as those produced by the 5-meC DNA glycosylases ROS1 and DME during the active DNA demethylation BER pathway (Martinez-Macias et al., 2012; Cordoba-Cañero et al., 2014). Mutants deficient in ZDP do not display any phenotypic alteration under normal growth conditions, but show hypersensitivity to MMS (Martinez-Macias et al., 2012). As indicated above, AP sites generated by nonenzymatic release of MMS-induced N7-meG are cleaved by AtFPG, and the generated 3′-P is processed by ZDP. In fact, *zdp*-deficient plants possessing an additional *fpg* mutation partially recover MMS resistance, suggesting that unrepaired AP sites are less toxic than downstream SSB repair intermediates with blocked 3′-P ends (Barbado et al., 2018).

Blocked 5′-Termini

When abasic sites are incised by AP endonucleases, a gap flanked by a 3′-OH group and a 5′-dRP blocked terminus is generated (**Figure 1**). To continue the repair pathway, the 5′-dRP end is processed to a 5′-P end by a dRP lyase activity. In mammals, the major dRP lyase activity is associated to DNA Polymerase β (Srivastava et al., 1998), through an N-terminal 8-kDa domain characteristic of Family X of DNA polymerases (Beard and Wilson, 2000). Processing of 5′-dRP may be rate limiting, and this blocking group may be also removed by strand displacement and incision during the LP-BER sub-pathway (**Figure 1**) (see the section *Gap Filling: Short-Patch and Long-Patch BER Sub-pathways*).

Unlike mammals, plants and yeast do not possess DNA polymerase β orthologs, but have related enzymes termed Pol λ and Pol IV, respectively. Pol λ, which is also present in mammalian cells, belongs to the X-family of DNA polymerases, shares more than 30% of sequence homology with mammalian Pol β (Garcia-Diaz et al., 2000) and also displays DNA polymerase and dRP lyase activities (Garcia-Diaz et al., 2000; Garcia-Diaz et al., 2002). Like Pol IV in yeast, Pol λ is the only member of the Family X of DNA Polymerases present in most plants. However, sequences with similarity to X-family members Pol μ and TdT have been identified in the unicellular alga *Chlamydomonas reinhardtii* (Morales-Ruiz et al., 2018). It has been shown that human Pol λ possesses dRP lyase activity (Garcia-Diaz et al., 2001), and it can function as a backup enzyme for DNA Pol β in BER (Braithwaite et al., 2010). The role of plant Pol λ has been studied in rice and *Arabidopsis* (Uchiyama et al., 2004; Amoroso et al., 2011; Roy et al., 2011). The rice Pol λ ortholog has been partially characterized, and biochemical analysis indicates that it possesses dRP lyase activity (Uchiyama et al., 2004). Although some biochemical properties of *Arabidopsis* Pol λ have been described, there is no evidence reported of its dRP lyase activity (Amoroso et al., 2011; Roy et al., 2011).

In addition to Pol β and Pol λ, Pol θ, other human DNA polymerase that belongs to Family A, possesses dRP lyase activity, and it has been demonstrated to function in human BER (Prasad et al., 2009). It has been suggested that although human Pol θ is not essential in BER, it may be a backup enzyme, and the same may be true in plants. In *Arabidopsis*, the gene *TEBICHI* (*TEB*) codes for a Pol θ homolog. Inactivation of *TEB* causes sensitivity to DNA-damaging agents, such as mitomycin C and MMS, that promote DNA crosslinks and SSBs/DSBs, respectively (Inagaki et al., 2006; Inagaki et al., 2009). Nevertheless, there is no data available supporting an implication of AtPolθ in dRP processing during BER in plants.

The *Arabidopsis* genome encodes two family-A DNA Polymerase paralogs, AtPolIA and AtPolIB, which are the only DNA Polymerases in plant organelles identified to date. Both have been implicated in organellar DNA replication, whereas only AtPolIB, but not AtPolIA, is involved in organellar DNA repair (Ono et al., 2007; Parent et al., 2011). Recently, the capacity of both AtPolIA and AtPolIB to remove the 5′-dRP moiety by an intrinsic lyase activity it has been described (Trasvina-Arenas et al., 2018).

GAP FILLING: SHORT-PATCH AND LONG-PATCH BER SUB-PATHWAYS

Gap filling during BER may proceed either *via* short-patch (SP), by incorporation of only a single nucleotide, or long-patch (LP), by insertion of 2 to 13 nucleotides (**Figure 1**). In mammalian cells the contribution of DNA Pol β and DNA Ligase III in SP-BER has been demonstrated (Kubota et al., 1996), and since plants lack homologs of both enzymes, it was initially accepted that plants only perform LP-BER (Uchiyama et al., 2008). Nevertheless, it has been confirmed that *Arabidopsis* cell extracts repair uracil and AP sites by both SP- and LP-DNA synthesis (Cordoba-Cañero et al., 2009; Cordoba-Cañero et al., 2011). As indicated above, Pol λ is the only member of Family X of DNA polymerases in plants. Although functions of plant Pol λ in nucleotide excision repair (Roy et al., 2011), oxidative DNA damage bypass (Amoroso et al., 2011), non-homologous end joining (Roy et al., 2013; Furukawa et al., 2015), and DSB repair (Sihi et al., 2015) have been stablished, its role, if any, in SP-BER remains to be clarified.

The alternative BER sub-pathway, LP-BER, occurs when two or more nucleotides are inserted in the repair gap. In mammals, Pol β is able to incorporate the first nucleotide in LP-BER (Podlutsky et al., 2001), but the elongation step is performed by replicative DNA Polymerases, such as DNA Pol δ and Pol ε. Plants possess orthologs of both DNA polymerases δ and ε, and evidences obtained in rice and *Arabidopsis* demonstrate the important role of Pol ε in DNA replication (Uchiyama et al., 2002; Ronceret et al., 2005). However, their involvement in LP-BER remains to be determined.

It has been suggested that the choice between SP- and LP-BER could be influenced by the nature of the lesion and/or the DNA glycosylase that initiates BER, and that the equilibrium between both sub-pathways may be additionally affected by the phase of the cell cycle (Fortini and Dogliotti, 2007). In *Arabidopsis*, the choice between SP- and LP-BER is affected by the nature of the 5′-end of the repair gap. When the 5′-end is a reduced dRP not amenable to β-elimination by dRP lyases, the SP-BER sub-pathway is abrogated, and repair is performed exclusively by LP-BER (Cordoba-Cañero et al., 2009; Cordoba-Cañero et al., 2011). Also, it has been demonstrated in *Arabidopsis* that AP sites generated by spontaneous depurination of N7-meG are repaired by SP-BER, whereas those generated enzymatically can be repaired by both SP- and LP-BER (Barbado et al., 2018).

DNA polymerases performing LP-BER promote strand displacement and generate a 5′-end single-stranded "flap" that needs to be removed by endonucleolytic cleavage. In mammals, this step is performed by Flap Endonuclease 1 (FEN1) (Kim et al., 1998), a structure-specific 5′ endo/exonuclease (Harrington and Lieber, 1994) belonging to the Rad2 nuclease family with essential roles in the processing of Okazaki fragments during replication and in LP-BER (Liu et al., 2004).

Plant homologs of FEN1 were first partially characterized in cauliflower (*Brassica oleracea* var. *botrytis*) inflorescences (Kimura et al., 1997) and later in rice [OsFEN1a and OsFEN1b (Kimura et al., 2000; Kimura et al., 2003)] and *Arabidopsis* [AtFEN1 (AT5G26680) (Zhang et al., 2016a; Zhang et al., 2016b)]. OsFEN1a and OsFEN1b proteins show a high degree of sequence similarity, and analysis of their expression revealed correlation with cell proliferation (Kimura et al., 2003). However, only OsFEN1a is able to complement *S. cerevisiae* null mutants deficient in the FEN1 homolog *rad27* (Reagan et al., 1995; Kimura et al., 2003). Similarly, *Arabidopsis* AtFEN1 partially complements a *rad27* mutant. OsFEN-1a possesses both 5′-endonuclease and 5′-exonuclease activities (Kimura et al., 2000), but AtFEN1 lacks exonuclease activity (Zhang et al., 2016a; Zhang et al., 2016b). Rice and *Arabidopsis* FEN1 homologs have been localized to the nucleus, and interaction between OsFEN-1a and PCNA has been reported (Kimura et al., 2001; Zhang et al., 2016a).

Whereas the knockout mutant of FEN1 causes early embryonic lethality in mice (Kucherlapati et al., 2002), yeast mutants are viable and show increased sensitivity to UV light and mutagens (Reagan et al., 1995; Vallen and Cross, 1995). In plants, AtFEN1 seems to be essential since no homozygous *Arabidopsis* mutants could be obtained from the progeny of a heterozygous *fen1-2* T-DNA insertion mutant (Zhang et al., 2016a). Shade avoidance mutant 6 (*sav6*) plants, which contain a single point mutation that affect mRNA splicing efficiency of AtFEN1, are hypersensitive to ultraviolet (UV)-C radiation and DSB-inducing agents (Zhang et al., 2016b). Furthermore, another AtFEN1 mutant, with a single nucleotide substitution (*fen1-1*), shows hypersensitivity to MMS and exhibits shortened telomeres (Zhang et al., 2016a). However, no evidence has been yet reported for a role of plant FEN1 homologs in BER.

NICK LIGATION

The SP and LP-BER sub-pathways converge by generating the same product: a nick flanked by 3′-OH and 5′-P termini. The culminating BER step is the action of a DNA ligase that seals the nick by catalyzing formation of a phosphodiester bond. DNA ligases are grouped into two families, ATP- and NAD^+-dependent ligases, according to whether catalysis is coupled with pyrophosphate hydrolysis of ATP or NAD cofactors. The NAD^+-dependent DNA ligases are highly conserved enzymes identified only in eubacteria, whereas most eukaryotic DNA ligases, together with archaeal and bacteriophage enzymes, are ATP-dependent DNA ligases (Ellenberger and Tomkinson, 2008).

In *E. coli*, the NAD^+-dependent DNA LigA functions in both DNA replication and BER. Eukaryotes generally possess three ATP-dependent DNA ligases (Lig I, Lig III, and Lig IV in mammals). Lig IV is implicated in non-homologous end joining

(Baumann and West, 1998) and seems to have no role in BER. The final ligation step during mammalian LP-BER is performed by Lig I, which is also essential in DNA replication, and the complex formed by Lig III and the X-ray repair cross-complementing 1 (XRCC1) protein participates in SP-BER (Cappelli et al., 1997; Timson et al., 2000; Sleeth et al., 2004).

Arabidopsis also possesses three ligases, AtLIG1, AtLIG4, and AtLIG6, but lack a Lig III homolog. AtLIG1 and AtLIG4 are orthologs of mammalian Lig I and Lig IV, respectively, whereas AtLIG6 is a plant-specific DNA ligase (Bonatto et al., 2005). AtLIG4 has been implicated in double-strand break repair (West et al., 2000; van Attikum et al., 2003) and, together with AtLIG6, seems to be critical for seed viability (Waterworth et al., 2010). *Arabidopsis* mutants in *AtLIG1* are lethal, and plants with a diminished expression display important phenotypic defects and deficiencies in the repair of single- and double-strand DNA breaks (Waterworth et al., 2009). Moreover, it has been demonstrated that AtLIG1 is essential for both SP- and LP-BER in *Arabidopsis* cell extracts (Cordoba-Cañero et al., 2011).

The mammalian *LIG3* gene, unlike the *LIG1* and *LIG4* genes, encodes different DNA ligase polypeptides by alternative translation initiation with different cellular functions and, notably, encodes the only mitochondrial DNA ligase (Tomkinson and Sallmyr, 2013). In contrast, in yeast and plants, different translation initiation sites generate distinct isoforms of DNA ligase 1 found in the nuclei and mitochondria (Donahue et al., 2001; Sunderland et al., 2006). No AtLIG1 targeting to chloroplasts has been detected in *Arabidopsis*.

ADDITIONAL PROTEINS INVOLVED IN BER

In addition to the BER factors discussed above, there are additional proteins (**Table 2**) that increase BER efficiency and/or function in the coordination of the various BER stages.

Proliferating Cell Nuclear Antigen (PCNA)

PCNA is an accessory factor that endows eukaryotic replicative polymerases with the high processivity required to duplicate an entire genome. Moreover, PCNA acts as a scaffold protein to facilitate recruitment of proteins to replication fork (Moldovan et al., 2007). In addition to DNA replication, PCNA plays also important roles in multiple DNA repair pathways (Maga and Hubscher, 2003). In eukaryotes PCNA is required for efficient DNA synthesis by Pol δ or Pol ε in LP-BER (Stucki et al., 1998) and also in SP-BER by interacting with Pol β and XRCC1 (Kedar et al., 2002; Fan et al., 2004). Interestingly, PCNA appears to be involved not only in the DNA synthesis step, since it interacts with multiple BER factors acting in other BER stages, such as UNG, MPG, MUTYH, NTHL1, APE1, APE2, FEN1, and Lig I (Maga and Hubscher, 2003).

Eukaryotic genomes possess at least one gene copy encoding PCNA. In mice and humans, one PCNA gene and several pseudogenes are present (Almendral et al., 1987; Ku et al., 1989; Travali et al., 1989; Yamaguchi et al., 1991). Plants such as *Oryza sativa* (rice) or *Pisum sativa* also contain a single-copy PCNA gene, but other species like *Arabidopsis* or *Zea mays* possess at least two PCNA paralogs (Lopez et al., 1997; Shultz et al., 2007; Strzalka and Ziemienowicz, 2011).

The *Arabidopsis* genome encodes two nearly identical PCNA genes. The AtPCNA1 (AT1G07370) and AtPCNA2 (AT2G29570) proteins have been purified and crystallized, and it has been demonstrated that they conserve a three-dimensional structure very similar to that of human PCNA (Strzalka et al., 2009). AtPCNA2 interacts with AtPolλ and enhances its bypass activity on oxidative DNA damage (Amoroso et al., 2011). However, no data have been yet reported on the involvement of plant PCNA homologs in BER.

The Scaffolding Protein X-Ray Cross-Complementation Group 1 (XRCC1)

XRCC1 does not exhibit any enzymatic activity but plays a major role in BER and SSBR pathways interacting with multiple components and facilitating repair (Caldecott, 2003). As mentioned above, mammalian XRCC1 functions in SP-BER (Cappelli et al., 1997) interacting with LigIIIα and enhancing its DNA ligase activity (Caldecott et al., 1994; Nash et al., 1997) In mammalian cells, additional interaction partners of XRCC1 in BER have been described, such as hOGG1 (Marsin et al., 2003), UNG2 (Akbari et al., 2010), hNEIL1 (Wiederhold et al., 2004), hNEIL2, MPG, hNTH1 (Campalans et al., 2005), PNKP (Whitehouse et al., 2001), APE1 (Vidal et al., 2001), or DNA Pol β (Kubota et al., 1996). Mammalian XRCC1 proteins possess two BRCT (BRCA1 C-terminal) domains (BRCT1 and BRCT2) implicated in protein–protein interactions between XRCC1 and poly (ADP-ribose) polymerase (PARP) proteins and DNA Ligase IIIα, respectively (Hanssen-Bauer et al., 2012). Interaction of XRCC1 through its BRCT2 domain with DNA Lig IIIα stimulates its DNA ligation activity (Caldecott et al., 1994; Nash et al., 1997).

TABLE 2 | Additional proteins involved in base excision repair in yeast, humans, and *Arabidopsis*.

Function	*S. cerevisiae*	*H. sapiens*	*Arabidopsis*		
			Name	**Gene ID**	**Reference**
Processivity factor	Pol30p	PCNA	AtPCNA1	AT1G07370	
			AtPCNA2	AT2G29570	(Amoroso et al., 2011)
Scaffolding		XRCC1	AtXRCC1	AT1G80420	(Martinez-Macias et al., 2013)
Nick sensing		PARP1	AtPARP1	AT2G31320	(Boltz et al., 2014)
		PARP2	AtPARP2	AT4G02390	(Song et al., 2015)
		PARP3	AtPARP3	AT5G22470	(Rissel et al., 2014)

XRCC1 knock-out mice are embryonic lethal and show increase DNA breakage (Tebbs et al., 1999; Thompson and West, 2000). In contrast, XRCC1 deficiency has no drastic consequences in plants. The *Arabidopsis* genome encodes an XRCC1 ortholog (AT1G80420) (Taylor et al., 2002), and plant *xrcc1* mutants develop normally, although they show radiosensitivity (Charbonnel et al., 2010). Rice OsXRCC1 interacts with ss- and ds-DNA, as well as with OsPCNA *in vivo* and *in vitro* (Uchiyama et al., 2008). The *Arabidopsis* XRCC1 protein stimulates uracil BER *in vitro* (Cordoba-Cañero et al., 2009) and is required for efficient DNA ligation, probably through interaction with AtLIG1 (Martinez-Macias et al., 2013). In agreement with the absence of a DNA ligase III homolog, plant XRCC1 lacks a BRCT2 domain (Taylor et al., 2002; Uchiyama et al., 2008). *Arabidopsis* XRCC1 also stimulates the 3′-DNA phosphatase activity of ZDP (Martinez-Macias et al., 2013).

Nick Sensors: Poly (ADP-Ribose) Polymerases (PARP)

Another type of proteins involved in the recruitment of BER enzymes are poly (ADP-ribose) polymerases (PARP). These proteins detect and bind tightly DNA strand breaks, signaling recruitment of repair proteins to the damaged site (Caldecott et al., 1996). The mammalian PARP family includes 17 proteins with homology to PARP1, its founding member (Schreiber et al., 2006; Hassa and Hottiger, 2008). In response to damage, PARP1 binds DNA strand breaks and is thereby activated to catalyze the synthesis of poly ADP-ribose (PAR) by transferring ADP-ribose from NAD^+ to both itself and nuclear target proteins (Schreiber et al., 2006). Mammalian PARP1 is the most extensively studied PARP protein, and evidences of its role in BER have accumulated. The participation of PARP1 in BER has been demonstrated in association with XRCC1 (Caldecott et al., 1996; Masson et al., 1998), and the requirement of PARP1 in both SP and LP-BER has been reported (Dantzer et al., 1999; Dantzer et al., 2000). Additionally, it has been found that PARP2 interacts with XRCC1 and belongs to a BER complex containing XRCC1, PARP1, DNA Pol β, and DNA LigIII (Schreiber et al., 2002). Both PARP1- and PARP2-deficient cells display a significant delay in resealing of DNA strand breaks (Trucco et al., 1998; Beneke et al., 2000; Schreiber et al., 2002). However, *in vitro* repair reactions using PARP1-deficient mice extracts showed to be partially compromised (Allinson et al., 2003), and since the pathway can be reconstituted with purified enzymes in the absence of PARP, it has been suggested that this protein is dispensable for BER, at least *in vitro*.

In contrast to mammals, the *Arabidopsis* genome contains only three genes encoding PARPs: AtPARP1 (AT2Gg31320), AtPARP2 (AT4G02390), and AtPARP3 (AT5G22470), with homology to human PARP1, PARP2, and PARP3, respectively (Babiychuk et al., 1998; Rissel et al., 2014; Vainonen et al., 2016). AtPARP1 and AtPARP2 seem to be broadly expressed, whereas AtPARP3 is detected mostly in developing seeds (Becerra et al., 2006). AtPARP1 and AtPARP2 localize to the nucleus and possess poly (ADP-ribose) polymerase activity, although AtPARP2 shows higher levels of activity than AtPARP1 (Feng et al., 2015). It has been suggested that variant residues at the active site in AtPARP3 could eliminate NAD^+ binding and, therefore, enzymatic activity (Lamb et al., 2012). Like in animals, plant PARPs play a role in DNA repair processes. In *Arabidopsis*, increasing levels of PARP expression after DNA damage have been described (Doucet-Chabeaud et al., 2001; Waterworth et al., 2010; Dubois et al., 2011), although it has been suggested that AtPARP2 plays the major role in response to ionizing radiation (Song et al., 2015). *Arabidopsis* single *atparp* null mutants are viable and, in contrast to animals, *atparp1 atparp2* double mutants are also viable (Boltz et al., 2014). Single mutant *atparp2* plants are more sensitive to DNA damaging agents than wild-type or *atparp1* plants (Song et al., 2015), whereas double *atparp1 atparp2* mutants exhibited further increased sensitivity (Boltz et al., 2014). A role of AtPARP3 in the repair of DNA damage accumulated during seed storage has also been suggested (Rissel et al., 2014). However, a function for plant PARP enzymes in BER has not yet been stablished.

OPEN QUESTIONS AND FUTURE CHALLENGES

Significant advances have been achieved in the biochemical and genetic analysis of plant BER. However, much remains to be elucidated regarding several important issues. A major unresolved question is the identity of the DNA polymerase(s) involved in gap filling. Although several indirect lines of evidence point to Pol λ, direct proof of its involvement in plant BER is still lacking, and the possible role of other DNA polymerases cannot be ruled out. An additional important area to be explored is the deployment of BER factors in a chromatin environment. Plant BER has been successfully studied *in vitro* with purified proteins or cell extracts using naked DNA substrates, but identification of additional BER factors will certainly require more complex approaches using nucleosome substrates. The interaction between BER proteins and factors that facilitate DNA accessibility in chromatin is likely to play an important role in BER efficiency and may dictate the spatial distribution of endogenous and exogenous DNA damage across the plant genome. It will also be important to clarify whether specific BER pathways operate in plant mitochondria and/or chloroplasts, as well as to identify the main proteins involved. As with BER studies in other organisms, advances in addressing these and other challenges could be accelerated by the development of novel BER assays with *in vivo*, rather than *in vitro*, endpoints. Additionally, increased BER knowledge will undoubtedly have an impact in the emerging field of CRISPR/Cas-mediated precision genome editing, which holds enormous potential for plant breeding and crop improvement (Puchta, 2017). For example, targeted C:G-to-T:A base pair substitution can be achieved by expressing dCas9–cytidine deaminase fusions, but lower than expected conversion efficiencies have been detected (Komor et al., 2016; Nishida et al., 2016). However, additional co-expression of the specific UDG inhibitor Ugi partially

inhibited endogenous BER of U:G intermediates, leading to increased levels of base substitution (Komor et al., 2016; Nishida et al., 2016). In summary, it is most likely that the near future will bring new and exciting results on this critical DNA repair pathway and its physiological roles in plants, as well as promising applications in existing and upcoming DNA technologies.

AUTHOR CONTRIBUTIONS

TR-A, RA, and DC-C jointly wrote the manuscript.

FUNDING

Funding was provided by the Spanish Ministry of Science, Innovation and Universities, as well as the European Regional Development Fund, under Grant BFU2016-80728-P.

ACKNOWLEDGMENTS

We are grateful to members of our lab for helpful criticism and advice. We apologize to authors whose relevant work could not be cited due to the lack of space.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 09 October 2019
doi: 10.3389/fpls.2019.01208

Translesion Synthesis in Plants: Ultraviolet Resistance and Beyond

*Ayako N. Sakamoto**

Department of Radiation-Applied Biology Research, National Institutes for Quantum and Radiological Science and Technology, Takasaki, Japan

Plant genomes sustain various forms of DNA damage that stall replication forks. Translesion synthesis (TLS) is one of the pathways to overcome stalled replication in which specific polymerases (TLS polymerase) perform bypass synthesis across DNA damage. This article gives a brief overview of plant TLS polymerases. In *Arabidopsis*, DNA polymerase (Pol) ζ, η, κ, θ, and λ and Reversionless1 (Rev1) are shown to be involved in the TLS. For example, AtPolη bypasses ultraviolet (UV)-induced cyclobutane pyrimidine dimers *in vitro*. Disruption of AtPolζ or AtPolη increases root stem cell death after UV irradiation. These results suggest that AtPolζ and ATPolη bypass UV-induced damage, prevent replication arrest, and allow damaged cells to survive and grow. In general, TLS polymerases have low fidelity and often induce mutations. Accordingly, disruption of AtPolζ or AtRev1 reduces somatic mutation frequency, whereas disruption of AtPolη elevates it, suggesting that plants have both mutagenic and less mutagenic TLS activities. The stalled replication fork can be resolved by a strand switch pathway involving a DNA helicase Rad5. Disruption of both AtPolζ and AtRAD5a shows synergistic or additive effects in the sensitivity to DNA-damaging agents. Moreover, AtPolζ or AtRev1 disruption elevates homologous recombination frequencies in somatic tissues. These results suggest that the Rad5-dependent pathway and TLS are parallel. Plants grown in the presence of heat shock protein 90 (HSP90) inhibitor showed lower mutation frequencies, suggesting that HSP90 regulates mutagenic TLS in plants. Hypersensitivities of TLS-deficient plants to γ-ray and/or crosslink damage suggest that plant TLS polymerases have multiple roles, as reported in other organisms.

Keywords: translesion synthesis, UV, mutation, DNA damage, genome stability

OPEN ACCESS

Edited by:
Alma Balestrazzi, University of Pavia, Italy

Reviewed by:
Wei Xiao, University of Saskatchewan, Canada
Carlos F. M. Menck, University of São Paulo, Brazil

***Correspondence:**
Ayako N. Sakamoto
sakamoto.ayako@qst.go.jp

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *20 February 2019*
Accepted: *03 September 2019*
Published: *09 October 2019*

Citation:
Sakamoto AN (2019) Translesion Synthesis in Plants: Ultraviolet Resistance and Beyond. Front. Plant Sci. 10:1208. doi: 10.3389/fpls.2019.01208

INTRODUCTION

Accurate replication of genomic DNA is vital for maintaining genome integrity. However, genomic DNA sustains various forms of damage caused by internal and external agents. Ultraviolet (UV) light is a major cause of DNA damage for land plants. It induces the formation of covalent bonds between the two adjacent pyrimidines. The two major products of UV damage, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4)PPs], are quickly repaired by the action of CPD and 6-4 photolyases in plant cells (Britt, 1999; Li et al., 2010; Hitomi et al., 2012). In addition, nucleotide excision repair (NER) plays an important role in removing UV damage (Kimura et al., 2004; Kunz et al., 2005; Canturk et al., 2016). Nevertheless, the remaining damage is toxic for cells because it distorts the template structure and prevents replication. This stalled replication creates a fragile single-strand region that easily leads to double-strand breaks

(DSBs), so organisms have multiple pathways to solve the stalled replication fork. Translesion synthesis (TLS) is one such pathway in which specific polymerases (TLS polymerase) are recruited to the replication machinery and perform the bypass synthesis across the DNA damage (Vaisman and Woodgate, 2017). **Figure 1A** illustrates the concept of TLS activity. When encountering DNA damage, the replicative polymerase (replicase) stalls because of distorted helix geometry. TLS polymerase, carrying a flexible active site, replaces the replicase and inserts one or more nucleotide(s) opposite the damage. Because of the relaxed constraints of these active sites, the TLS polymerase has a low fidelity and often incorporates one or more incorrect nucleotide(s) that can be removed by the exonuclease activity of replicases or the mismatch repair mechanism. However, unremoved errors result in base substitutions, frameshifts, or other types of mutation. This mutagenic nature of TLS has been linked to the senescence, carcinogenesis, and evolution of organisms.

It is more than a decade since the first report of TLS in plants. The accumulation of reports from multiple groups has clarified the roles and importance of TLS not only in UV resistance but also in the maintenance of genome stability in plants. This mini-review aims to summarize 1) TLS activity in plants in comparison with that in other organisms, 2) the contribution of TLS activity to plant responses to DNA-damaging stresses, and 3) possible other functions of TLS polymerases, which may unveil novel damage-resistant mechanisms in plants.

DNA POLYMERASE FAMILY MEMBERS IN PLANTS

DNA polymerases are classified into seven families based on their amino acid sequence similarity (Ishino and Ishino, 2014). Eukaryotes have Family A, B, X, and Y polymerases, whereas Family C polymerases are only seen in bacteria and Family D and E polymerases only in archaea. *Arabidopsis* has at least 11 polymerases classified into five families based on comparisons with human and yeast homologs (**Table 1**). The representative member of Family A polymerases is *Escherichia coli* polymerase I, which was the first DNA polymerase to be identified (Kornberg et al., 1956). Eukaryotic members of this group are polymerase γ (Polγ) and DNA polymerase θ (Polθ). *Arabidopsis* also has homologs of two prokaryotic-type DNA polymerases, PolI-like A and B (Parent et al., 2011), as well as AtPolθ, which was originally isolated as the causative gene of the short-root mutant *tebichi* (Inagaki et al., 2006). Family B polymerases include *E. coli* Pol II and eukaryotic polymerases α (Polα), δ (Polδ), and ε (Polε), which are involved in the replication of nuclear DNA. Polα, δ, and ε are conserved in *Arabidopsis* (Ronceret et al., 2005; Shultz et al., 2007; Liu et al., 2010; Iglesias et al., 2015; Pedroza-Garcia et al., 2016). This family includes DNA polymerase ζ, the first identified TLS polymerase that is also conserved in *Arabidopsis* (Sakamoto et al., 2003). Family X is only conserved in eukaryotes: its representative polymerase is Polymerase β, which is involved in base excision repair. Humans have four members in Family X (Polβ, Polλ, Polμ, and terminal deoxytransferase), whereas plants only have Polλ, which is phylogenetically distant from the Polλ of other organisms (Filée et al., 2002; Pavlov et al., 2006). Family Y carries the largest number of TLS polymerases, including *E. coli* Pol IV and V; eukaryotic Polη, Polκ, Polι; and Rev1 (Ohmori et al., 2001). Rev1 was originally isolated as a responsible gene for yeast *reversionless1* mutant, which carries a deoxycytidyl transferase activity (Nelson et al., 1996). Homologs of Polη, Polκ, and Rev1 are found in *Arabidopsis* (Takahashi et al., 2007).

Most recently, it has been shown that some members of the Archea-Eucaryotic Primase superfamily, such as human PrimPol, perform bypass synthesis across DNA damage (Iyer et al., 2005; Bianchi et al., 2013; Guilliam et al., 2015). *Arabidopsis* has a herpes-pox type primase (Iyer et al., 2005), although its function has not yet been investigated.

ISOLATION OF TRANSLESION SYNTHESIS POLYMERASES BASED ON ULTRAVIOLET RESISTANCE

A UVB-sensitive mutant *rev3* was isolated in *Arabidopsis* by screening ion-beam mutagenized seedlings under non-photoreactivating conditions (Sakamoto et al., 2003). The responsible gene, *AtREV3,* encodes a homolog of the catalytic subunit of DNA polymerase ζ (Polζ). DNA replication in the *rev3* root meristem was reduced after UVB irradiation (Sakamoto et al., 2003). *AtREV7* and *AtREV1* encode a regulatory subunit of Polζ and a Family Y polymerase, respectively (Takahashi et al., 2005). The *rev7* and *rev1* plants showed reduced growth compared with wild-type plants under chronic UVB irradiation (Takahashi et al., 2005). *AtPOLH* encodes a homolog of DNA polymerase η (Polη) that complements the yeast *rad30* mutant (Santiago et al., 2006). Disruption of Polζ and Polη had an additive effect on *Arabidopsis* root growth after UVB treatment (Anderson et al., 2008). Moreover, cell death was induced at root stem cells, and the number of mitotic cells was reduced severely in the UV-irradiated Polζ- and Polη-deficient plants (Curtis and Hays, 2007). This series of studies showed that these polymerases are important in plant UV resistance. The polymerases allow DNA replication to continue, saving the stem cell from cell death and maintaining growth in the presence of harmful UV irradiation.

DAMAGE BYPASS ACTIVITIES OF TRANSLESION SYNTHESIS POLYMERASES

TLS activity has been investigated *in vitro* using purified or recombinant polymerases and synthetic damage-inducing templates, such as cyclobutane TT dimer (CTD) and (6-4)TT photoproducts [(6-4)TP]. These analyses revealed that the bypass efficiency is dependent on both the type of damage and the polymerases involved (**Figure 1B**). For example, yeast and human Polη bypasses CTD efficiently (Johnson et al., 1999; Masutani et al., 1999), but Polη only inefficiently bypasses (6-4)TP (Johnson et al., 2001). In humans, DNA polymerase ι (Polι) inserts a nucleotide opposite 3′-T in the (6-4)TP (Vaisman et al., 2003). The 3′-end is thought to be elongated by the second polymerase (Polζ, Polκ, or

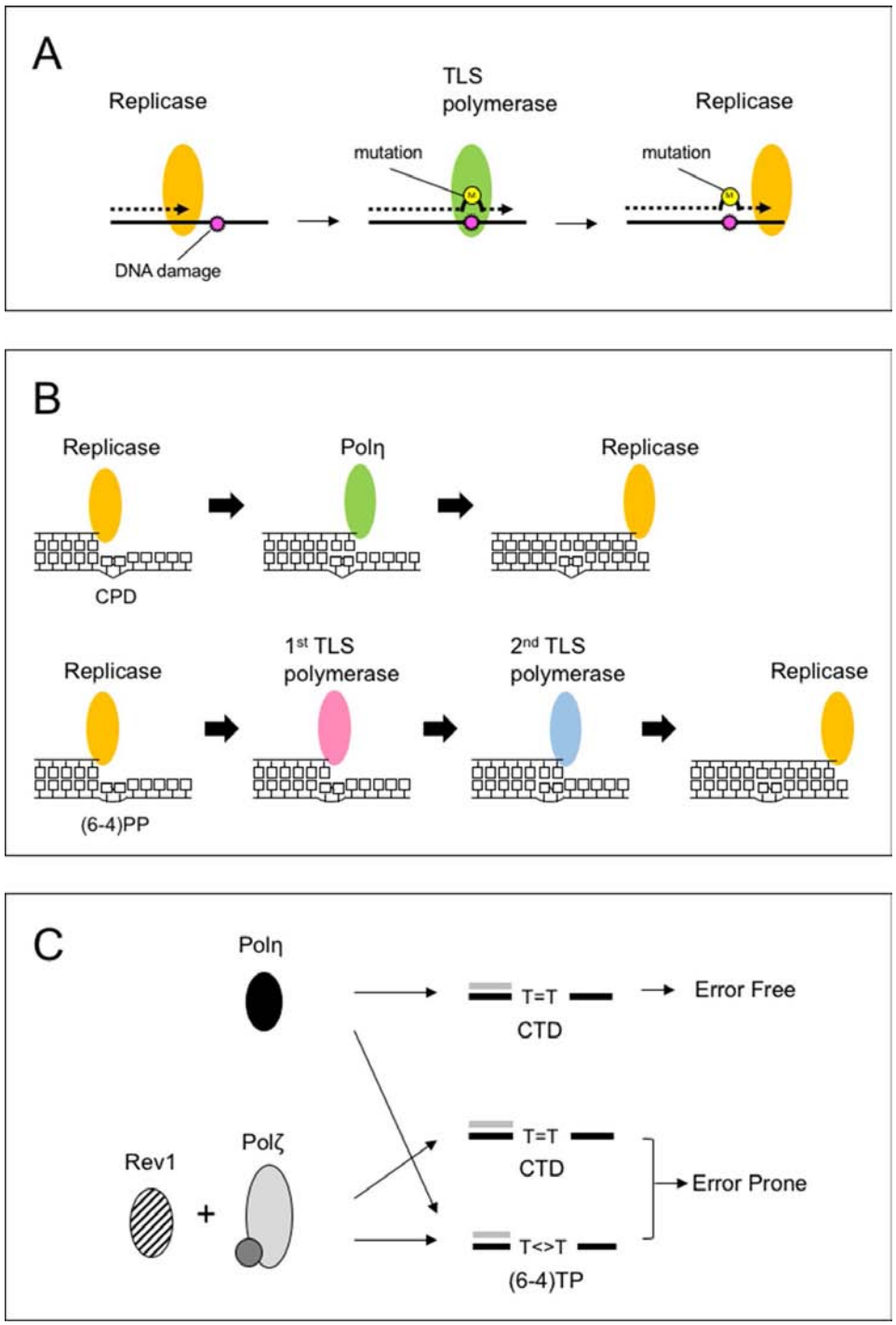

FIGURE 1 | Continued

Polθ), which has 3′-end elongation activity (Prakash et al., 2005, Seki and Wood, 2008). The subsequent *in vivo* analyses suggest that the UV damage at CC or CT sequence are also bypassed by a similar one- or two-step mechanism. Thus, TLS involves the multiple switching of polymerases at the replication site (**Figure 1B**; Prakash and Prakash, 2002; Bebenek and Kunkel, 2004).

The bypass activity of AtPolη for the major UV damage was examined by two groups who showed that AtPolη bypasses the CTD *in vitro* (Anderson et al., 2008; Hoffman et al., 2008). The activity of AtPolη is comparable to that of human Polη when examined at optimum salt concentration and temperature, and HsPolη, ScPolη, and AtPolη do not bypass (6-4)TP (Hoffman et al., 2008).

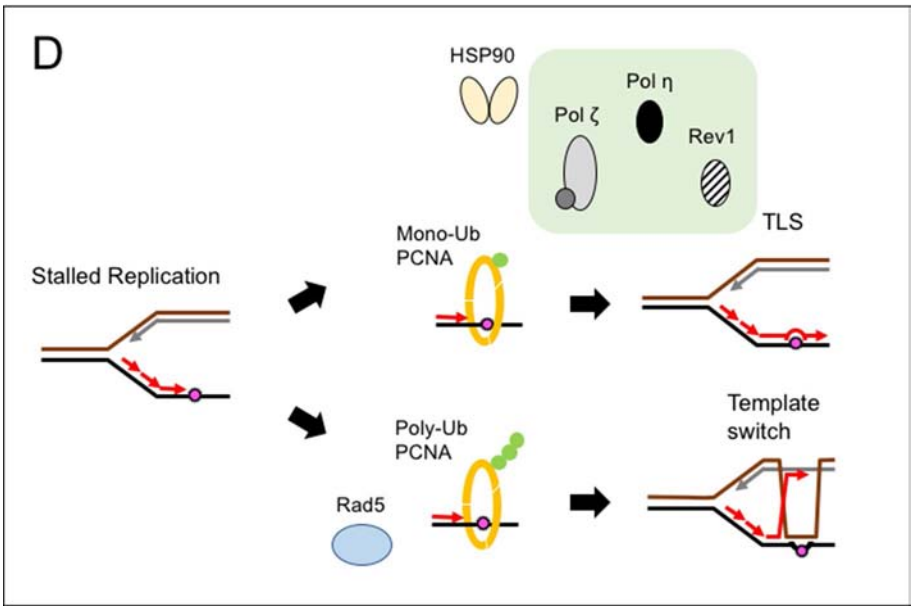

FIGURE 1 | Schematic of translesion synthesis (TLS). **(A)** Concept of TLS. When encountering DNA damage, the replicase stalls before the damage. TLS polymerase replaces the replicase and inserts one or more nucleotides opposite the damage. Because of the low fidelity, TLS polymerase incorporates one or more incorrect nucleotides, resulting in base substitutions, frameshifts, or other types of mutation. **(B)** Proposed model for the bypass of two major forms of ultraviolet (UV) damage. The model was proposed from the biochemical activities of TLS polymerases. The cyclobutane pyrimidine dimer (CPD) is efficiently bypassed by Polη (upper). However, no polymerase can complete the bypass of (6-4) photoproducts [(6-4)PP] by itself. Thus, (6-4)PPs may be bypassed by two polymerases, incorporating nucleotides one after the other (lower). **(C)** A model for UV-induced mutagenesis at the TT site in plants. The cyclobutane TT dimer (CTD) is efficiently bypassed by Polη in an error-free manner; any misincorporation is removed by replicases. In contrast, Polζ and Rev1 are involved in the error-prone bypass for both CTD and (6-4) TT photoproducts [(6-4)TP]. Polη cannot complete the bypass of (6-4)TP, so error-prone bypass is achieved by Polζ. **(D)** A model for damage tolerance mechanism in plants. The stalled replication fork is processed by either of two pathways: mutagenic synthesis by specific TLS polymerases or accurate synthesis using an intact template (template switch). The stalled replication fork signals the modification of PCNA. When PCNA is monoubiquitinated, the TLS polymerases interact with the Ub-PCNA and are recruited to the replication fork. The stalled replication fork also signals the transfer of Polη and Rev1. The 90-kDa heat shock protein (HSP90) promotes TLS activity through interaction with TLS polymerases. When TLS is deficient or reduced by depletion of HSP90, Rad5-dependent polyubiquitination of PCNA leads to a template switch, which causes genome instability.

Research has also been done on other types of DNA damage: AtPolκ inserted an A/C opposite 8-oxoG, a common form of oxidative damage induced by reactive oxygen species (García-Ortiz et al., 2007). Deletion of the C-terminal domain elevates the processivity and fidelity of AtPolκ, suggesting that the C-terminal domain regulates the activities of this polymerase through interactions with other proteins (García-Ortiz et al., 2004; García-Ortiz et al., 2007). DNA polymerase λ bypassed 8-oxoG in both error-free (dC insertion) and error-prone (dA insertion) manners (Amoroso et al., 2011). AtRev1 inserted a C opposite an apurin/apyrimidine (AP) site (Takahashi et al., 2007), which is formed by spontaneous depurination or occurs as an intermediate in the base excision repair process (Boiteux and Guillet, 2004). AtPolIA and AtPolIB have also been shown to bypass the AP site *in vitro* (Baruch-Torres and Brieba, 2017).

DETECTION OF MUTATIONS INDUCED BY TRANSLESION SYNTHESIS

Mutations induced by TLS have been investigated in *in vivo* assay systems (Lawrence and Christensen, 1978; Lawrence and Christensen, 1979; Roche et al., 1994; Harfe and Jinks-Robertson, 2000; Yu et al., 2001; Bresson and Fuchs, 2002; Kozmin et al., 2003; Gibbs et al., 2005; Szüts et al., 2008; Yoon et al., 2009; Yoon et al., 2010). In yeast, the deletion of Polζ or Rev1 reduces the UV-induced mutation frequency (Lawrence and Christensen, 1978, Lawrence and Christensen, 1979), whereas the deletion of Polη increases the frequency (Yu et al., 2001; Kozmin et al., 2003). These observations are not consistent with the *in vitro* characteristics of Polζ and Polη because Polη is less accurate than Polζ when replicating undamaged DNA (McCulloch et al., 2007; Zhong et al., 2006). Comprehensive analysis of *in vitro and in vivo* data suggested that Polη bypasses CTD with some errors, which are removed by the exonuclease activity of other polymerase(s) (McCulloch and Kunkel, 2008). Prakash et al. (2005) suggest that yeast Polη bypasses CPD at CC or CT sequence in an error-free manner. However, Pol η seems to induce C to T transition by inserting dA opposite deaminated C or mC in CPD (Ikehata and Ono, 2011). Yeast and mammalian Polη bypass (6-4)TP in an error-prone manner (Bresson and Fuchs, 2002; Yoon et al., 2010). It is suggested that Polζ contributes to the mutagenic bypass of (6-4)PP by extending the mismatched primer end caused by the action of Polη or other polymerases (Prakash et al., 2005; Hirota et al., 2010). Thus, the mutation frequency depends on the polymerases available, damage type, sequence context, and the assay system, and so on.

TABLE 1 | DNA polymerases in *Arabidopsis*[a,b].

Family	Category	Subunit	*A. thaliana* Gene ID	Reference	Function
A	DNA polymerase IA	POLIA	At3g20540	Parent et al., 2011	Replication of organellar DNA, TLS
	DNA polymerase IB	POLIB	At1g50840		Replication of organellar DNA, TLS
	DNA polymerase θ	POLQ	At4g32700	Inagaki et al., 2006	Repair of crosslink damage DSB repair TLS
B	DNA polymerase α	POLA1	At5g67100	Shultz et al., 2007; Liu et al., 2010	Replication
		POLA2	At1g67630		
		POLA3	At1g67320		
		POLA4	At5g41880		
	DNA polymerase δ	POLD1	At5g63960	Shultz et al., 2007; Iglesias et al., 2015	Replication
		POLD2	At2g42120		
		POLD3	At1g78650		
		POLD4	At1g09815		
	DNA polymerase ε	POLE1	At1g08260	Ronceret et al., 2005; Pedroza-Garcia et al., 2016	Replication
		POLE2	At2g27120		
		POLE3	At5g22110		
		POLE4	At1g07980		
			At5g43250		
			At2g27470		
	DNA polymerase ζ	REV3	At1g67500	Sakamoto et al., 2003; Takahashi et al., 2005	TLS, Repair of crosslink damage
		REV7	At1g16590		DSB repair
X	DNA polymerase λ	POLL	At1g10520	Uchiyama et al., 2004	Repair synthesis TLS
Y	DNA polymerase η	POLH	At5g44740	Santiago et al., 2006	TLS, Repair of crosslink damage
	DNA polymerase κ	POLK	At1g49980	García-Ortiz et al., 2004	TLS
	Rev1	REV1	At5g44750	Takahashi et al., 2005	TLS, Repair of crosslink damage

[a]*Homologs for DNA polymerase σ are not listed here because opinions are divided whether Polσ has a DNA polymerase activity or not.* [b]*Organellar DNA primases are not listed here.*

In plants, the reversion frequencies in *Arabidopsis* plants were measured using β-glucuronidase (GUS)-based markers (Kovalchuk et al., 2000; Nakagawa et al., 2011a; Nakagawa et al., 2011b).

The markers carry a G-T mutation, which corresponds to the 3'-T of TT sequence, a possible target of UV dimer. A misincorporation of dC opposite 3'-T leads to detect a reversion (a T to G transversion). When irradiated with UVB, the Polζ- and Rev1-deficient plants made fewer reversions in somatic cells compared with wild-type plants. By contrast, the Polη-deficient plant showed higher reversion frequencies than wild-type plants, which were reduced in Polζ and Polη double-deficient plants. From these results, the authors proposed a model in which *Arabidopsis* has two TLS pathways for responding to UV damage: a more mutagenic pathway involving Polζ and Rev1 and a less mutagenic pathway involving Polη (Nakagawa et al., 2011a). Polη bypasses CTD in an error-free manner (**Figure 1C**). Polζ and Rev1 bypass both CTD and (6-4)TP in an error-prone manner. The Polη inserts a nucleotide opposite (6-4)TP, which is extended by Polζ and causes the mutation. Since the bypass activity across (6-4)TP is low anyway, the minor dC insertions would be detected in this assay system. However, other explanations are possible, for example, when UV induces a double-strand break near the TT sequence, which is wrongly repaired and causes a mutation. Also, further analysis by employing a C-containing marker is necessary to profile UV-induced mutations in plants.

REGULATION OF TRANSLESION SYNTHESIS

Maintenance of the replication fork is crucial because stalled replication forks easily lead to strand breaks. It has been suggested that a stalled replication fork signals the modification of proliferating cell nuclear antigen (PCNA), which triggers the switching of replicase to TLS polymerase (Stelter and Ulrich, 2003; Kanao and Masutani, 2017). That is, when the PCNA is monoubiquitinated, the replicase detaches from PCNA and TLS polymerases are recruited to the replication site to perform the bypass of damaged DNA, whereas polyubiquitinated PCNA leads to the strand switch pathway. The mammalian Polη, Polκ, and Rev1 have been shown to interact with monoubiquitinated PCNA through the UBZ or UBM motif located in the C-terminal (Bienko et al., 2005; Wood et al., 2007). Moreover, Rev1 has also been shown to interact with other TLS polymerases (Guo et al., 2003) and is suggested to function as a bridge through which the best polymerase for TLS is selected (Boehm et al., 2016).

Arabidopsis has two copies of PCNA, but only AtPCNA2 complements the yeast *pol30* mutant (Anderson et al., 2008). The AtPolη has a UBM motif and two PIP repeats but does not have a UBZ motif conserved in animal and yeast Polηs. The mutant AtPolη disrupted in PIP1, PIP2, or UBM still interacts with *Arabidopsis* PCNA2 but does not fully complement yeast *rad30* cells (Anderson et al., 2008). Both *Arabidopsis* PCNAs interact with ubiquitin in *N. benthamiana* cells and are ubiquitinated *in vitro*

(Strzalka et al., 2013). The AtREV1 interacts with PCNA2, AtPolη, and AtREV7, a regulatory subunit of AtPolζ in yeast (Sakamoto et al., 2018). The processivity of rice Polλ is stimulated in the presence of PCNA (Uchiyama et al., 2004). Moreover, when *Arabidopsis* Polλ bypasses 8-oxoG, the ratio of error-free (dC insertion) to error-prone (dA insertion) bypass changed depending on its interaction with PCNA2 (Amoroso et al., 2011). These results suggest that the modification of PCNA leads to the switching from replicase to the appropriate TLS polymerase in plants.

It has been suggested that stalled replication in plants is also resolved by a Rad5-dependent strand switch pathway (Wang et al., 2011). The *rev3* and *rad5a* mutations caused synergistic or additive effects on root growth in plants exposed to UV, MMS, or crosslink agents compared with plants containing each single mutation (Wang et al., 2011). The *rad5a* plant failed to induce homologous recombination events after bleomycin treatment (Chen et al., 2008). By contrast, *rev3* and *rev1* plants induced significantly more recombination events after UV irradiation (Sakamoto et al., 2018). If AtRAD5a and AtREV3 work *via* two alternative pathways, the elevation of recombination activities in *rev3* and *rev1* plant could be due to the activation of a RAD5-dependent pathway (**Figure 1D**).

TRANSLESION SYNTHESIS AND HEAT SHOCK PROTEIN 90

The 90-kDa heat shock protein (HSP90) is an evolutionarily conserved molecular chaperone that stabilizes and activates various proteins involved in homeostasis, transcriptional regulation, chromatin remodeling, and DNA repair (Pennisi et al., 2015). The *Arabidopsis* genome has four copies of cytosolic HSP90 and three copies of organellar HSP90 (Krishna and Gloor, 2001). Queitsch et al. (2012) reported that the application of geldanamycin, a specific inhibitor of HSP90, to *Arabidopsis* plants elevated homologous recombination (HR) frequencies, suggesting that the HSP90s are involved in genome maintenance in plants.

Human HSP90 interacts with HsPolη and HsRev1 and regulates the TLS activities (Sekimoto et al., 2010; Pozo et al., 2011). The frequency of UV-induced supF mutation in hPolη-proficient cells is elevated by applying 17-AAG, an HSP90 inhibitor, due to the inhibition of error-free bypass of UV damage (Sekimoto et al., 2010). Conversely, in HsPolη-deficient cells, 17-AAG treatment reduces mutation due to the inhibition of the REV1-dependent error-prone bypass (Pozo et al., 2011). In contrast with the results in mammals, treatment with geldanamycin reduces mutation frequencies in wild-type plants, which are AtPolη-proficient (Sakamoto et al., 2018). This suggests that HSP90 mainly regulates the error-prone TLS pathway, involving AtRev1, in *Arabidopsis*.

TRANSLESION SYNTHESIS AND CELL CYCLE CHECKPOINT

In *Arabidopsis*, UVB or gamma irradiation induces programmed cell death of stem and progenitor (StPr) cells in the root meristem that depends on *ataxia-telangiectasia mutated* (*ATM*), *ataxia-telangiectasia and Rad3-related* (*ATR*), and *SUPPRESSOR OF GAMMA RESPONSE1* (Curtis and Hays, 2011; Furukawa et al., 2010). Curtis and Hays (2011) investigated the time course of cell death in UV-irradiated Polζ-deficient (*rev3*) and Polη-deficient (*polh*) roots as well as the roots of damage checkpoint kinase *atm* and *atr* mutants. They found that the cells in *polh* plants started dying at around 16 h after UV treatment, but the cells in *rev3* plant started to die at around 20 h. The time courses of cell death in *atr* and *rev3 atr* plants were similar to that in *rev3* plants, whereas the UV dose-dependency plots of *atr, rev3 atr*, and *rev3* fitted similar slopes. Thus, they hypothesized that there are two types of TLS in *Arabidopsis* StPr cells: rapid TLS involving Polη and slow TLS involving Polζ. No Polη or a failure of rapid TLS results in the accumulation of single-stranded DNA, which activates a damage checkpoint and Polζ bypasses the damage (slow TLS). If both Polη and Polζ are absent, or if Polη and ATR are absent, then the stalled replication fork collapses to produce DSB, and the ATM activates DSB repair pathways. A similar epistatic relationship between ATR and Polζ was observed in yeast; the Polζ-dependent mutation requires the yeast ATR homolog Mec1 (Pagès et al., 2009). Therefore, some, but not all, of the TLS activities appear to be controlled by checkpoint activation in both plants and microorganisms.

REV7, the regulatory subunit of Polζ, contains a HORMA (Hop1, Rev7, and MAD2) domain (Aravind and Koonin, 1998). Based on its homology to MAD2, the key component of the mitotic-spindle-assembly checkpoint, and Hop1, a meiotic-synaptonemal complex component, it has been speculated that REV7 acts as an adaptor for DNA repairs and the spindle assembly checkpoint (Aravind and Koonin, 1998). Human REV7 makes a homodimer, and REV7-MAD2 a heterodimer, *in vitro* (Murakumo et al., 2000). In the absence of REV7, human cells arrest in the G2/M-phase and display increased monoastral and abnormal spindles with misaligned chromosomes (Bhat et al., 2015). Crystal structure and NMR analyses showed that two copies of REV7 bind to the canonical REV7-binding motifs (RBMs) of REV3 (Rizzo et al., 2018). In plants, *Arabidopsis* REV7 makes a homodimer in both the nucleus and the cytosol (Sakamoto et al., 2018). However, there is only one repeat of RBM in AtREV3 sequences, which is similar to yeast REV3 (Tomida et al., 2015). Therefore, the conformation of active Polζ in plants could be different from that in mammals.

TRANSLESION SYNTHESIS DNA POLYMERASE IN THE REPAIR OF DOUBLE-STRAND BREAKS OR CROSSLINK DAMAGE

Substantial evidence points to the involvement of TLS polymerases in the DSB repair pathway. For example, chicken REV3(−/−) cells and *Arabidopsis rev3* plants are sensitive to ionizing radiation (Sakamoto et al., 2003; Sonoda et al., 2003). *Arabidopsis* Polλ-disruption plants are hypersensitive to ionizing radiation and bleomycin (Furukawa et al., 2016). In yeast, Polζ and Rev1 are associated with the homing endonuclease

(HO)-induced DSB end (Hirano and Sugimoto, 2006). Moreover, ScREV3 is responsible for mutations near the HO-induced cleavage site (Holbeck and Strathern, 1997; Rattray et al., 2002). These results show that some TLS polymerases, at least, have a role in DSB-repair processes in both animals and plants. Recently, DNA polymerase θ was shown to be involved in the alternative end-joining (Alt-EJ) pathway in animals (Chan et al., 2010; Wood and Doublié, 2016; Schimmel et al., 2017) and in moss (Mara et al., 2019). Polθ-deficient *Arabidopsis* cannot integrate T-DNA, suggesting that the Polθ stabilizes two minimally paired 3' overhanging DNA ends during the T-DNA integration process (van Kregten et al., 2016). It is possible that TLS polymerases work in DSB repair pathway in some context.

Polθ is the best understood polymerase involved in the repair of interstrand crosslink (ICL) damage (Harris et al., 1999; Shima et al., 2003; Beagan et al., 2017). Other TLS polymerases have also been suggested to work in the process of ICL damage repair. For example, REV3(−/−) cells or organisms are sensitive to ICL-inducing treatments in mammals, chickens, yeast, and plants (Grossmann et al., 2000; Sakamoto et al., 2003; Nojima et al., 2005; Takahashi et al., 2005; Sarkar et al., 2006; Sharma et al., 2012). Disruption of Rev1 and Polη makes the cell or organism hypersensitive to ICL treatment (Takahashi et al., 2005; Sharma et al., 2012), and hPolη can bypass the ICL adduct *in vitro* (Vaisman et al., 2000). It has been suggested that ICL damage is processed by the Fanconi anemia complementation group A (FANCA)-dependent pathway, which includes nucleolytic incision, TLS, and HR (Kim and D'Andrea, 2012). Several TLS polymerases have been shown to bypass ICL damage if the DNA around the ICL is appropriately trimmed (Ho et al., 2011; Roy et al., 2016). These data suggest that TLS activities are important in overcoming ICL damage. In conclusion, TLS polymerases have multiple roles, which are critical for the genome stability of animals, plants, and microorganisms.

CONCLUSION AND FUTURE PERSPECTIVES

The deletion of TLS polymerase often causes lethal or severe phenotypes in animals (Esposito et al., 2000; O-Wang et al., 2002; Wittschieben et al., 2006; Dumstorf et al., 2006; Stallons and McGregor, 2010). By contrast, almost all TLS polymerase activities can be disrupted in plants without severe reduction of fertility. Therefore, the plant system is ideal for analyzing the function, regulation, and interaction of TLS polymerases. Information on the structure and catalytic fidelity of TLS polymerases can assist us to build a novel genome-editing enzyme with elaborate specificities. Plant cells can provide a good platform for developing these upcoming technologies.

AUTHOR CONTRIBUTIONS

ANS wrote all the parts of this mini-review.

FUNDING

This work was partially supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant number 24241028, 25440147, and 17K00561 to AS.

ACKNOWLEDGMENTS

The author thanks Shinya Takahashi for his critical reading of the manuscript. She also thanks Ann Seward and Sarah Williams, ELS, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor is currently co-organizing a Research Topic with the author, and confirms the absence of any other collaboration.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 October 2019
doi: 10.3389/fpls.2019.01299

ATR, a DNA Damage Signaling Kinase, Is Involved in Aluminum Response in Barley

*Miriam Szurman-Zubrzycka[1], Malgorzata Nawrot[1], Janusz Jelonek[1], Mariusz Dziekanowski[1], Jolanta Kwasniewska[2] and Iwona Szarejko[1]**

[1] Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Katowice, Poland, [2] Department of Plant Anatomy and Cytology, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Katowice, Poland

OPEN ACCESS

Edited by:
Kaoru Okamoto Yoshiyama, Tohoku University, Japan

Reviewed by:
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Min Yu, Foshan University, China

***Correspondence:**
Iwona Szarejko
iwona.szarejko@us.edu.pl

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *12 April 2019*
Accepted: *18 September 2019*
Published: *22 October 2019*

Citation:
Szurman-Zubrzycka M, Nawrot M, Jelonek J, Dziekanowski M, Kwasniewska J and Szarejko I (2019) ATR, a DNA Damage Signaling Kinase, Is Involved in Aluminum Response in Barley. Front. Plant Sci. 10:1299. doi: 10.3389/fpls.2019.01299

Ataxia Telangiectasia and Rad-3-related protein (ATR) is a DNA damage signaling kinase required for the monitoring of DNA integrity. Together with ATM and SOG1, it is a key player in the transcriptional regulation of DNA damage response (DDR) genes in plants. In this study, we describe the role of ATR in the DDR pathway in barley and the function of the *HvATR* gene in response to DNA damages induced by aluminum toxicity. Aluminum is the third most abundant element in the Earth's crust. It becomes highly phytotoxic in acidic soils, which comprise more than 50% of arable lands worldwide. At low pH, Al is known to be a genotoxic agent causing DNA damage and cell cycle arrest. We present barley mutants, *hvatr.g* and *hvatr.i*, developed by TILLING strategy. The *hvatr.g* mutant carries a G6054A missense mutation in the *ATR* gene, leading to the substitution of a highly conserved amino acid in the protein (G1015S). The *hvatr.g* mutant showed the impaired DDR pathway. It accumulated DNA damages in the nuclei of root meristem cells when grown in control conditions. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis revealed that 60% of mutant nuclei possessed DNA nicks and breaks, whereas in the wild type only 2% of the nuclei were TUNEL-positive. The high frequency of DNA damages did not lead to the inhibition of the cell cycle progression, but the mutant showed an increased number of cells in the G2/M phase. In response to treatments with different Al doses, *hvatr.g* showed a high level of tolerance. The retention of root growth, which is the most evident symptom of Al toxicity, was not observed in the mutant, as it was in its parent variety. Furthermore, Al treatment increased the level of DNA damages, but did not affect the mitotic activity and the cell cycle profile in the *hvatr.g* mutant. A similar phenotype was observed for the *hvatr.i* mutant, carrying another missense mutation leading to G903E substitution in the HvATR protein. Our results demonstrate that the impaired mechanism of DNA damage response may lead to aluminum tolerance. They shed a new light on the role of the ATR-dependent DDR pathway in an agronomically important species.

Keywords: aluminum, ATR, DDR pathway, barley, TILLING

INTRODUCTION

Aluminum (Al) is the most abundant metal and the third most abundant chemical element (after oxygen and silicon) in the Earth's crust that comprises approximately 8% of its mass (Bojórquez-Quintal et al., 2017). It is a highly reactive element that in a neutral pH is incorporated into various non-toxic minerals, mainly in the form of aluminum oxides and aluminosilicates. In acidic conditions, aluminum solubilizes into highly phytotoxic $[Al(H_2O)_6]^{3+}$ molecules, referred to as Al^{3+} cations, which can be easily absorbed by plants (Mossor-Pietraszewska, 2001; Rahman et al., 2018). The toxicity of Al^{3+} in acidic soils is an important agricultural problem that has been identified more than 100 years ago (Miyake, 1916). Acidic lands (pH 5.5 and lower) are spread worldwide—it is assumed that 50% of the world's potentially arable soils have low pH (Kochian et al., 2015). In tropical and subtropical regions, soil acidity is one of the most important constrains that hinder the increase of food production, but also in the temperate zones of eastern North America and throughout Europe (where acidic soils reach up to 80% of the total area), Al stress may significantly affect crop yields (von Uexküll and Mutert, 1995; Aggarwal et al., 2015). Furthermore, modern farming practices, such as application of ammonium-based fertilizers, as well as industrial pollution unceasingly increase the acidification of soils (Kochian et al., 2005). The first and the most evident symptom and the important effect of aluminum toxicity is the reduction of root growth caused by inhibition of cell divisions in the root tip and decreased elongation of root cells. Additionally, Al^{3+} ions reduce the uptake of water and nutrients and consequently reduce plant growth and yield. Taken together, aluminum toxicity is considered as the main growth-limiting factor in acidic soils and the second, after drought, most serious abiotic stress to crop production worldwide (Kochian et al., 2015).

The best-known mechanism of aluminum tolerance is Al exclusion that is based on the exudation of organic acids (OAs) from the root tip to the rhizosphere. The OA transporters localized in the plasma membrane are activated by the presence of Al^{3+} ions in the environment. The excreted OAs, mainly citrate and/or malate, act as chelators of Al^{3+} ions, forming compounds that do not enter the root and are not toxic to plants. Another mechanism of tolerance, when Al^{3+} cations enter the root cells, is based on the internal formation of OAs and other organic compounds that form complexes with Al. Such complexes are sequestered and detoxified in vacuoles or translocated away from the root tip to the less Al-sensitive parts of the plant (reviewed in Kochian et al., 2015; Riaz et al., 2018).

The mechanisms of Al tolerance involving OAs are well understood; however, the true biochemical targets of Al^{3+} ions and the mechanisms of Al toxicity have not been fully characterized. The primary targets of Al^{3+} in apoplast are negatively charged compounds of the cell wall, such as hemicellulose or pectins (Yang et al., 2011). Aluminum alters the cell wall properties and causes cell wall rigidity, which affects cell elongation. However, Al^{3+} ions may interfere with multiple sites in both symplast and apoplast, and therefore, the exact multilevel molecular mechanisms underlying Al toxicity remain elusive (Singh et al., 2017, Riaz et al., 2018).

Studies carried out on many plant species, including *Arabidopsis* and barley, show that aluminum causes DNA double-strand breaks in root meristem cells, which indicates that DNA is a target for Al^{3+} ions (Nezames et al., 2012; Jaskowiak et al., 2018). Further work performed on *Arabidopsis* has clearly shown that the DNA damage response (DDR) pathway is involved in the Al response (Eekhout et al., 2017). The *Arabidopsis* Al-hypersensitive mutant *als3-1* has been used for suppressor screening to find mutations that can reverse its phenotype. The *als3-1* mutant carries a loss-of-function mutation in the *AtALS3* gene encoding an ABC transporter involved in the translocation of aluminum away from the root tip (Larsen et al., 1997; Larsen et al., 2005). The second-site mutagenesis revealed four suppressor genes: *ATR—Ataxia Telangiectasia and Rad3-related* (Rounds and Larsen, 2008), *ALT2—Aluminum Tolerant 2* (Nezames et al., 2012), *SOG1—Suppressor of Gamma response 1* (Sjogren et al., 2015), and *SUV2—Sensitive to UV 2* (Sjogren and Larsen, 2017), all of them involved in the DDR pathway. The mutations identified in these suppressor genes reversed a severe Al hypersensitivity observed in the *als3-1* and increased aluminum tolerance in the wild-type plants (summarized in Eekhout et al., 2017).

In general, in response to the DNA damage, the DDR pathway coordinates a transient cell cycle arrest and DNA repair. ATR is a key cell cycle checkpoint regulator that is required for the monitoring of DNA integrity (Culligan et al., 2004). It is a serine/threonine kinase that, together with Ataxia Telangiectasia Mutated (ATM), transmits the DNA damage signals to the downstream effectors by phosphorylating SOG1 (Rounds and Larsen, 2008). It was shown that ATR is activated when persistent ssDNA is accumulated in the nucleus, whereas ATM is activated in the presence of DSBs (DNA double-strand breaks) (Hu et al., 2016). SOG1, which may be phosphorylated by both ATR and ATM, is a central DDR transcription factor that activates the expression of hundreds of genes involved in DNA repair and cell cycle inhibition (Yoshiyama et al., 2009; Yoshiyama et al., 2013). ALT2 is a WD-40 protein that has, so far, an undefined role; however, it is required for the assessment of DNA integrity, including the monitoring of DNA crosslinks (Nezames et al., 2012). *SUV2* encodes a putative ATRIP (ATR-interacting protein) homologue that co-localizes with ATR and helps in the ATR recruitment to the persistent single-stranded DNA in *Arabidopsis* (Sakamoto et al., 2009; Sweeney et al., 2009). Taken together, the identified genes arrest the cell cycle progression after Al exposure in *Arabidopsis*; thus, their loss-of-function mutations induced in the *als3-1* background led to the progression of cell divisions regardless of the presence of aluminum. The factors encoded by these genes are thought to work together in detecting Al-induced DNA damage. They inhibit the cell cycle progression in order to repair the DNA damage and, eventually, to promote terminal differentiation and endoreduplication (Sjogren et al., 2015; Eekhout et al., 2017).

Due to the fact that soil acidification is a global problem in agriculture, it is important to broaden the knowledge on the mechanisms of Al toxicity in agronomically important crops. Barley (*Hordeum vulgare* L.), which is the fourth cereal crop in regard to cultivation area and production tonnage, is considered as the most Al-sensitive species among the cereals (Wang et al., 2006). In this study, we indicate for the first time the role of ATR

in the DDR pathway in barley and the function of the *HvATR* gene in response to Al toxicity. Using TILLING strategy and our *Hor*TILLUS population (Szurman-Zubrzycka et al., 2018), we have developed a barley mutant *hvatr.g* carrying a G6054A missense mutation that leads to the substitution of a highly conserved amino acid in the ATR protein (G1015S). The *hvatr.g* mutant showed an increased tolerance to Al treatment and a high accumulation of DNA damages in root meristem cells, both in control and Al treatment conditions. Despite the accumulation of DNA damages in response to Al, the cell cycle progression was not arrested in the mutant, while the cell cycle profile in root meristems differed between the mutant and its parent. We confirmed the hypothesis on the involvement of *HvATR* in response to DNA damages in barley through the analysis of another mutant in the *HvATR* gene (*hvatr.i*), with a similar to *hvatr.g*, though weaker phenotype. Our results demonstrate that *hvatr* mutants have an impaired mechanism of DNA damage response that leads to aluminum tolerance.

MATERIAL AND METHODS

Plant Material

The *Hor*TILLUS (*Hordeum*-TILLING-University of Silesia) population, which is a barley TILLING population developed at the Department of Genetics, University of Silesia in Katowice, has been used as the material for mutational screening in the *HvATR* gene. This population has been created after double treatment of seeds of spring barley cultivar 'Sebastian' with sodium azide (NaN_3) and *N*-methyl-*N*-nitrosourea (MNU) (Szurman-Zubrzycka et al., 2018).

Mutational Screening of the *HvATR* Gene Using the TILLING Strategy

The whole genome sequence of barley (second version) has been screened in order to find potential homolog(s) of the *Arabidopsis ATR* gene. Based on bioinformatics analysis with the use of Ensembl Plants datasets and tools (http://plants.ensembl.org/index.html), we have identified the putative barley *ATR* gene with acc. no HORVU7Hr1G118750. The gene is located on chromosome 7 and encodes a 2,575-aa protein. The *HvATR* gene has no paralogs in barley genome. The domains and motifs in a putative HvATR protein were computationally predicted with the use of InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) and Conserved Domain Search Service at the NCBI website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Four domains characteristic of ATR proteins were identified: 1) the UME domain of unknown function, 2) the FAT domain, 3) the PIKKc domain that is a phosphatidylinositol 3-/4-kinase catalytic domain, and 4) the FATC domain at the C-terminus. We have selected two conserved fragments of *HvATR* for mutational screening (**Supplementary Material 1**):

1) A 930-bp fragment encoding the UME domain that was proven to be involved in Al response in *Arabidopsis* (Rounds and Larsen, 2008) (T1 amplicon) and
2) A 865-bp fragment encoding a part of the PIKKc domain that includes a kinase catalytic center (T2 amplicon).

The PCR reactions for the T1 and T2 amplicons were optimized for specific primers labeled with IRDye-700 and IRDye-800 (forward and reverse primers, respectively) (**Supplementary Material 2**). Eight-fold DNA pools of M_2 *Hor*TILLUS plants were used as initial templates for mutation detection. TILLING screening was based on Celery Juice Extract (CJE) digestion of heteroduplexes followed by electrophoresis on polyacrylamide gels in the LI-COR DNA sequencers, according to the protocol described elsewhere (Szurman-Zubrzycka et al., 2017; Jost et al., 2019). All putative mutations were confirmed by sequencing.

Aluminum Treatment in Hydroponics

The system for studying the effect of Al treatments on root growth was based on the method described by Jaskowiak et al. (2018). The hydroponic setup consisted of plastic containers with a capacity of 4.5 L covered with lids containing 12 openings and air distributors with 12 outlets connected to the air pump. The Magnavaca solution was used as a medium for treatment with $AlCl_3$ (Magnavaca et al., 1987). The fraction of the bioavailable Al^{3+} ions was calculated using GEOCHEM-EZ software (Shaff et al., 2010). In the presented study, the Al concentration always refers to the bioavailable fraction of Al^{3+} ions. The Al concentrations used in our assays were 0, 5, 10, and 15 μM of bioavailable Al^{3+}, which correspond to 0, 25, 50, and 75 μM of nominal $AlCl_3$ added to the medium. The pH of the medium was determined each day and maintained at 4.0 throughout the whole experiment.

The seeds of the analyzed genotypes were surface-sterilized in 5% sodium hypochlorite and put on Petri dishes filled with wet filter paper for imbibition at 4°C for 72 h in the dark, and then transferred to 25°C for another 48 h. Afterwards, the seedlings with roots approximately 1.5–4 cm long (depending on the genotype) were implanted into the openings on the container lids, 12 seeds per container, in such a way that their roots were submerged in the medium. The experiments were carried out in a growth room under controlled conditions: light intensity, 250 μM m^{-2} s^{-1}; temperature, 20°C/18°C (day/night); and photoperiod, 16/8 h. The seedlings were grown in hydroponics for 7 days. One container was assumed as one biological repetition, with three repetitions per treatment.

Flood-and-Drain Semi-Hydroponics for Evaluation of Root System Growth

In order to characterize the root system of the *hvatr.g* mutant and its wild-type 'Sebastian' grown under optimal conditions, the germinated seeds (prepared as described above) were transferred into a flood-and-drain semi-hydroponic system described by Slota et al. (2016). Briefly, the seedlings were grown individually in acrylic tubes filled with soda lime-glass beads (MEGAN, Poland) and supplemented with a Hoagland medium (Hoagland and Arnon, 1950) through an automatic drip irrigation system. The medium was delivered to the tubes every 15 min through an afferent pump controlled remotely by a programmable logic controller. The acrylic tubes had a bottom drainage opening to ensure draining of the medium. The experiment was carried out for 14 days under conditions of a growth room described above.

The experiment was conducted in four repetitions with 10 plants per one biological repetition.

Root System Scanning and Image Analysis

After 7 days of the Al hydroponic experiment or 14 days of the flood-and-drain root system experiment, seedlings were preserved in 50% ethyl alcohol and their root systems were scanned in waterproof trays filled with water using a specialized scanner with a dual-lens system (EPSON PERFECTION V700 PHOTO) and a WinRHIZO software (Regent Instruments). The root parameters were evaluated for each seminal root separately using WinRHIZO and SmartRoot (https://smartroot.github.io/) software. The calculated root parameters included the length of the longest root, the number of seminal roots, the length of all seminal roots, the average diameter of seminal roots, the number of lateral roots, the length of all lateral roots, and the total root system length, surface, and volume. Statistical analyses were performed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$).

Analysis of Mitotic Activity

The Al treatment was performed as described in the previous section ("Aluminum Treatment in Hydroponics"). The mitotic activity of the meristematic root cells was analyzed in the *hvatr.g* and *hvatr.i* mutants and their wild-type parent after 7-day treatment with 10 μM Al^{3+}. Additionally, for *hvatr.g*, the frequency of anaphases with chromosomal aberrations and the frequency of cells with micronuclei were estimated. Seedlings grown in the Magnavaca medium at pH 4.0 without Al^{3+} ions were used as a control. The roots were fixed in the ethanol/glacial acetic acid (3:1, *v*/*v*) solution and cytogenetic slides were prepared using the Feulgen's squash technique. The experiment was carried out in three biological repetitions, with three plants per repetition. The cytogenetic parameters listed above were counted for minimum 10,000 cells for each *hvatr.g* experimental combination and for approximately 5,000 cells per *hvatr.i* experimental combination. Statistical analyses were performed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$).

Analysis of Cell Cycle Profile Using Flow Cytometry

The Al treatment of the *hvatr.g* mutant and cv. 'Sebastian' was performed as described in the previous section. Cell cycle analysis was performed for control roots and roots treated with 10 μM Al for 7 days. For one experimental replication, 20–30 root meristems were analyzed and three replications per treatment were used. The root tips were mechanically fragmented in a nuclei extraction buffer (CyStain® UV Precise P, 05-5002, Sysmex) and the suspension of nuclei was filtered through a 30-μm nylon mesh in order to remove any debris and stained with a staining buffer (CyStain® UV Precise P, 05-5002, Sysmex). Samples were analyzed with a CyFlow Space flow cytometer (Sysmex, Japan) with a 365-nm UV LED diode as the light source. The flow rate was adjusted to 20–40 nuclei per second. To determine the cell cycle phase, FloMax software with the Cell Cycle Analysis application was used.

TUNEL Test

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) test was used to detect and quantitatively analyze Al-induced DNA breaks in *hvatr.g*, *hvatr.i*, and 'Sebastian' roots. Al treatment was performed as described in the previous section. Control roots and roots treated with 5 and 10 μM Al for 7 days were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and then washed three times in PBS. Meristematic tissues were squashed in the PBS buffer. The prepared slides were frozen at −70°C. Prior to the TUNEL analysis, the slides were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate at 4°C for 2 min and rinsed in PBS. DNA fragment labeling was performed using a TUNEL reaction mixture (*in situ* Cell Death Detection Kit, Fluorescein, Roche) according to the provided protocol. The reaction containing an enzyme solution (terminal transferase) and a label solution (FITC-labeled nucleotides) in a 1:9 ratio (*v*/*v*) was applied to the preparations that were incubated at 37°C in the dark in a humid chamber. After 1 h, the preparations were rinsed three times in PBS and stained with DAPI (2 μg/ml) and then mounted in a Vectashield medium (Vector Laboratories). Preparations were examined with a Zeiss Axio Imager.Z.2 wide-field fluorescence microscope equipped with an AxioCam Mrm monochromatic camera (Zeiss, Germany). The frequency of TUNEL-positive FITC-labeled nuclei with DNA fragmentation was established based on analysis of 2,000 cells on two slides (each prepared from one root meristem) for the one repetition. For each experimental combination, two repetitions were analyzed. In total, 8,000 nuclei were analyzed for one combination. Statistical analyses were performed using Student's *t* test with $P < 0.05$.

RESULTS

Identification of Induced Mutations in the *HvATR* Gene

Seventeen mutations have been found in the two fragments of the *HvATR* gene screened using TILLING strategy (**Table 1**). The T1 amplicon (930 bp) encoding the UME domain (whose function has not yet been established) was analyzed in 6,144 M_2 plants of the *Hor*TILLUS population. In total, five mutations were found within this fragment: three missense mutations (*hvatr.f*, *hvatr.g*, and *hvatr.i*) and two silent mutations. The mutation density calculated based on analysis of the T1 fragment was 1 mut./1,143 kb.

The T2 amplicon (865 bp) encoding a fragment of the PIKKc domain responsible for kinase activity was screened in 6,189 M_2 plants of the *Hor*TILLUS population. In total, 12 mutations were identified within this fragment. Among them, four were missense (*hvatr.d*, *hvatr.m*, *hvatr.o*, and *hvatr.p*), six were silent, and two occurred in the non-coding intron region. The mutation density calculated for the T2 fragment was 1 mut./446 kb, thus two times higher than in the UME domain-encoding fragment.

TABLE 1 | Mutations identified in the *HvATR* gene.

Allele	Mutation position in gDNA (in CDS)	State of mutation in M_2 plant	Type of mutation	Effect in protein	Mutated domain
hvatr.a	G11111A; G7524A	Homozygous	Silent	–	
hvatr.b	C11060T; C7473T	Heterozygous	Silent	–	
hvatr.c	G10792A; G7317A	Heterozygous	Silent	–	
hvatr.d	**C11104T; C7517T**	**Heterozygous**	**Missense**	**A2506V (alanine to valine)**	**PIKKc**
hvatr.e	C6023T; C3012T	Homozygous	Silent		
hvatr.f	**G5682A; G2767A**	**Homozygous**	**Missense**	**E923K (glutamic acid to lysine)**	**UME**
hvatr.g	**G6054A; G3043**	**Heterozygous**	**Missense**	**G1015S (glycine to serine)**	**UME**
hvatr.h	G6146A; G3135A	Homozygous	Silent	–	
hvatr.i	**G5623A; G2708A**	**Heterozygous**	**Missense**	**G903E (glycine to glutamic acid)**	**UME**
hvatr.j	G10693A; G7218A	Heterozygous	Silent	–	
hvatr.k	G10945A; -	Heterozygous	Intronic	–	
hvatr.l	G10486A; G7011A	Homozygous	Silent	–	
hvatr.m	**C10978T; C7391T**	**Heterozygous**	**Missense**	**A2464V (alanine to valine)**	**PIKKc**
hvatr.n	G10453A; G6978A	Homozygous	Silent	–	
hvatr.o	**C10574T; C7099T**	**Homozygous**	**Missense**	**L2367F (leucine to phenylalanine)**	**PIKKc**
hvatr.p	**T10604C; T7129C**	**Homozygous**	**Missense**	**W2377R (tryptophan to arginine)**	**PIKKc**
hvatr.r	C10871T;-	Heterozygous	Intronic	–	

The alleles that carry missense mutations are bolded.

The mutations identified in the *HvATR* gene were confirmed by sequencing, and all of them, except for one (*hvatr.p*), were G/C to A/T transitions. Nine mutations were identified in the heterozygous state and eight mutations were in the homozygous state in M_2 plants (**Table 1**).

Among all the mutations identified in this study, the missense mutations were chosen for functional analysis of the *HvATR* gene as they may affect protein activity and function. Mutants carrying the changes that led to the amino acid substitutions in the encoded protein were developed into homozygous lines and the M_3 seed material was increased to M_4 or, if needed, further generation to perform phenotyping and Al treatment experiments.

The *hvatr.g* Mutant and Its Response to Aluminum

Of all the barley *atr* mutants that were preliminary tested for aluminum response, only one, *hvatr.g*, showed a strong Al-tolerant phenotype in all Al^{3+} concentrations used. The *hvatr.g* mutant carries a G6054A missense mutation in the UME domain that changes glycine-1015 to serine (G1015S) at the protein level. The multiple alignment of the ATR proteins from a broad spectrum of species showed that the glycine-1015 altered in the *hvatr.g* mutant is conserved among plants, animals, and humans (**Figure 1**). This suggests that the described mutation might have a significant impact on the protein function.

Detailed evaluation of root system growth after aluminum treatment was performed for the *hvatr.g* mutant and its parent variety 'Sebastian' grown in the Magnavaca solution without Al and supplemented with 5, 10, and 15 μM Al. The *hvatr.g* mutant was characterized by a shorter root system than the wild type in the control combination; however, the growth of its roots was not affected by Al, contrary to 'Sebastian' roots (**Figures 2A**, **B**). The length of the longest root of 'Sebastian' was reduced by 25%, 55%, and 60% in 5, 10, and 15 μM Al, respectively, whereas the length of the longest root of the mutant was not reduced in any of the Al doses tested (**Figure 2B**). The total root length that is the sum of the length of all seminals and laterals was significantly reduced in 'Sebastian' in all Al concentrations (up to 80%), while in the mutant it was reduced only in the higher Al concentrations and only by approx. 30% (**Figure 2B**). Aluminum caused an increase of the seminal root diameter in 'Sebastian' (which is a typical symptom of Al toxicity), whereas in the mutant the root diameter was not affected by any of the Al concentrations used (**Figure 2B**). As a result, the surface and volume of the whole root system of 'Sebastian' were significantly reduced, up to 70% and 50%, respectively, while these parameters in the *hvatr.g* mutant were reduced only to a small extent, up to 20% (data not shown).

Detailed Analysis of Root System Architecture in the *hvatr.g* Mutant

Due to the fact that the root system of the *hvatr.g* mutant was Al-tolerant, but significantly reduced, compared to the wild type, after 7 days of growth in a hydroponic culture in Magnavaca solution at pH 4.0 (control in the Al experiment), we decided to analyze the mutant root phenotype after a longer growth period in more optimal conditions. The seedlings of the mutant and the wild type were grown for 14 days in a flood-and-drain semi-hydroponic system and were irrigated with a full-strength Hoagland solution (pH ~6.0), rich in nutrients. We observed that also under these optimal conditions, the seminal roots of the *hvatr.g* were in general shorter than in the case of 'Sebastian' and the length of the longest root was even twice reduced (11 and 23 cm for the mutant and the wild type, respectively; **Figure 3**). However the *hvatr.g* mutant produced more seminal roots (eight and six for the mutant and the wild type, respectively), and they were characterized by an increased diameter. Moreover, *hvatr.g* developed longer lateral roots (the total lateral root length was significantly higher compared to 'Sebastian'), which all resulted

FIGURE 1 | Multiple alignments of a fragment of the ATR protein sequences from various species, with the position of the substituted amino acids in the *hvatr.g* and *hvatr.i* mutants indicated by a *red frame*. *Hs, Homo sapiens*; *Mm, Mus musculus*; *Ha, Helianthus annuus*; *At, Arabidopsis thaliana*; *Zm, Zea mays*; *Hv, Hordeum vulgare*; *Os, Oryza sativa* (*X1* and *X2* represent two isoforms of this protein in rice). An * indicates positions which have a fully conserved residue. (A : indicates conservation between groups of strongly similar properties. A . indicates conservation between groups of weakly similar properties).

in no difference of the length, surface, and volume of the whole root system between the mutant and its wild type (**Figure 3**).

Influence of Al Treatment on the Mitotic Index and Cell Cycle Profile of the *hvatr.g* Mutant

We have analyzed the mitotic activity of the meristematic root cells of the *hvatr.g* mutant and its wild type after 1 week of 10 μM Al treatment. The mitotic indices in the root meristems of both 'Sebastian' and *hvatr.g* mutant were at the same level, approx. 4.5% in control conditions (Magnavaca solution, pH 4.0). Aluminum treatment caused the reduction of the mitotic index in 'Sebastian' to 2%, whereas it did not affect the mutant (**Figure 4A**). The frequencies of the particular mitotic phases were calculated and showed that the majority of the dividing cells were in the prophase stage; however, in the mutant, this fraction of mitotically active cells was higher than in the wild type (77.5% and 69%, for *hvatr.g* and 'Sebastian', respectively). Aluminum treatment increased slightly the percentage of prophase cells and decreased the number of cells in the other phases of mitosis (metaphases and anaphases/telophases) in both genotypes (**Figure 4B**).

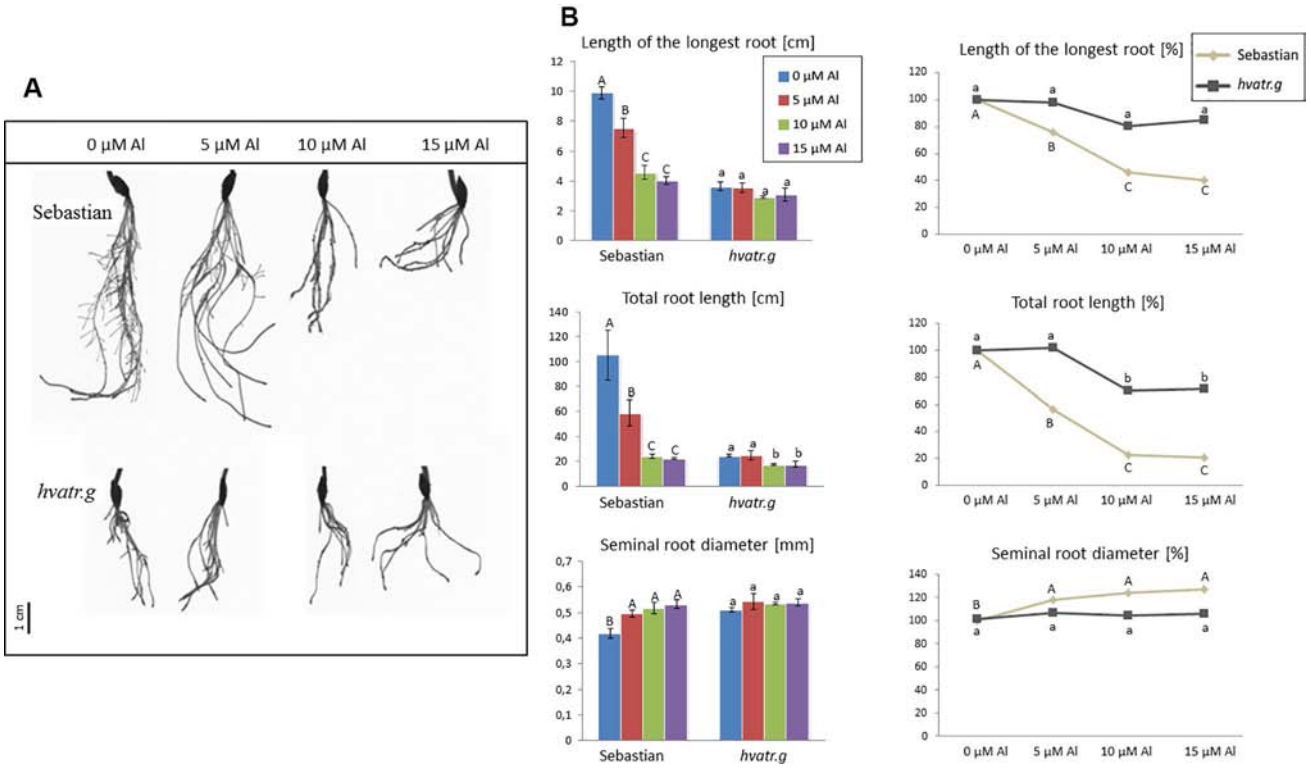


FIGURE 2 | (A) Comparison of a root system of the *hvatr.g* mutant and cv. 'Sebastian' grown for 7 days in Magnavaca solution at pH 4.0 without Al and with 5, 10, and 15 µM of bioavailable Al^{3+} ions. **(B)** Main root parameters of 'Sebastian' and the *hvatr.g* mutant grown for 7 days in Magnavaca solution at pH 4.0 without Al and with 5, 10, and 15 µM of bioavailable Al^{3+} ions. The parameters are presented as direct values (in centimeters) and as percentage, where 100% is the value for untreated roots of the genotype. Presented parameters: length of the longest root, total length of the whole root system (seminals + laterals), and average diameter of seminal roots. Statistically significant differences between different doses of Al within each genotype were assessed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$) and are indicated by different uppercase letters for the 'Sebastian' variety and by different lowercase letters for the *hvatr.g* mutant.

Analysis with the use of flow cytometry was performed in order to check the effect of Al treatment on the cell cycle in the roots of the analyzed genotypes. The cell cycle profile in control conditions differed between the *hvatr.g* mutant and its wild type. The mutant was characterized by a higher frequency of cells in the G2/M phase (61%) and a lower frequency of cells in the S phase (20%) than the wild type (43% and 32% for the G2/M and S phases, respectively). The aluminum treatment did not change the cell cycle profile in the *hvatr.g* mutant, while it caused the decrease of the cells in the S phase (to 22%) and the increase of cells in the G2/M phase (to 62%) in 'Sebastian' (**Figure 5**).

Assessment of DNA Damage Induced by Aluminum in the *hvatr.g* Mutant

The TUNEL test was applied in order to analyze the frequency of nuclei with DNA breaks in the root meristems of the *hvatr.g* mutant and its wild-type parent after 7 days of treatment with 5 and 10 µM Al. To determine the percentage of damaged nuclei, all cells were simultaneously stained with DAPI. The nuclei that had a green fluorescence detected in the FITC channel were characterized by DNA damage (**Figures 6A**, **B**). The analysis revealed that as much as 60% of the *hvatr.g* mutant nuclei showed TUNEL-specific fluorescence in the control conditions, while the parent variety 'Sebastian' in the control hydroponics had 1.9% of TUNEL-positive nuclei. Treatment with 5 or 10 µM Al significantly increased the frequency of damaged nuclei: by about 10% in the mutant, whereas by 14% and 20% in 'Sebastian' root cells, respectively (**Figure 6C**).

Additionally, we have calculated the frequency of cells with micronuclei, which arise from unrepaired double-strand DNA breaks, as well as the frequency of chromosome aberrations during anaphase. Similar to the results of the TUNEL test, the *hvatr.g* mutant showed a five times higher frequency of cells with micronuclei under control conditions than the wild type. Al treatment increased the number of such cells in both genotypes, almost twice in the mutant and five times in the wild-type parent (**Supplementary Material 3**). Correspondingly, under control conditions, the chromosomal aberration index was also higher in the mutant compared to the wild-type parent, and it increased significantly after Al treatment in both genotypes (**Supplementary Material 4**).

Analysis of the *hvatr.i* Mutant Carrying Another Mutation in the *HvATR* Gene

The *hvatr.g* mutant was induced by chemical mutagenesis; therefore, it might carry other mutations affecting the traits analyzed in this study. In order to confirm that the Al-tolerant

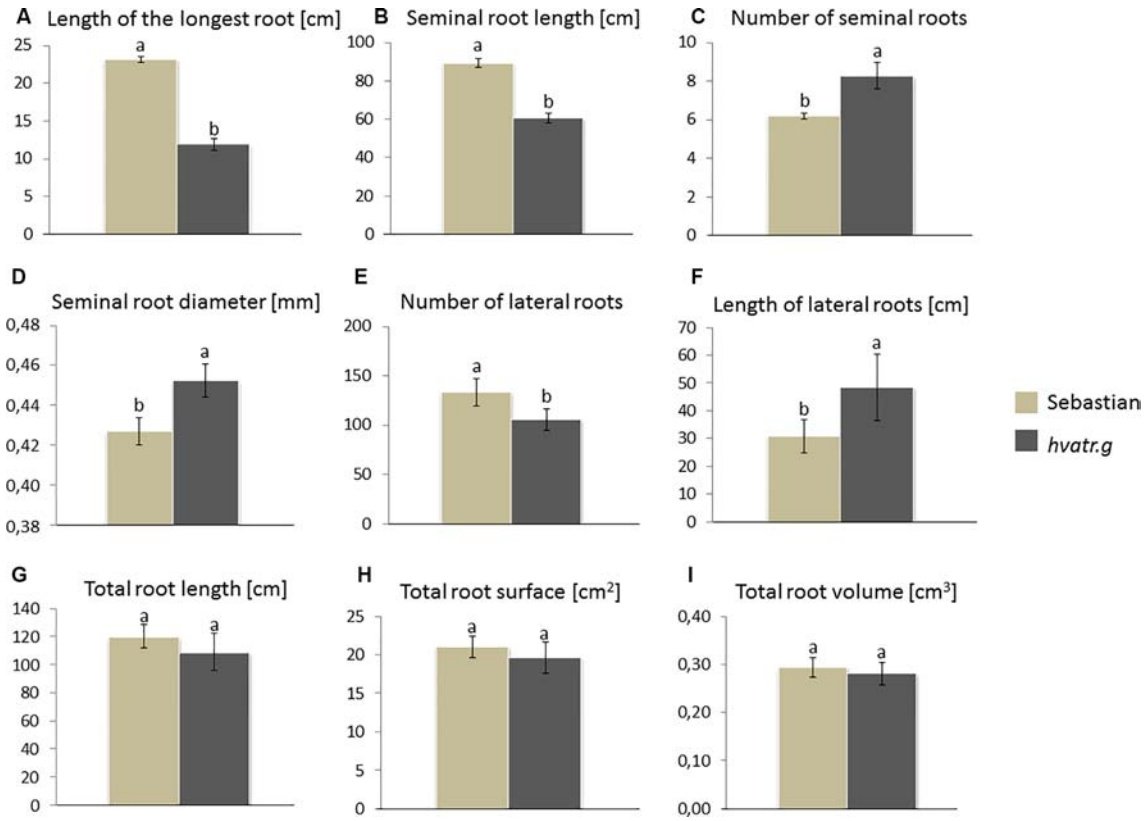
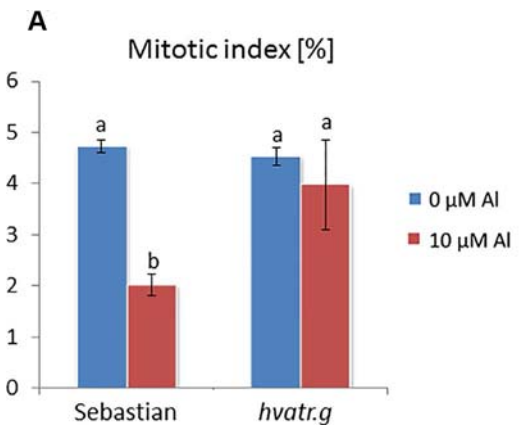


FIGURE 3 | Main root parameters of 'Sebastian' and the *hvatr.g* mutant grown for 2 weeks in a flood-and-drain semi-hydroponics irrigated with a full-strength Hoagland solution. **(A)** Length of the longest root. **(B)** Length of all seminal roots. **(C)** Number of seminal roots. **(D)** Average diameter of seminal roots. **(E)** Number of lateral roots. **(F)** Length of all lateral roots. **(G)** Length of the whole root system (seminals + laterals). **(H)** Surface of the whole root system (seminals + laterals). **(I)** Volume of the whole root system (seminals + laterals). Statistical analyses were performed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$) to assess the differences between two genotypes. Statistically significant differences are indicated by different letters.



B

Mitosis phase		Sebastian		*hvatr.g*	
		-Al	+Al	-Al	+Al
P		69%	78%	77.5%	83.5%
M		12.5%	9%	12%	8%
A/T		18.5%	13%	10.5%	8.5%

FIGURE 4 | Cytological effects of Al in the root cells of cv. 'Sebastian' and the *hvatr.g* mutant. **(A)** Mitotic activity in the root meristems of untreated and Al-treated plants. **(B)** Frequency of mitosis phases (prophases, metaphases, and anaphases/telophases). Statistical analyses were performed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$). Statistically significant differences are indicated by different letters.

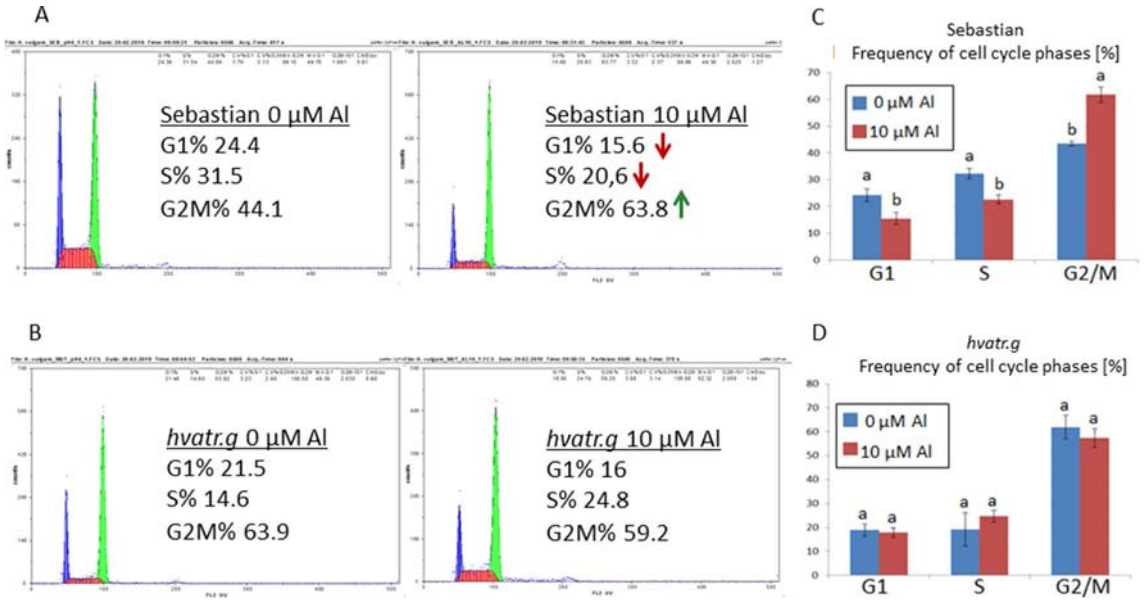


FIGURE 5 | Flow cytometric analysis of the cell cycle profiles in root meristematic cells of 'Sebastian' and the *hvatr.g* mutant. **(A)** Examples of representative histograms for 'Sebastian' grown in control conditions and treated with 10 µM Al. **(B)** Examples of representative histograms for the *hvatr.g* mutant grown in control conditions and treated with 10 µM Al. **(C)** Frequencies of cell cycle phases in the root meristems of 'Sebastian' untreated and treated with 10 µM Al. **(D)** Frequencies of cell cycle phases in the root meristems of the *hvatr.g* mutant untreated and treated with 10 µM Al. Statistical analyses were performed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$) in order to assess the differences between treated and untreated samples. Statistically significant differences are indicated by different letters.

phenotype of the *hvatr.g* mutant (described in the previous sections) was indeed caused by the identified G6043A mutation in the *HvATR* gene and not by other mutations present in the *hvatr.g* genome, we screened all other TILLING mutants carrying different missense mutations in the analyzed gene for their response to Al treatment. We found that one of these mutants, *hvatr.i*, showed a similar to *hvatr.g*, but a weaker Al-tolerant phenotype. The *hvatr.i* mutant carries a G5623A transition leading to the change of glycine-903 to glutamic acid (G903E) in the UME domain (**Figure 1**).

Evaluation of *hvatr.i* root system growth after aluminum treatment was performed in the same way as described for the *hvatr.g* mutant. Two concentrations of Al were tested: 5 and 10 µM Al^{3+}. The *hvatr.i* mutant, similarly to *hvatr.g*, was characterized by a shorter root system than the wild type when grown in the control medium without Al; however, the growth of its roots was much less affected by Al treatment than the growth of 'Sebastian' roots (**Figures 7A, B**). The length of the longest root of 'Sebastian' was reduced by 12.5% and 54% after treatment with 5 and 10 µM Al, respectively, whereas the length of the longest root of the *hvatr.i* mutant was not affected (or even slightly increased) by 5 µM Al and was reduced by 36% in the medium with 10 µM Al (**Figure 7B**). The total root length of all seminals and laterals was significantly reduced in 'Sebastian' in both Al concentrations tested (by 27% and 72% in 5 and 10 µM Al, respectively), while in the *hvatr.i* mutant it was also reduced, but to a lesser extent (by 7% and 53% in 5 and 10 µM Al, respectively; **Figure 7B**). In this experiment, we have not observed any differences in the average seminal root diameter between the analyzed genotypes.

We have analyzed the mitotic activity of the root cells in the meristematic zone of *hvatr.i* seedlings grown in the medium with 10 µM Al and without Al at pH 4.0. The preliminary analysis revealed that in the wild-type cv. 'Sebastian', the mitotic index was reduced by 40% after Al treatment, whereas in the analyzed mutant the frequency of dividing cells was reduced by 24% (**Figure 8A**).

To assess the level of DNA damage caused by Al, the TUNEL test was applied. Similarly to the *hvatr.g* mutant, the percentage of TUNEL-positive nuclei in the *hvatr.i* mutant was much higher than in 'Sebastian', even under control conditions, and it was further increased by aluminum treatment. Almost 60% of the *hvatr.i* mutant nuclei was damaged in the control medium (at pH 4.0); however, their fluorescence was lower than that in the *hvatr.g* mutant. Under the same control conditions, only 2% of 'Sebastian' nuclei were TUNEL-positive. Treatment with 5 and 10 µM Al significantly increased the frequency of damaged nuclei in both genotypes, but to a much lesser extent in the *hvatr.i* than in the parent cultivar: by about 6% and 8% in the mutant, whereas by 15% and 22% in 'Sebastian' root cells, respectively (**Figure 8B**). All these observations indicate that the *hvatr.i* mutant expresses similar to *hvatr.g*, though a weaker phenotype in regard to the Al response and DNA damage repair.

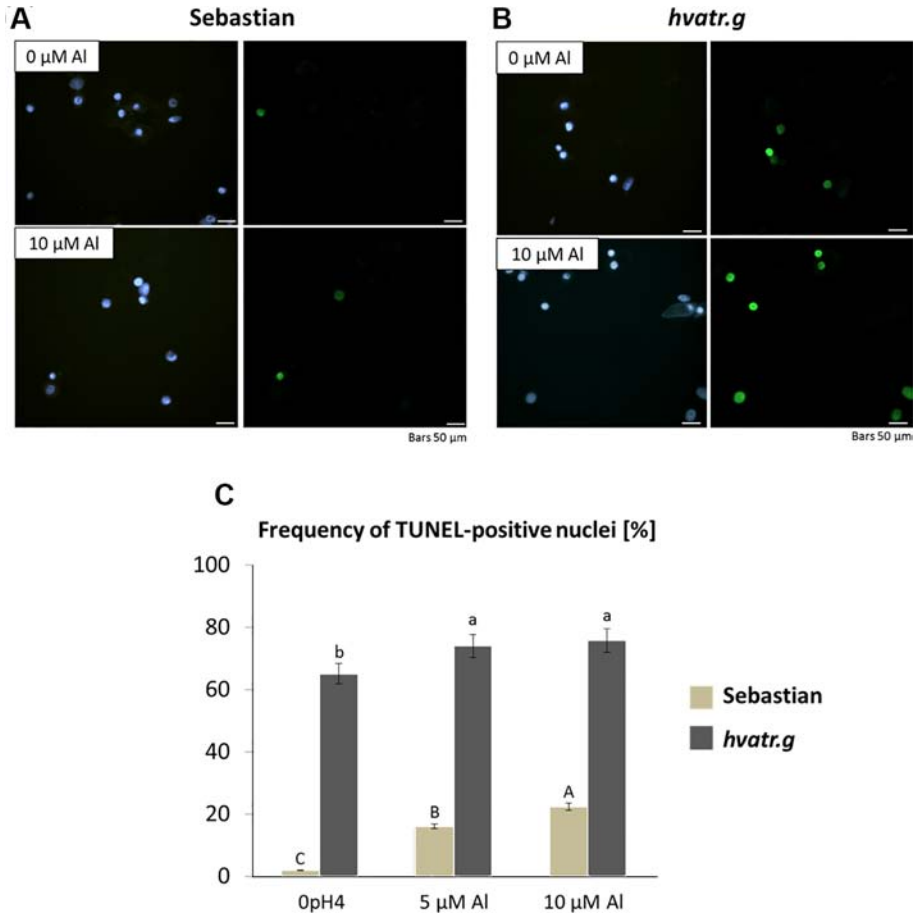


FIGURE 6 | Results of the TUNEL test in the root meristematic cells of 'Sebastian' and the *hvatr.g* seedlings treated with Al. **(A)** Examples of damaged nuclei observed in control 'Sebastian' roots and roots treated with 10 µM Al. Left Images of DAPI-stained nuclei. Right Images from the FITC channel. **(B)** Examples of damaged nuclei observed in control *hvatr.g* roots and roots treated with 10 µM Al. Left Images of DAPI-stained nuclei. Right Images from the FITC channel. **(C)** Frequency of labelled nuclei in the root cells of analyzed genotypes treated with 5 and 10 µM Al. The significant differences ($P < 0.05$) between the groups are indicated by different letters (uppercase letters for 'Sebastian' and lowercase letters for *hvatr.g* mutant).

DISCUSSION

In essence, activation of the DDR pathway in response to DNA damage leads to cell cycle stoppage, the activation of DNA repair processes, and to programmed cell death (reviewed in Kim et al., 2019). We have identified mutants, named *hvatr.g* and *hvatr.i*, that carry missense mutations in the *HvATR* gene. ATR is a serine/threonine kinase that acts as a central regulator in the DDR pathway. Together with another kinase, ATM, it is responsible for the activation of this pathway and transduction of a signal in response to DNA damage (Culligan et al., 2004). The mutations identified in our TILLING mutants lead to the substitution of very conserved amino acids, glycine-1015 to serine in the *hvatr.g* mutant and glycine-903 to glutamic acid in the *hvatr.i* mutant. Both altered amino acids are within the UME domain of the ATR protein. This domain has not yet been functionally characterized; however, it is predicted to be required for protein–protein interactions (Rounds and Larsen, 2008).

Here, we present that both mutants, *hvatr.g* and *hvatr.i*, are impaired in the DDR pathway. They accumulated DNA damages in control hydroponic conditions, when seedlings were grown in the Magnavaca medium at pH 4.0. The damages have been proven by TUNEL analysis, which revealed that approx. 60% of both mutants' nuclei possessed DNA nicks and breaks, whereas in the wild type only approx. 2% of nuclei were TUNEL-positive. Additionally, in the *hvatr.g* mutant, which was characterized in more detail, the frequencies of micronuclei and chromosome aberrations were statistically higher in the mutant than in its wild-type parent under control conditions. The work performed on *Arabidopsis* has also shown, based on a comet assay, that the *atr* mutant (in the *als3-1* background) was characterized by a higher DNA damage level in control conditions than Col-0; however, this difference was not that substantial (Rounds and Larsen, 2008). Interestingly, even though the frequency of dividing cells was not altered in the *hvatr.g* mutant, the cell cycle profile differed from that of 'Sebastian'. The mutant possessed fewer cells in the S phase and more cells in the G2/M phase. Moreover, the cells in the stage of prophase represented a greater percentage of dividing cells in the mutant than in 'Sebastian'. Taken together, these results show that the transduction of a signal of DNA damage

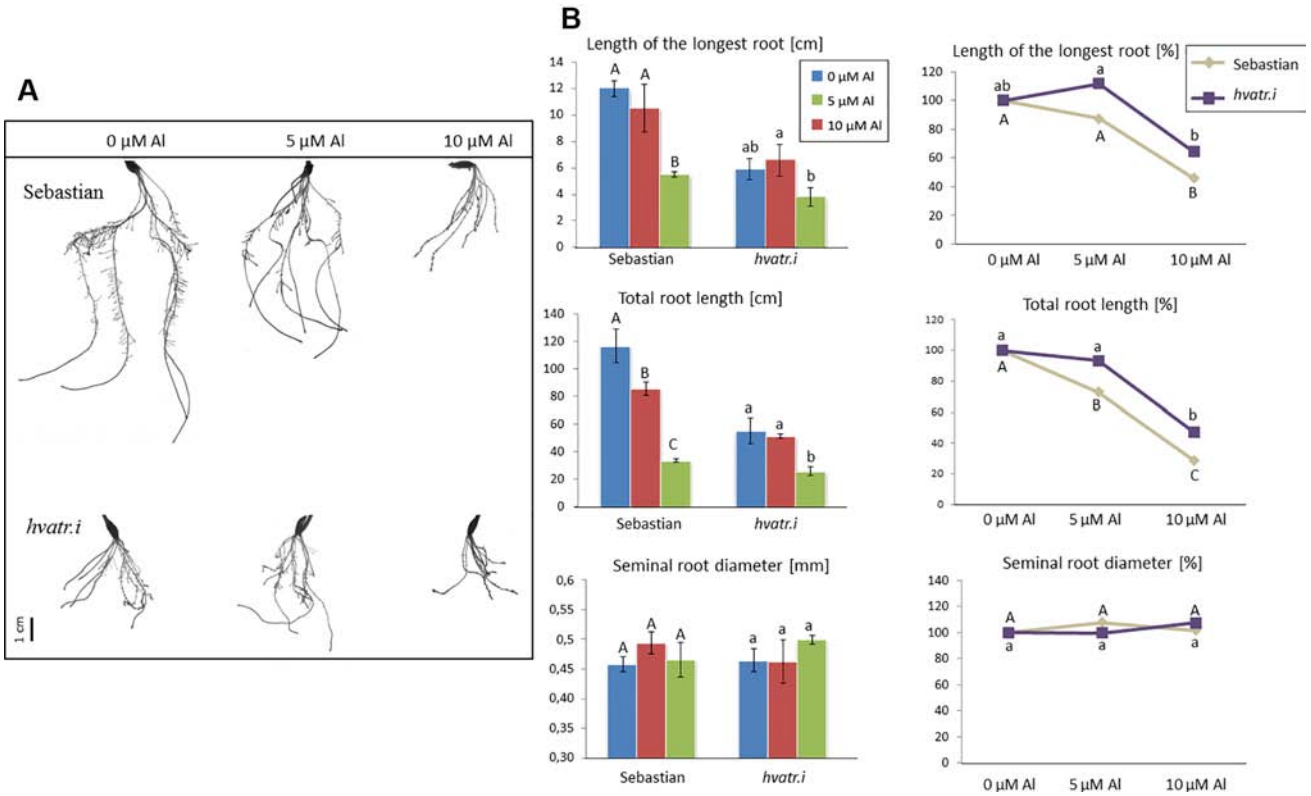


FIGURE 7 | (A) Comparison of the root system of the *hvatr.i* mutant and cv. 'Sebastian' grown for 7 days in Magnavaca solution at pH 4.0 without Al and with 5 and 10 µM of bioavailable Al^{3+} ions. **(B)** Main root parameters of 'Sebastian' and the *hvatr.i* mutant grown for 7 days in Magnavaca solution at pH 4.0 without Al and with 5 and 10 µM of bioavailable Al^{3+} ions. The parameters are presented as direct values (in centimeters) and as percentage, where 100% is the value for untreated roots of the genotype. Presented parameters: length of the longest root, total length of the whole root system (seminals + laterals), and average diameter of seminal roots. Statistically significant differences between different doses of Al within each genotype were assessed using ANOVA ($P < 0.05$) followed by Tukey's honestly significant difference test (Tukey HSD test, $P < 0.05$) and are indicated by different uppercase letters for the 'Sebastian' variety and by different lowercase letters for the *hvatr.i* mutant.

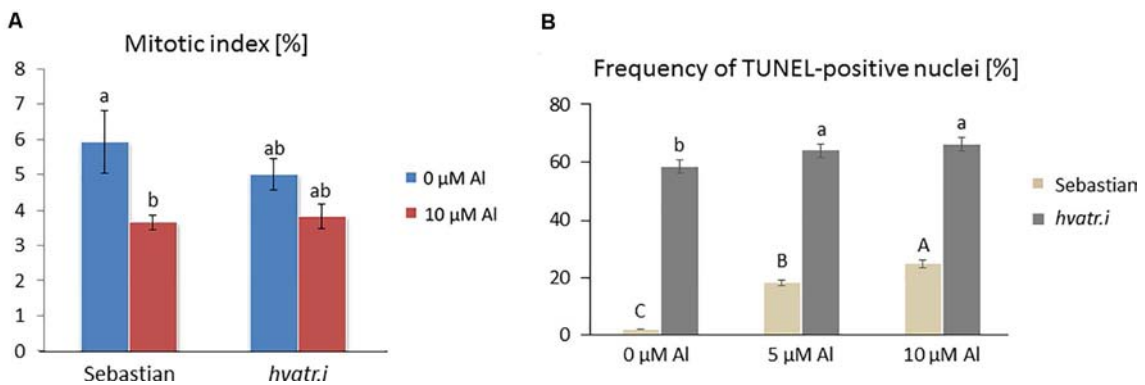


FIGURE 8 | Cytological analysis of the *hvatr.i* mutant and its parent variety 'Sebastian'. **(A)** Mitotic activity in the root meristems of untreated and Al-treated plants. **(B)** Results of the TUNEL test—frequency of labeled nuclei in the root cells of analyzed genotypes untreated and treated with 5 and 10 µM Al. The significant differences ($P < 0.05$) between the groups are indicated by different letters (uppercase letters for 'Sebastian' and lowercase letters for *hvatr.g* mutant).

does not function properly in the *hvatr.g* mutant, confirming the crucial role of ATR in the DDR pathway in barley.

Aluminum toxicity is considered as one of the most important agricultural problems worldwide. The first evidence that the activation of the DDR pathway is, at least in part, responsible for Al-induced root growth inhibition came from studies on *Arabidopsis* (summarized in Eekhout et al., 2017). Here, we confront this knowledge with an agronomically important

species and present the evidence that the change in the DDR pathway may affect response to aluminum in barley.

Our *hvatr.g* and *hvatr.i* mutants manifested Al-tolerant phenotypes. The longest seminal root of *hvatr.g* was not reduced even by the highest aluminum concentration applied, whereas the root system of the cultivar 'Sebastian' was severely reduced in all Al concentrations tested. These results are consistent with the work performed on *Arabidopsis*, where analysis of the root growth of *atr* mutants showed a high level of Al tolerance. One of the described *Arabidopsis* mutants possessed mutation within the UME domain, which confirms that this domain is necessary for proper activity of the ATR protein (Rounds and Larsen, 2008).

In our study, the reduction of root length in the wild-type cv. 'Sebastian' after exposure to Al might result, at least in part, from the decreased mitotic activity in the root meristems and the increased level of DNA damage. Our previous work performed for the same genotype treated with aluminum in Hoagland solution has shown that Al treatment significantly reduced the mitotic activity of the root tip cells (Jaskowiak et al., 2018). Similar results were obtained in Al studies of other species, for example *Helianthus annuus* (Kumar and Srivastava, 2006; Li et al., 2015), *Vicia faba* (Zhang et al., 2018), and *Allium cepa* (Qin et al., 2010). Nonetheless, the root meristematic cells of the barley *hvatr.g* mutant studied here were dividing despite the presence of this genotoxic agent in the medium, which further increased the level of DNA damage observed in control conditions. The frequency of dividing cells has not been changed after Al treatment. The same tendency was observed in the *hvatr.i* mutant—although the frequency of dividing cells decreased after Al treatment, this reduction was not that high as in 'Sebastian'. Moreover, the cell cycle profile of the *hvatr.g* mutant was not affected by Al treatment, whereas in the wild type the aluminum treatment caused an increase in the number of cells arrested in the G2 phase. Similarly, the inhibition of root growth caused by Al in *Arabidopsis* correlated with the cell cycle arrest in the G2 stage, which was accompanied by the accumulation of Cyclin B1;1 in the root tips of the wild type and Al-hypersensitive *als3-1* mutant. In the *atr* mutant, no concomitant increase in the activity of *CycB1;1* was observed, which indicated that cell division was not arrested at the G2 stage (Rounds and Larsen, 2008). Correspondingly, in another *Arabidopsis* Al-hypersensitive mutant, *star1* (with T-DNA insertion in *STAR1* encoding a nucleotide binding domain of ABC transporter), the expression of *CycB1;1* dramatically increased in root meristems after exposure to even low doses of Al, which did not cause inhibition of root growth in the wild type. This suggests that the cell cycle progression was halted in *star1* in response to the low level of Al (Zhang et al., 2018).

The DNA-damaging effect of Al was observed in our study for all genotypes tested. Using TUNEL, micronuclei and chromosome aberration tests, we demonstrated the increase in the frequency of root tip cells carrying DNA damages after Al treatment. Our data support other studies showing that aluminum has an impact on DNA integrity, probably through a direct binding to the DNA phosphate backbone (Silva et al., 2000; Jaskowiak et al., 2018). Studies performed in several Gramineae species showed DNA fragmentation after Al treatment in rye, barley, and oat roots, but not in maize and wheat (Vardar et al., 2016). These observations indicate that plants differ in response to phytotoxic Al^{3+} ions. Barley is known to be the most sensitive to Al among cereals; however, it shows some genotype variation (Ma et al., 2004; Ma et al., 2016), which makes it possible to breed more Al-tolerant cultivars. Our barley *atr* mutants with the impaired DDR pathway may serve as a tool to study Al tolerance in this important crop species. It should be noted that they are fully fertile, which indicates that the mutants do not carry DNA damages in generative cell lines, where the DNA lesions might be potentially repaired through a different pathway (not ATR-dependent).

The fact that TILLING mutants possess a high number of mutations in their genomes might be a limitation in using them for functional gene studies. However, it should be noted that a vast majority of mutations after classical chemical mutagenesis occur in non-coding regions (Kurowska et al., 2012). The barley genome is very large (approx. 5.3 Gbp) and coding sequences constitute only 1.3% of its size (Mascher et al., 2017). Moreover, many mutations in the coding sequences are silent or do not affect the protein function. Therefore, the probability of obtaining knockdown or knockout mutations in genes involved in the same process of interest (for example, DNA repair through the DDR pathway) in the same mutated plant is very low (Szurman-Zubrzycka et al., 2018). Nevertheless, here, we described two independent TILLING mutants carrying different mutations in the *HvATR* gene. These mutants showed similar phenotypes related to aluminum response and DNA repair, which confirms that, indeed, the disruption of ATR function is responsible for the observed alterations and that these mutants, together, are a useful tool for functional analysis of the *ATR* gene in barley.

CONCLUSIONS

We have identified barley TILLING mutants, *hvatr.g* and *hvatr.i*, carrying different missense mutations in the *HvATR* gene that showed an impaired repair of DNA lesions, but the Al-tolerant phenotype. The high frequency of DNA damages observed in the mutants already in the control conditions did not lead to the inhibition of cell cycle progression. Al treatment increased the level of DNA damages, but did not affect the mitotic activity and the cell cycle profile in the *hvatr.g* mutant. The *hvatr.i* mutant showed a similar, although a weaker, Al-tolerant phenotype. We demonstrate that ATR is required for detection of DNA damage caused by toxic Al^{3+} ions in barley. We conclude that the reduction of root growth in response to aluminum is, at least in part, triggered by the ATR-dependent activation of DDR response leading to the arrest of cell cycle. The identified *hvatr.g* and *hvatr.i* mutants may serve as a useful tool in further studies on the DDR pathway in cereal species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

IS conceived and supervised the project. MS-Z and JJ identified TILLING mutants. MS-Z, MN and MD conducted hydroponic experiments and analyzed the root system. MS-Z and MD performed cytological analysis. JK performed TUNEL analysis. MS-Z and IS analyzed the data and wrote the manuscript.

FUNDING

This work was supported by grant from ERA-NET for Coordinating Plant Sciences. The authors gratefully acknowledge financial support of the research by the National Centre for Research and Development (grant ERA-CAPS-II/2/2015).

ACKNOWLEDGMENTS

We would like to thank our partners in ERA-CAPS Consortium, Drs. Lieven de Veylder (Belgium), Paul Larsen (United States of America), and Arp Schnittger (Germany), for fruitful discussions about the DDR pathway and aluminum toxicity. We thank Jon E. Shaff (United States of America) for many tips on choosing the right medium for Al treatment. We thank Dr. Magdalena Rojek for assistance during analysis on flow cytometer.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01299/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 January 2020
doi: 10.3389/fpls.2019.01723

DNA Damage Inducible Protein 1 is Involved in Cold Adaption of Harvested Cucumber Fruit

Bin Wang[1,2], *Guang Wang*[1] *and Shijiang Zhu*[1]*

[1] Guangdong Province Key Laboratory of Postharvest Science of Fruits and Vegetables/Key Laboratory of Biology and Germplasm Enhancement of Horticultural Crops in South China, Ministry of Agriculture, College of Horticulture, South China Agricultural University, Guangzhou, China, [2] College of Ying-Tong Agricultural Science and Engineering, Shaoguan University, Shaoguan, China

Chilling stress can cause cellular DNA damage, affecting the faithful transmission of genetic information. Cold acclimation enhances chilling tolerance, but it is not clear that the process of cold adaption involves DNA damage responses, as cold acclimation does not form real chilling stress. Here we showed with cucumber fruit that pre-storage cold acclimation (PsCA) reduces chilling injury and upregulates DNA damage inducible protein1 (*CsDDI1*), suggesting that the chilling tolerance induced by cold acclimation involves *CsDDI1* transcription. Application of nitric oxide (NO), abscisic acid (ABA) or H_2O_2 biosynthesis inhibitor before PsCA treatment downregulates *CsDDI1* and aggravates chilling injury, while H_2O_2 generation inhibition plus exogenous NO or ABA application before PsCA treatment restores chilling tolerance, but does not restore *CsDDI1* expression, suggesting H_2O_2 plays a crucial role in triggering cold adaption. *CsDDI1* overexpression *Arabidopsis* lines show faster growth, stronger chilling tolerance, lower reactive oxygen species levels, enhanced catalase and superoxide dismutase activities and higher expression of nine other *Arabidopsis* defense genes under chilling stress, suggesting *CsDDI1* strengthens defenses against chilling stress by enhancing antioxidant defense system. Taken together, *CsDDI1* positively regulates chilling tolerance induced by cold acclimation in cucumber. In addition, H_2O_2 is involved in initiation of cold acclimation. While *CsDDI1* upregulation requires H_2O_2 as a key signaling molecule, the upregulation of *CsDDI1* activates an antioxidant system to reduce biotoxic accumulation of H_2O_2 and helps in DNA repair.

Keywords: DNA damage response, cold acclimation, chilling tolerance, H_2O_2, cucumber fruit

OPEN ACCESS

Edited by:
Ayako N. Sakamoto, National Institutes for Quantum and Radiological Science and Technology, Japan

Reviewed by:
Daisuke Takahashi, Saitama University, Japan
Kentaro Nakaminami, Plant Genomic Network Research Team, Japan

***Correspondence:**
Shijiang Zhu
sjzhu@scau.edu.cn

Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *27 June 2019*
Accepted: *09 December 2019*
Published: *24 January 2020*

Citation:
Wang B, Wang G and Zhu S (2020) DNA Damage Inducible Protein 1 is Involved in Cold Adaption of Harvested Cucumber Fruit. Front. Plant Sci. 10:1723. doi: 10.3389/fpls.2019.01723

INTRODUCTION

Plants have evolved the ability to cope with various environmental stresses to ensure survival and proliferation, including solar UV and ionizing radiation, chemical mutagens, heavy metals, droughts, heat, pathogenic attacks, and chilling. Although different environmental stresses may cause different disorders or symptoms in plants, they can all cause cellular DNA damage (Durrant et al., 2007; Gichner et al., 2008; Ciccia and Elledge, 2010; Achary et al., 2012; Cvjetko et al., 2014;

Roy, 2014; Daghino et al., 2016; Ding et al., 2016; Maric et al., 2017; Shim et al., 2018). DNA damage may result in changes to both the chemical and physical structures of DNA, which can seriously threaten the survival and the faithful transmission of genetic information in plants (Zhang et al., 2015a; Ding et al., 2016). For example, chilling stress leads to DNA damage in root stem cells and their early descendants in *Arabidopsis* (Hong et al., 2017).

The organisms may initiate responses to defend itself against DNA damage. DNA damage-inducible (DDI) proteins are usually related to plant defense responses and play important roles in DNA repair pathways (Maric et al., 2017). *Arabidopsis* DNA damage-inducible protein 1 (AtDDI1) participates in plant defense responses against abiotic stresses such as drought and salt by regulating the expression of defense genes (Ding et al., 2016).

DNA damage binding (DDB) proteins are involved in damage recognition in global genomic repair (GGR), a sub-pathway of nucleotide excision repair (NER) (Shuck et al., 2008). DDB may function to alter chromatin structure and recruit NER factors to DNA damage sites (Gillet and Scharer, 2006). AtDDB1 is involved in DNA damage protection; overexpression of *DDB1A* and *DDB2* increases UV resistance, while *ddb1a* knockout and *AtDDB2* loss of function lead to increased UV sensitivity (Al Khateeb and Schroeder, 2009; Biedermann and Hellmann, 2010).

DNA-damage repair/tolerance (DDR or DRT) proteins are members of the RecA protein family and are involved in RecA-mediated homologous recombination (HR), which may play a role specifically in the repair/reduction of abasic sites and DNA single-strand breaks in plants (Pang et al., 1992; Fujimori et al., 2014). *VvDRT100-L* overexpressing plants remain noticeably healthier under lethal UV radiation, suggesting that VvDRT100-L may enhance plants' tolerance against UV (Fujimori et al., 2014).

One of the most challenging environmental stresses that a plant faces is chilling during its development. Fruits, in particular, either as reproductive organs (Valenzuela et al., 2017) or as commercial products (Wang et al., 2018a) are susceptible to chilling injury because of the large water content in their tissues. Therefore, chilling injury, which is usually caused by suboptimal non-freezing low temperature, is one of the leading factors that affects fruit quality and seed development.

The occurrence of chilling injury is often concomitant with the production of reactive oxygen species (ROS) (Asada, 2006; Zhang et al., 2016a). If the generated ROS are not scavenged in a timely manner, they may cause DNA damage and are one of the primary causes of DNA decay in plants (Roldan-Arjona and Ariza, 2009). On the other hand, ROS have been established as signal molecules in plant defense responses against abiotic stresses (Baxter et al., 2014; Lamotte et al., 2015; Farnese et al., 2016). H_2O_2 is the only stable ROS species in solution and can diffuse across cell membranes, making H_2O_2 a fit signaling molecule (Farnese et al., 2016).

Plants have evolved the ability to acquire chilling and freezing tolerance after being exposed to low non-freezing temperatures (Park et al., 2015; Jiang et al., 2017), a process referred to as cold acclimation (Thomashow, 1999). ROS, nitric oxide (NO) and abscisic acid (ABA) are essential for plant cold acclimation (Gusta et al., 2005; Shapiro, 2005; Zhao et al., 2009; Zhou et al., 2012; Yu et al., 2014). The elevated ROS concentrations result in the activation of nitrate reductase (NR) (Lin et al., 2012; Sewelam et al., 2016). The increased NR-mediated NO production in turn regulates NADPH oxidase activity and antioxidant systems, resulting in reduced H_2O_2 accumulation (Yun et al., 2011; Groß et al., 2013; Sevilla et al., 2015; Begara-Morales et al., 2016). ABA induces production of H_2O_2 and NO, which in turn induce the transcription/translation and activity of antioxidant enzymes (Zhou et al., 2005; Zhang and Wang, 2009). NO and ROS interact each other to regulate ABA biosynthesis and then to modulate stomatal closure (Sewelam et al., 2016). These suggest a subtle interaction among ROS, NO and ABA in the regulation of the defense responses of plants. However, it is unclear whether and how these three signal molecules are involved in the regulation of *CsDDI1* in response to cold stress.

We previously demonstrated that cold acclimation enhances chilling tolerance in cucumber fruit through the activation of antioxidant systems (Wang and Zhu, 2017; Wang et al., 2018b). Using proteomic analysis, we demonstrated that cold acclimation significantly increased CsDDI1 accumulation in cucumber (Wang et al., 2018a). However, it is not clear whether CsDDI1 plays a role in initiating cold acclimation. In the current study, we used physiological, biochemical and genetic approaches to show that cold acclimation significantly upregulates *CsDDI1* expression in a H_2O_2-mediated manner, which in turn upregulates a CsDDI1-activated antioxidant system to reduce biotoxic accumulation of H_2O_2 and to alleviate chilling injury.

MATERIALS AND METHODS

Plant Materials and Treatment

Cucumber (*Cucumis sativus* L. cv Huaqing) fruit harvested at commercial maturity from a farm in Yinan County, Shandong Province, China, were transported to the laboratory within 24 h of harvest. All fruit were selected for uniform size and were free of blemishes, without mechanical damage and disease symptoms. During the years 2013 through 2017, three experiments were conducted, each repeated at least two times. The results presented here were from one set of experiments.

The first experiment was conducted to investigate the effects of pre-storage cold acclimation (PsCA) on chilling tolerance in relation to expression of DNA damage related genes. There were two treatments in this experiment: storage at 5°C (Control) and incubation at 10°C for 3 d followed by storage at 5°C (PsCA).

The second experiment was conducted to investigate the roles of endogenous abscissic acid (ABA) or nitric oxide (NO) in PsCA-induced tolerance and whether *CsDDI1* expression is regulated by endogenous NO and ABA. In this experiment, there were four treatments: Control, PsCA, TS (tungstate, ABA biosynthesis inhibitor)+PsCA, and L-NAME(L-nitro-arginine

methyl ester, NO biosynthesis inhibitor)+PsCA. For the application of the combination treatments, the fruit were first sprayed with TS or L-NAME and then were air-dried at ambient temperature for 3 h before exposure to cold acclimation at 10°C.

The third experiment was conducted to unravel the relationships among H_2O_2, NO and ABA. In this experiment, there were five treatments: Control, PsCA, DPI (diphenylene iodonium, a NADPH oxidase inhibitor)+PsCA, DPI+ABA +PsCA, DPI+SNP (sodium nitroprusside, nitric oxide donor) +PsCA. For application of the combination treatments, the fruit were first sprayed with DPI, incubated in plastic bags for 3 h, and then air-dried at ambient temperature before the next treatment was applied. After the application of all reagents, the fruit were then cold acclimated at 10°C for 3 d.

Concentrations of chemicals used in the above experiments were as follows: TS at 50 μM, L-NAME 100 μM, ABA 100 μM, SNP 10 μM, DPI 10 μM (Zhang et al., 2015b; Liu et al., 2016). The solutions were applied in a fine mist until runoff to 90 fruit per treatment.

Each treatment was replicated three times, each time with 90 fruit. Following treatment, fruit were wrapped with perforated polyethylene film in the dark at 95% RH for 12 d of cold storage. Peel tissues from 3 fruit for each treatment were collected at 0 h (or 0 d, untreated), 3 h, 6 h, 12 h, 24 h, 48 h, 72 h (3 d) and every 2 days afterward. The peel tissues from each sample were then pooled and ground to powder in liquid nitrogen and stored at −80°C. Of the 90 fruit, 30 fruit of each treatment were labeled for observation of chilling injury severity and the rest for sampling.

Evaluation of Chilling Injury, Secondary Disease, and Electrolyte Leakage

Chilling injury and secondary disease symptoms of the fruit surface were evaluated for 30 fruit for each replicate using a subjective scale of visual symptoms described previously (Liu et al., 2016). Chilling injury development was observed during storage at 5°C and secondary disease development was observed at ambient temperature (20°C) following 12 d of cold storage. Chilling injury or secondary disease severity scores range from 0 to 4, where 0 represents no pitting (chilling injury) or decay (secondary disease), 1 represents very slight pitting or decay (25% or less), 2 represents minor pitting or decay (25–50%), 3 represents medium pitting or decay (50-75%) and 4 represents severe pitting or decay (>75%). Chilling injury indices (CII) or secondary disease indices (SDI) were calculated using the following formula: Σ[pitting or decay scales (0–4) × number of corresponding fruit within each category]/total number of fruit evaluated.

Electrolyte leakage (EL) was measured as previously described (Liu et al., 2016). Briefly, the exocarp of cucumber fruit was separated with a vegetable peeler and 20 discs of cucumber peel tissue or *Arabidopsis* leaf were excised with a stainless steel cork borer (5 mm in diameter). The excised samples were rinsed three times with double distilled water before being incubated for 2 h at room temperature (25°C) in 25 ml of double distilled water. After 2 h of incubation, conductivity was measured using a conductance bridge (DDS-307, Leici Electron Instrument Factory, Shanghai). Total conductivity was determined after boiling the flasks for 30 min and cooling to room temperature. The EL was expressed as percentage of total conductivity.

Analysis of Chlorophyll Fluorescence

Recently, maximal quantum yield of PSII (*Fv/Fm*) has been widely used to reflect chilling severity in harvested vegetables (Wang and Zhu, 2017; Fan et al., 2018; Tan et al., 2018). In the current study, *Fv/Fm* was measured using an imaging pulse amplitude modulated fluorometer (IMAG-K7, Walz, Germany) (Wang et al., 2018b). *Arabidopsis* plants were dark-adapted for 30 min to ensure sufficient opening of the reaction center before measurement. Minimal fluorescence (Fo) was measured during the weak measuring pulses and maximal fluorescence (Fm) was measured by a 0.8-s pulse light, and images for chlorophyll fluorescence were taken at the same time. *Fv/Fm* was calculated using the equation: *Fv/Fm* = (Fm − Fo)/Fm.

RNA Extraction and Gene Expression Analysis

Total RNA from cucumber peels or *Arabidopsis* plants were extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions (Zhang et al., 2016b). The concentration and quality of total RNA was determined by spectrophotometry and visualized using 1.1% agarose gel. Genomic DNA was digested by RNase-free DNaseI (Promega, USA) and the RNA remaining in the sample was then used to synthesize first-strand complementary DNA (cDNA). The cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's instructions (Liu et al., 2016).

Quantitative real time PCR (qRT-PCR) was carried out using the SYBR Green PCR Master Mix (Bio-Rad, USA) as described previously (Wang and Zhu, 2017). The PCR reactions were performed by initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 30 s, using CFX96-Optics Module Real-Time PCR apparatus (Bio-Rad, USA). Expression values were normalized using cucumber *Actin* (*CsActin,* accession no. AB698859) or *Arabidopsis Actin* (*AtActin,* AGI code: AT3G12110). The relative expression levels of target genes were calculated using the formula ($2^{-\triangle\triangle Ct}$) after normalization (Livak and Schmittgen, 2001). The specific primers (**Table S1**) were designed according to cDNA sequences using the Primer-BLAST tool of NCBI (National Center for Biotechnology Information) database.

Isolation and Bioinformatics Analysis of *CsDDI1*

The Open Reading Frame (ORF) of *CsDDI1* was obtained from the Cucumber Genomic database (http://cucurbitgenomics.org/). cDNA from cucumber fruit peels was used as template for amplifying the full length of *CsDDI1*. The specific primers (forward, F1; reverse, F2) used for PCR amplification are listed in **Table S1**. Conditions for PCR amplification were as follows: 35 cycles of 94°C for 0.5 min, 60°C for 0.5 min and 72°C for 1 min, then a final step of 72°C for 10 min.

Gene sequence data was analyzed using the programs provided by BLASTN on the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular weight (MW) and isoelectric point (*pI*) of CsDDI1 were obtained using the ExPASy program (http://www.expasy.org/tools). Multiple alignments of amino acid sequences were analyzed using CLUSTALX (version 2.0) and mapped with the program DNAMAN (version 6.0). A phylogenetic tree of DDI1 from five plant species was constructed using the Neighbor-Joining (NJ) method in the MEGA5 program.

Subcellular Localization of CsDDI1 Protein

The full-length CDS without the stop codon of *CsDDI1* was amplified by RT-PCR and was ligated into the C terminus of the green fluorescent protein (GFP) of a transient expression vector (*pCAMBIA2300-GFP*) between *Kpn I* and *Spe I* sites, driven by a cauliflower mosaic virus (CaMV) 35S promoter. The specific primers (F3 and F4) are listed in **Table S1**. The fusion constructs and control vector were electroporated into *Agrobacterium tumefaciens* strain GV3101 using Gene PulserXcellTM Electroporation Systems (Bio-Rad, USA) and then transformed into tobacco (*Nicotiana benthamiana*) leaf using the infiltration method. GFP fluorescence in tobacco leaf was observed 48 h after transfection using a fluorescence microscope (Zeiss Axioskop 2 Plus) (Wang et al., 2018a).

Over-expression of CsDDI1 in *Arabidopsis*

To generate *35S::pCAMBIA 2300-CsDDI1* transgenic *Arabidopsis* plants, the coding sequence was subcloned into *pCAMBIA 2300* between the *Kpn I* and *Spe I* sites using T4 ligase, fused with 35S CaMV promoter. The construct *pCAMBIA 2300-CsDDI1* was then electroporated into GV3101 and transformed into *Arabidopsis* using the floral dip method (Zhang et al., 2006). The seeds were harvested and then sown onto MS selection medium containing kanamycin (50 μg/ml) for identification of the transgenic plants using the method as described previously (Zhang et al., 2016b). Two independent *35S::CsDDI1* overexpression lines were obtained. Plants were grown in growth chambers with a photoperiod of 16 h (13,000 lx)/8 h, the light/dark cycle at temperatures of 23/16°C. DNA and total RNA extracted from the kanamycin-resistant transformants of T1, T2 and T3 plants were used as templates to perform PCR using *CsDDI1* gene specific primers (F1 and F2, see **Table S1**). All PCR products were visualized on a 1.1% agarose gel containing 0.05‰ (v/v) gold view (Bio-Rad, USA). T3 homozygous seedlings of two transgenic lines were used for analysis.

Phenotype Analysis of Transgenic *Arabidopsis* Plants

Phenotype analysis was performed as we described previously (Zhang et al., 2016b). Germination assays were carried out on three replicates of 50 seeds. Seeds were sterilized with 75% (v/v) ethanol solution for 1 min and with 2% (v/v) chlorine solution for 10 min, and then rinsed four to five times in sterile distilled water. The sterilized seeds were then sown on MS medium, and the plates were incubated at 4°C for 2 d in the dark before germination and were subsequently grown in a growth chamber at 23°C with 16/8 h light/dark photoperiod. Germination rates were scored at times with one day intervals within 10 d of incubation. Fifteen d-old seedlings grown on MS medium were used to determine root length, hypocotyl length and seedling height. Twenty-eight d-old plants were used to measure rosette leaf number. Flowering required time was recorded from 10 plants from each line when the inflorescence grew to 1 cm. Twenty-two d-old plants were used to determine leaf growth rates during a one week period when plants were incubated under normal (23°C) or chilling (0°C) temperature growth conditions.

Chilling Tolerance Test of Transgenic *Arabidopsis* Plants

To determine chilling tolerance of the transgenic plants, a cold treatment assay was performed as described previously (Wang et al., 2018b). Twenty-two d-old *Arabidopsis* plants from WT and transgenic plants were used to test chilling tolerance. Plants were subjected to chilling stress for 6 days at 0°C with 16/8 h light/dark photoperiod. *Fv/Fm* and EL were measured at one day intervals during chilling condition. The cold treatment experiment was performed in triplicate.

Determination of ROS Accumulation and antioxidant enzyme activities

ROS accumulations and antioxidant enzyme activities in *Arabidopsis* plants were measured following the method described previously (Wang et al., 2018b). The excised *Arabidopsis* leaves were incubated in a 1 mg/ml nitro blue tetrazolium (NBT) solution (pH 3.8) or in a 1 mg/ml diaminobenzidine (DAB) solution (Sigma, Germany) for 8 h in the dark to determine the localization of hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\bullet-}$), respectively. H_2O_2 concentration was assayed by monitoring the absorbance of the titanium-peroxide complex at 415 nm and the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$ at 530 nm. The determined H_2O_2 and $O_2^{\bullet-}$ concentrations were expressed on fresh weight basis as μmol/g FW. Superoxide dismutase (SOD) activity was assayed by measuring the reduction of nitroblue tetrazolium chloride (NBT) at 560 nm. Catalase (CAT) activity was assayed by measuring the initial rate of H_2O_2 decomposition at 240 nm in a reaction with 10 mM H_2O_2. SOD and CAT activities were calculated and expressed on fresh weight basis as U/g FW. The experiments were performed in triplicate.

Statistical Analysis

The experiments were completely randomized designs. All data are presented as the means ± standard error (± *SE*) of at least three replicates. Statistical analyses of two groups were performed by student's t-test and significant differences were

indicated by "**"($P \leq 0.01$) or "*" ($P \leq 0.05$), while statistical comparisons between more than three groups were performed by one-way analysis of variance (ANOVA) and significant differences ($P \leq 0.05$) were indicated by different letters above bars.

RESULTS

Expression of *CsDDI1*, *DDR1*, and *DDB1* Genes in Response to Cold Acclimation

We investigated expression patterns during pre-storage cold acclimation (PsCA) treatment and cold storage for DDI1, DDR1 and DDB1, which are all involved in DNA repair responses (Al Khateeb and Schroeder, 2009; Fujimori et al., 2014; Maric et al., 2017). In fruit exposed to cold stress from the very beginning (the control), *CsDDI1* expression remained almost unchanged until 6 d, while that of the PsCA-treated cucumber significantly increased following 3 d of cold acclimation and kept increasing even after the fruit were placed in chilling stress condition (**Figure 1A**). As for *CsDDR1* and *CsDDB1,* they did not show obvious increased expression in fruit exposed to the control treatment, but were highly up-regulated following 3 d of PsCA treatment. However, the expression decreased dramatically when the fruit were removed to cold stress (**Figures 1B, C**). These results suggest that PsCA triggered a kind of long-term expression for *CsDDI1* in cold stress, but not for *CsDDR1* and *CsDDB1*. Therefore, further experiments were conducted to address the role of *CsDDI1* in chilling tolerance.

Biosynthesis of Both ABA and NO are Involved in Cold Acclimation

Cold acclimation induces endogenous ABA and NO accumulation, which are positively related to chilling tolerance in *Arabidopsis* plants (Cuevas et al., 2008; Zhao et al., 2009). To investigate whether endogenous NO and ABA accumulation is necessary for chilling tolerance induced by cold acclimation, TS (tungstate, an ABA biosynthesis inhibitor) and L-NAME (L-nitro-arginine methyl ester, a nitric oxide biosynthesis inhibitor) were applied to cucumber fruit before exposure to cold acclimation. Compared with the control treatment, pre-storage cold acclimation (PsCA) significantly reduced chilling injury index (CII), electrolyte leakage (EL) and secondary disease index (SDI) (**Figures 2A–C**), suggesting PsCA enhances strong chilling tolerance in cucumber fruit. However, the application of TS or L-NAME significantly aggravated chilling injury, as was reflected by increased CII, EL and SDI relative to PsCA treatment (**Figures 2A–C**). This strongly indicates that PsCA-induced chilling tolerance involves biosynthesis of endogenous ABA and NO.

Measuring the expression levels of *CsDDI1* after these four treatments confirmed that *CsDDI1* is expressed at significantly higher levels after PsCA than the control treatment, in agreement with the results in **Figure 1A**. However, compared with PsCA treatment, application of L-NAME or TS before cold acclimation reduced *CsDDI1* expression for up to 9 d of exposure to cold stress (**Figure 2D**), suggesting that ABA and NO are involved regulating expression of *CsDDI1*.

H_2O_2 Plays Crucial Roles in Initiating Cold Acclimation

H_2O_2 is considered a central signaling molecule in plant responses to biotic and abiotic stresses (Yoshioka et al., 2003; Xia et al., 2009). As H_2O_2 generated by NADPH oxidase is involved rapid systemic signaling associated with responses to abiotic stresses (Miller et al., 2009; Ben Rejeb et al., 2015), to check the role of endogenous H_2O_2 generated at the early stage of cold acclimation, DPI (diphenylene iodonium), an NADPH oxidase inhibitor (Cross and Jones, 1986), was applied before cold acclimation. CII, EL, and SDI of DPI+PsCA treatment were not obviously lower than those of the control, but significantly

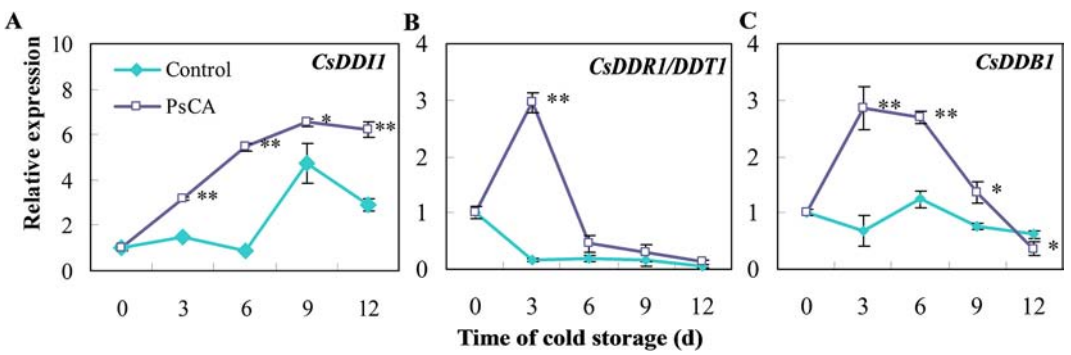

FIGURE 1 | Effects of pre-storage cold acclimation (PsCA) on relative expression of three DNA damage- or repair-related genes in cold-stored cucumber. **(A)**, relative expression of *CsDDI1*; **(B)**, relative expression of *CsDDR1/DDT1*; **(C)**, relative expression of *CsDDB1*. Fruit were either directly placed at 5°C (Control) or were first incubated at 10°C for 3 d and then stored at 5°C (PsCA). The relative expression was evaluated by quantitative real-time PCR (qRT-PCR) using gene-specific primers (**Table S1**) and the expression data were all normalized to 100% (1.0) at 0 d of the control. Significant differences between the control and PsCA are indicated by "**"($P \leq 0.01$) or "*" ($P \leq 0.05$). Data are presented as means ± standard errors (± *SE*) (n = 3).

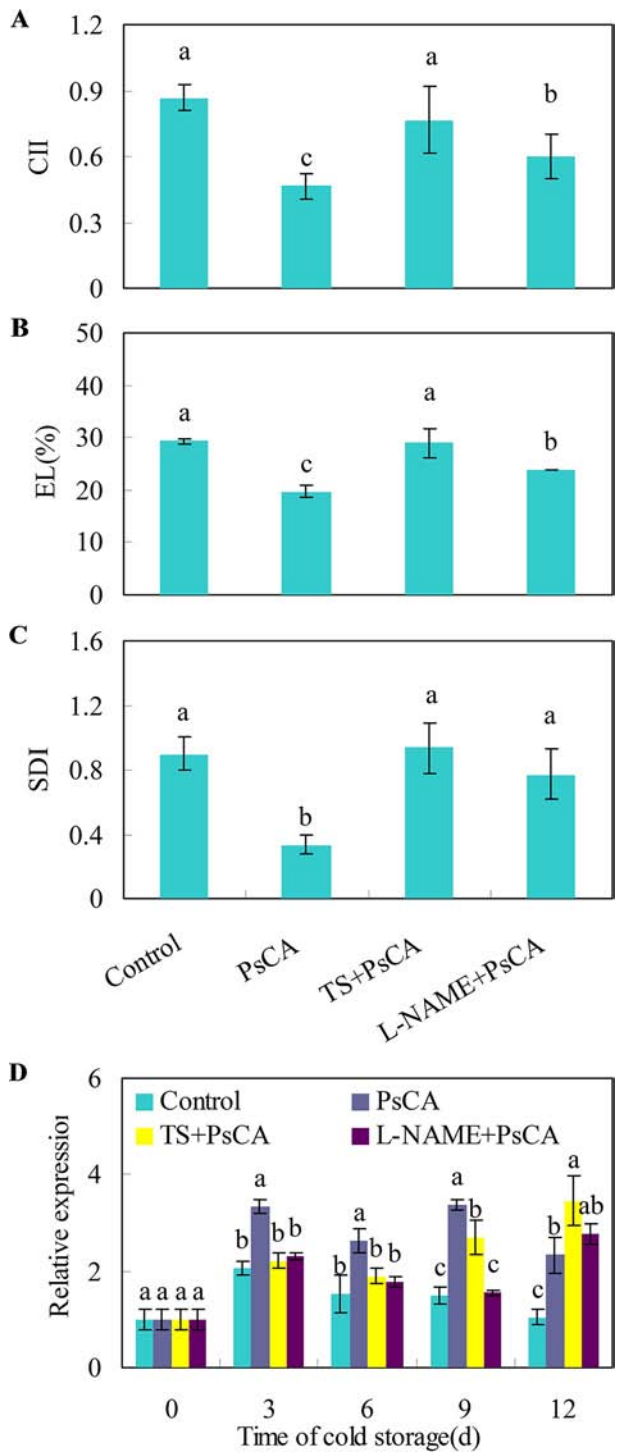

FIGURE 2 | Continued

FIGURE 2 | Effects of L-NAME and TS on chilling tolerance and *CsDDI1* expression as affected by PsCA in cold-stored cucumber. For the control treatment, fruit were directly placed at 5°C. For PsCA treatment, fruit were first incubated at 10°C for 3 d and then stored at 5°C. For the application of the combination treatments, the fruit were first sprayed with TS at 50 μM or L-NAME at 100 μM and then air-dried at ambient temperature for 3 h before exposure to cold acclimation at 10°C for 3d. Following cold acclimation, the fruit were then placed 5°C for 12 d. Chilling injury indices (CII) **(A)** and electrolyte leakage (EL) **(B)** were evaluated after storage at 5°C for 12 d. Secondary disease indices (SDI) **(C)** were evaluated after the cucumbers were transferred to 20°C following 12 d of storage at 5°C. The relative expression of *CsDDI1* **(D)** was evaluated by quantitative real-time PCR (qRT-PCR) using specific primers (**Table S1**) and the expression data were all normalized to 100% (1.0) at 0 d of the control. L-NAME, L-nitro-arginine methyl ester, nitric oxide biosynthesis inhibitor, TS, tungstate, ABA biosynthesis inhibitor. Significant differences between the control and treatments are indicated by different letters above each bar ($P \leq 0.05$). Data are presented as means ± standard errors (± *SE*) (n = 3).

higher than those of PsCA treatment alone (**Figures 3A–C**), suggesting that endogenous H_2O_2 generation is required for initiation of cold acclimation. Measuring the expression levels of *CsDDI1* again confirmed that PsCA enhances expression of *CsDDI1* (**Figure 3D**). Furthermore, it showed that H_2O_2 biosynthesis inhibition before cold acclimation (DPI+PsCA) significantly reduced expression of *CsDDI1* on 3 d, 6 d and 9 d, suggesting that H_2O_2 is involved in cold acclimation.

To further confirm the involvement of H_2O_2 in PsCA-induced defense against chilling stresses, we analyzed the expression of six more defense genes related to chilling resistance: *ASR1*, *GSH-Px*, *Prd-2B*, *SOD(Cu-Zn)*, *L-APX6* and *POD* (Yoshimura et al., 2004; Xia et al., 2017; Wang et al., 2018a). The results showed DPI application before cold acclimation strongly downregulated all the six genes compared to PsCA treatment alone (**Figure S6**). These results suggest that H_2O_2 is necessary during the process of cold acclimation.

To investigate the relationship between H_2O_2, ABA and NO, exogenous ABA or NO was applied following inhibition of endogenous H_2O_2 by DPI. Either ABA or SNP were capable of restoring chilling tolerance compromised by DPI (**Figures 3A–C**). However, *CsDDI1* gene expression was not restored by either compound (**Figure 3D**). In other words, when endogenous H_2O_2 is inadequate, neither ABA nor NO is sufficient to upregulate *CsDDI1* gene. This implies that H_2O_2 plays a crucial role in regulating *CsDDI1* expression and that the role of ABA and NO in regulating *CsDDI1* expression and cold acclimation was independent of H_2O_2, or the function of CsDDI1 might be complemented by other family members of DDI proteins that could be regulated by ABA or NO.

It is noted that TS+PsCA and L-NAME+PsCA treatments in **Figure 2** and DPI+PsCA in **Figure 3** had lower chilling tolerance but higher *CsDDI1* gene expression than PsCA treatment on 12 d. This raises the question about whether upregulation of *CsDDI1* gene during cold acclimation and early during cold stress really contributes to chilling tolerance. To address this question, full-length cDNA of *CsDDI1* was isolated and used to generate *Arabidopsis* lines overexpressing *CsDDI1*.

Bioinformatics Analysis and Localization of *CsDDI1*

Plant DNA damage inducible genes play a critical role in defense responses in a number of different plants. However, few *DDI1* genes have so far been identified in plants. To clone the *CsDDI1* gene from cucumber fruit, the coding sequence of *CsDDI1* was

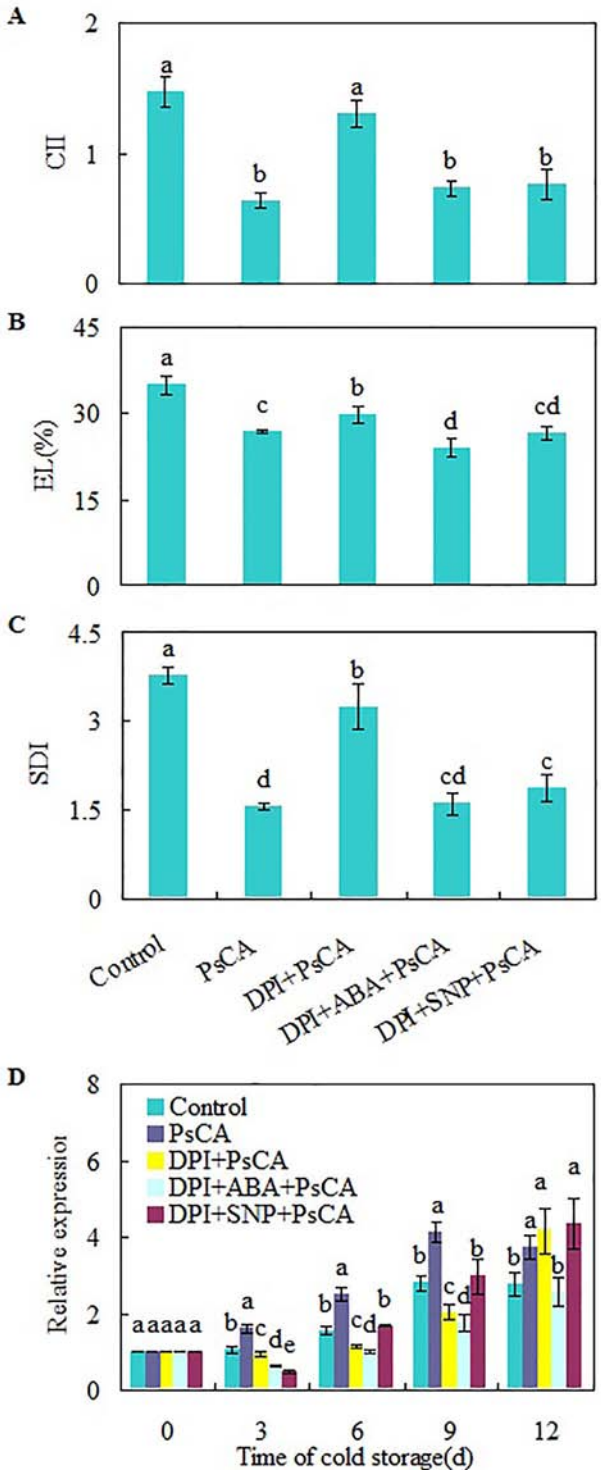

FIGURE 3 | Continued

FIGURE 3 | Regulation of endogenous H_2O_2, NO and ABA on chilling tolerance and *CsDDI1* expression of cold-stored cucumbers. For the control treatment, fruit were directly placed at 5°C. For PsCA treatment, fruit were first incubated at 10°C for 3 d and then stored at 5°C. For the application of the combination treatments, the fruit were first sprayed with DPI, incubated in plastic bags for 3 h, air-dried at ambient temperature before ABA (at 100 μM) or SNP (at 10 μM) was applied. After all reagents were sprayed, then the fruit were cold acclimated at 10°C for 3 d before being finally placed at 5°C for 12 d. Chilling injury indices (CII) **(A)** and electrolyte leakage (EL) **(B)** were evaluated after storage at 5°C for 12 d. Secondary disease indices (SDI) **(C)** were evaluated after the cucumbers were transferred to 20°C following 12 d of storage at 5°C. The relative expression of *CsDDI1* **(D)** was evaluated by quantitative real-time PCR (qRT-PCR) using specific primers (**Table S1**) and the expression data were all normalized to 100% (1.0) at 0 d of the control. DPI, diphenylene iodonium, NADPH oxidase inhibitor; SNP, sodium nitroprusside, nitric oxide (NO) donor. Significant differences between the control and treatments are indicated by letters above each bar ($P \leq 0.05$). Data are presented as means ± standard errors (± *SE*) (n = 3).

obtained from Cucumber Genomic database. The full length cDNA of *CsDDI1* contained an ORF of 1,224 bps coding for 15 exons separated by 14 introns (**Figure S1A**), as validated by PCR amplification and sequencing. The predicted ORF encodes a protein of 407 amino acid residues (**Figure S1B**) with an estimated MW of 44.76 kDa and a *pI* of 4.92 according to the computed *pI*/MW program. Multiple alignments of CsDDI1 and DDI1 proteins from five other plants including melon, *Arabidopsis*, rice, tomato and tobacco show shared sequence identities between 34.3% and 99.3% (**Table S2**) with Cucumber CsDDI1. CsDDI1 contains three conserved domains, UBQ (1–70 aa, ubiquitin homologues), RVP (181–304 aa, RP_DDI: retropepsin-like domain of DNA damage inducible protein) and UBA (369–405 aa, UBA/TS-N domain) (**Figure S1** and **Figure 4**), the typical domains of DDI1 proteins in living organisms (Nowicka et al., 2015).

A phylogenetic tree was reconstructed using the deduced amino acid sequence of CsDDI1 and five other plant DDI1 proteins, revealing they were clustered as one clade and CsDDI1 is most closely related to CmDDI1 from melon (**Figure 5A**). Transient expression of *CsDDI1* in tobacco leaf indicated that CsDDI1 proteins were distributed in the nucleus and cytoplasm of tobacco leaf cells (**Figure 5B**), suggesting that they may function to protect cytoplasmic and nuclear DNA.

Phenotype of Transgenic *Arabidopsis* Plants Overexpressing *CsDDI1*

Two transgenic lines (OE1 and OE2) overexpressing the full-length of *CsDDI1* under the CaMV 35S promoter were generated (**Figures S2** and **S3A**). PCR analysis using DNA from T1 and T2 generations as templates confirmed that the *CsDDI1* gene was successfully transformed into *Arabidopsis* plants (**Figures S3B, C**). Semi-quantitative PCR, using cDNA from T3 generations as templates, confirmed that *CsDDI1* was stably expressed in *Arabidopsis* plant (**Figure S3D**).

The transgenic plants showed higher germination rate on 4 d and 6 d after been sown in the media (**Figure S4A**), higher root length (**Figure S4D**), more rosette leaves (**Figure S4E**) and shorter time required for flowering (**Figure S4F**) than the wild-types. Hypocotyl and seedlings of transgenic plants were longer than those of wild-types, although the differences were not statistically significant. (**Figures S4B, C**). Furthermore, transgenic plants showed faster leaf growth rates compared with wild-type plants (**Figures S4G, H**).

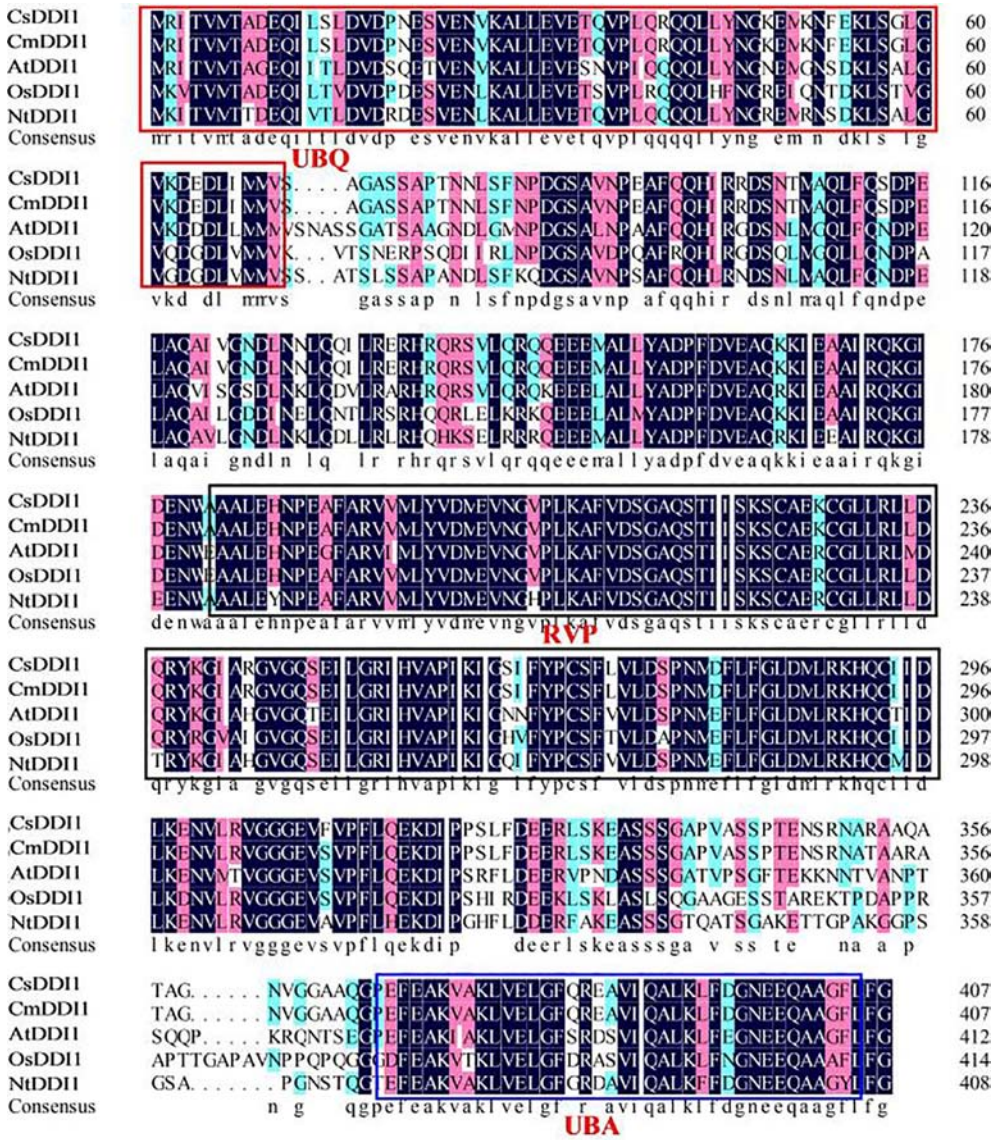

FIGURE 4 | Multiple alignment of the amino acid sequences of CsDDI1s with DDI1s from four other plant species. Species names are abbreviated as follows: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Cs, *Cucumis sativus*; Cm, *cucumis melo*; Nt, *Nicotiana tabacum;* Sl, *Solanum lycopersicum*. Protein names and the corresponding Genbank accession numbers of the proteins are: CsDDI1 (XP_004139767.1), CmDDI1 (XP_008447792.1), AtDDI1 (AT3G13235), OsDDI1 (Os02g0198600), NtDDI1 (XP_016465827.1). The UBQ, RVP and UBA conserved domains are showed in the red, black and blue boxes, respectively.1.

CsDDI1 Overexpression Confers Chilling Tolerance in *Arabidopsis* Plants

Chilling tolerance was assessed for 22 d-old *Arabidopsis* seedlings. When 22 d-old plants were subjected to 0°C for 6 days, the transgenic plants showed stronger chlorophyll fluorescence (**Figure 6A**), higher *Fv/Fm* ratios (**Figure 6B**) and lower EL (**Figure 6C**), than the wild-type plants. Moreover, no leaf expansion was observed in wild-type plants, whereas leaves on the transgenic lines continued to grow under chilling stress (0°C) (**Figures S4G, H**).

CsDDI1 Overexpression Enhanced Antioxidant Capacity in Transgenic *Arabidopsis* Lines under Chilling Stress

$O_2^{\bullet-}$ and H_2O_2 are the major ROS in plants under chilling temperatures (Sharma et al., 2012; Del Río, 2015). Plant superoxide dismutase (SOD) and catalase (CAT) are part of the major ROS scavenging network (Ding et al., 2018). For example, decreases in the activities of SOD and CAT correlates with greater accumulation of $O_2^{\bullet-}$ and H_2O_2 and higher chilling damage in cold-stored cucumber fruit (Wang and Zhu, 2017).

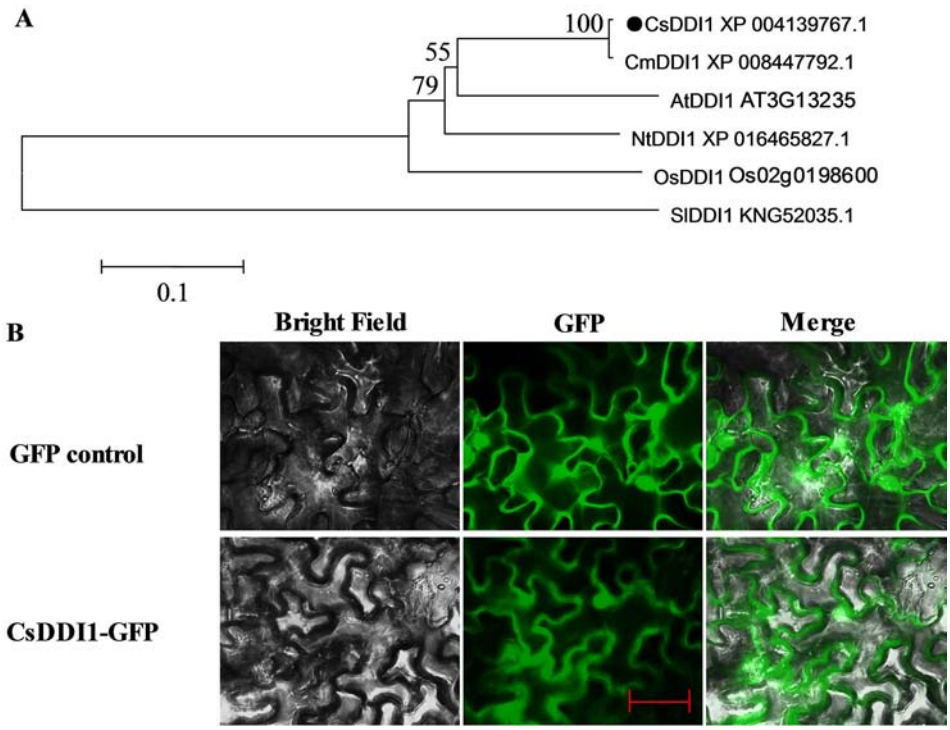

FIGURE 5 | Phylogenetic analysis and subcellular localization of CsDDI1. **(A)** Phylogenetic tree based on comparison between protein sequences of CsDDI1 and DDI1 from five other plant species. The phylogenetic tree was produced using the Neighbor-Joining (NJ) method in the MEGA5 program. Species names are abbreviated as follows: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Cs, *Cucumis sativus*; Cm, *cucumis melo*; Nt, *Nicotiana tabacum;* Sl, *Solanum lycopersicum*. The accession numbers are indicated following protein name. CsDDI1 is marked by a black dot. **(B)** Subcellular localization of CsDDI1 in tobacco leaves. The coding sequence of *CsDDI1* without stop codon was cloned into a transient expression vector (*pCAMBIA2300-GFP*) driven by the CaMV 35S promoter. The fusion constructs and control vector were electroporated into *Agrobacterium tumefaciens* strain GV3101, which were then infiltrated into tobacco (*Nicotiana benthamiana*) leaves. After 72 h of the infiltration, GFP fluorescence was imaged using a fluorescence microscope. The length of the red bar is 50 μm.

Here, we showed that the accumulation of $O_2^{\bullet-}$ and H_2O_2 in leaves of transgenic *Arabidopsis* lines overexpressing *CsDDI1* was reduced compared with the wild-type under chilling temperature (**Figures 7A–D**) and that the gene expression and enzyme activities of CAT and SOD were significantly enhanced in the transgenic plants (**Figures 7E–H**). These indicate that the transgenic plants overexpressing *CsDDI1* had higher antioxidant capacity than the wild-type plantlets.

CsDDI1 Overexpression Enhances Expression of Multiple Defense-Related Genes in *Arabidopsis* Under Chilling Stress

To explore how *CsDDI1* coordinates the regulation of chilling tolerance, the expression of nine genes involved in various defense pathways in response to cold stress were analyzed using qRT-PCR. The overexpression line 1 (OE1) was used for gene expression assay. The expression levels of *AtCOR47*, *AtCOR15b*, *AtPR1*, *AtHSP20*, *AtCML30*, *AtRD29A*, *AtNIA2*, *ATRH9*, and *AtPHR1* did not increase in wild-type plants, but all genes were highly upregulated in transgenic plants exposed to chilling stress (**Figure S5**).

DISCUSSION

Fruits, as reproductive organs, serve to provide the stable internal environment needed for seeds to develop and keep their genetic composition intact, even in adverse external environments. Therefore, the fruits are required to quickly respond to any environmental changes in order to stay physiologically healthy and genetically stable. Harvested fruits continue as living organs and play a critical role in protecting seeds inside (Wang et al., 2018a). Therefore, the fruits must have the capacity to initiate defenses against environmental stresses well before the onset of the real stress. Cold acclimation is a mechanism that can enable a plant or organ to gain tolerance to much more severe low temperature stress. We have previously showed that cold acclimation significantly reduces chilling injury in harvested cucumber fruit exposed to 5°C compared with the control (Wang et al., 2018a) and the results are confirmed in this study with two more independent sets of experiments (**Figures 2** and **3**), suggesting that cold acclimation enabled harvested fruit to adapt to chilling stress before the real chilling approaches.

Cold acclimation is a complex process that includes signal transduction and regulation of transcription (Thomashow, 1999).

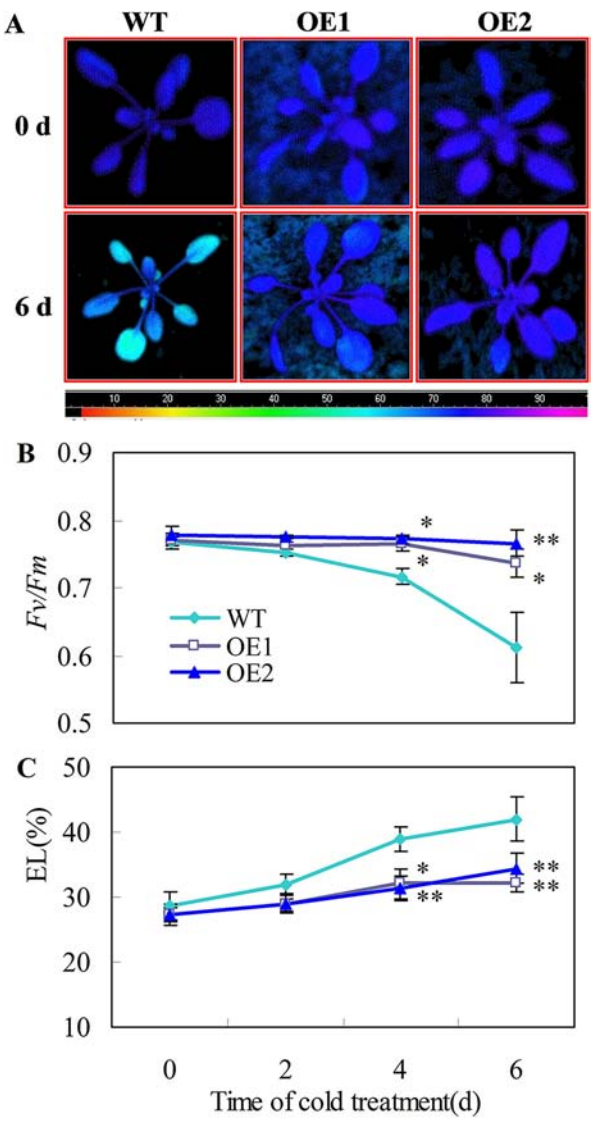

FIGURE 6 | Transgenic *Arabidopsis* plants overexpressing *CsDDI1* display tolerance to chilling stress. **(A)** Images of chlorophyll fluorescence of chilling temperature (0°C)-treated leaves. Photographs show a representative picture of three repeated experiments. The color code depicted at the bottom of the image ranged from 0 (left) to 1.0 (right). **(B)** The maximum PSII quantum yield (*Fv/Fm*) values. **(C)** Electrolyte leakage (EL). WT, wild-type; OE1 and OE2, two transgenic lines over-expressing *CsDDI1*. For evaluating chilling tolerance of transgenic plants, 22-d old plants of the WT and *35S::CsDDI1* transgenic plants (OE1 and OE2) were subjected to 0°C for 6 d. Significant differences between the WT and OE lines are indicated by "**"($P \leq 0.01$) or "*" ($P \leq 0.05$). Data are presented as means ± standard errors (± *SE*) (n = 3).

Studies have been focused on the role of the CBF (CRT/DRE-binding factor) pathway in the acquisition of cold tolerance (Park et al., 2015). However, it remained unclear whether DNA damage inducible proteins are involved in cold acclimation, as cold acclimation is normally initiated at critical temperatures which do not cause chilling injury. In this study, *CsDDI1* expression in the control fruit remained largely unchanged until the 6th day in cold stress (**Figure 1A**), but was highly increased in PsCA-treated cucumber following 3 d of cold acclimation (**Figure 1A**) and remained higher than the control during the remaining period in cold stress. Two more lines of evidences largely confirmed this trend (see **Figures 2** and **3**). These suggest that *CsDDI1* is involved in cold acclimation. In this study, the expression patterns of three genes involved in DNA repair pathways were induced by PsCA (**Figure 1**), implying non-chilling-stress temperature activated DNA repair responses. However, it is worth noting that, after cucumber fruit were transferred to chilling stress condition, *CsDDI1* expression continued to increase, while the transfer caused a sudden drop in *CsDDR1/CsDDT1* and *CsDDB1* transcription, which stayed at very low levels during cold stress (**Figures 1B, C**), implying *CsDDI1* could play a role in PsCA-induced chilling tolerance, but *CsDDR1/CsDDT1* and *CsDDB1* might not.

Recognition of cold signals lead to increased biosynthesis and accumulation of H_2O_2, NO and ABA (Cuevas et al., 2008; Zhao et al., 2009; Zhou et al., 2012; Xia et al., 2015), the early generation of which are required to trigger defense responses in plants (Rejeb et al., 2015; Chan et al., 2016; Yao et al., 2017). We conducted two sets of experiments to explore how these signal molecules are involved in regulation of cold acclimation in relation to *CsDDI1* expression. In the first one, inhibition of biosynthesis of either ABA or NO before PsCA treatment significantly aggravated chilling injury of cucumber compared with PsCA treatment alone, suggesting ABA and NO are necessary for initiating cold acclimation of cucumber fruit. In addition, inhibition of endogenous ABA or NO biosynthesis reduced *CsDDI1* transcription except for the last day (**Figure 2**), suggesting ABA or NO is necessary for activating transcription of *CsDDI1*.

In the second experiment, inhibition of H_2O_2 generation before PsCA treatment significantly reduced chilling tolerance and *CsDDI1* gene expression except on the last day relative to PsCA alone (DPI+PsCA vs PsCA, **Figure 3**), suggesting H_2O_2 is required for initiating cold acclimation and for activating *CsDDI1* expression. However, addition of ABA or NO after inhibition of H_2O_2 restores chilling tolerance, but did not restore *CsDDI1* expression levels to that of PsCA alone (DPI +ABA+PsCA and DPI+SNP+PsCA, **Figure 3**). Considering that inhibition of NO or ABA downregulated *CsDDI1* (see TS+PsCA and L-NAME treatments in **Figure 2D**), the results suggest that H_2O_2 is required for NO and ABA to induce *CsDDI1* expression and that chilling tolerance restored by ABA or NO was not dependent on DNA damage response. These results together suggest that H_2O_2 plays a crucial role in activating transcription of *CsDDI1*. It could be that the H_2O_2 induced by cold acclimation may serve as DNA damage signal, as ROS may cause DNA damage in plants (Roldan-Arjona and Ariza, 2009). Therefore, that DPI+ABA+PsCA or DPI+SNP+PsCA treatment did not upregulate *CsDDI1* could be resulted from the fact that DPI quenched the DNA damage signal.

To further study the role of *CsDDI1* in cold acclimation-induced chilling tolerance, we cloned the full-length cDNA and

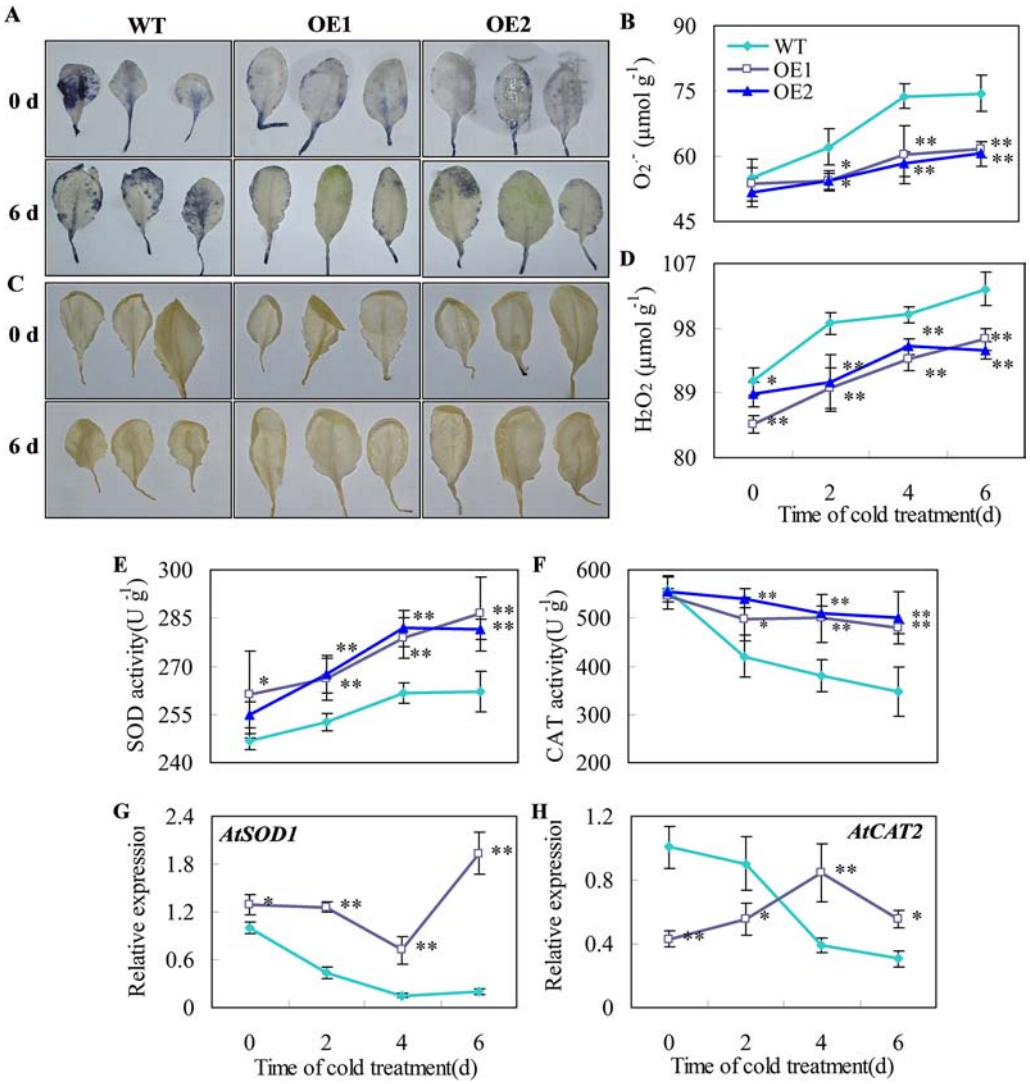

FIGURE 7 | Effects of *CsDDI1* overexpression on $O_2^{\bullet-}$ and H_2O_2 accumulations, and CAT and SOD expression and activities in *Arabidopsis* plants. Twenty-two d old seedlings were subjected to 0°C for 6 days. $O_2^{\cdot-}$ location **(A)** and concentration changes **(B)** in *Arabidopsis* leaves were assayed with nitro blue tetrazolium (NBT). H_2O_2 location **(C)** and concentration changes **(D)** were assayed with diaminobenzidine (DAB). Superoxide dismutase (SOD) activity **(E)** was assayed by measuring the reduction of NBT. Catalase (CAT) activity **(F)** was assayed by measuring the initial rate of H_2O_2 decomposition. Gene expression of *AtSOD1* **(G)** and *AtCAT2* **(H)** were assayed by qRT-PCR. The gene expression data were normalized to 100% (1.0) at 0 d of the wild-type plants. Gene names and the corresponding AGI codes are: *AtSOD1*, AT1G08830 and *AtCAT2*, AT1G20630. WT, wild-type; OE1 and OE2, two transgenic lines overexpressing *CsDDI1*. Significant differences between the WT and OE lines are indicated by "**"($P \leq 0.01$) or "*" ($P \leq 0.05$). Data are presented as means ± standard errors (± *SE*) (n = 3).

generated *Arabidopsis* plant lines overexpressing *CsDDI1*. The deduced CsDDI1 protein contains ubiquitin-like domains at its C and N termini and a retropepsin-like domain (RVP) (**Figure 4** and **Figure S1**), which are typical domains of DDI proteins involved in an ubiquitin-dependent pathway (Nowicka et al., 2015). It has been shown that DDI1 interacts with Ub through the UBA domain (Nowicka et al., 2015). Here we show that CsDDI1 protein was distributed in the nucleus and cytoplasm (**Figure 5**), implying CsDDI1 could play a role in DNA repair, as in yeast and mammals, ubiquitination have been discovered to be involved in DNA repair (Tian and Xie, 2013).

In eukaryotic organisms, ubiquitin is a small 8.5 kDa regulatory protein. Ubiquitination, the addition of ubiquitin to a substrate protein, mainly affects protein stabilization, including protein degradation, cellular location, activity, and interactions (Glickman and Ciechanover, 2002; Mukhopadhyay and Riezman, 2007). Moreover, ubiquitination plays important roles in the regulation of the cell cycle, stress tolerance, phytohormone levels, and cell differentiation (Lyzenga and Stone, 2012; Dametto et al., 2015). Overexpression or knockdown of *DDI1* in tomato plants did not show an aberrant developmental phenotype (Miao et al., 2014), while

transgenic tobacco lines overexpressing a wheat ubiquitin gene (Ta-Ub2) showed earlier germination and enhanced growing (Guo et al., 2008).

In this study, two lines of transgenic *Arabidopsis* plants showed very similar phenotypes. Overexpression of *CsDDI1* in *Arabidopsis* showed earlier germination, faster growth and earlier flowering compared with the wild-type plants (**Figures S4A–F**). This implies that *CsDDI1* has similar function as ubiquitin, which enables the fruits and seeds to get mature faster in chilling stress by enhancing cell cycle.

Overexpression of *Ta-Ub2* in tobacco resulted in high tolerance to drought (Guo et al., 2008) and the deletion of *AtDDI1* increased susceptibility to pathogenic bacteria (Ding et al., 2016). In this study, transgenic *Arabidopsis* lines overexpressing *CsDDI1* showed higher *Fv/Fm* ratios (**Figures 6A, B**), lower EL (**Figure 6C**), and higher leaf growth rate (**Figures S4G, H**). These results, together with the results that PsCA upregulated *CsDDI1*, and TS, L-NAME and DPI downregulated *CsDDI1*, strongly suggest that *CsDDI1* positively regulate chilling tolerance of harvested cucumber fruit.

Antioxidant defense machinery is an important mechanism against abiotic stresses in plants (Asada, 2006; Miller et al., 2010). Accumulation of ROS under abiotic stress is regarded as inducer of DNA damage, such as double strand breaks, base deletion, and base modification (Jahnke et al., 2010; Sharma et al., 2012; Yao et al., 2013). These lead to increased homologous recombination and mutation frequency in plants under stress conditions (Shim et al., 2018). In plants, the major forms of ROS include superoxide radical ion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Mittler, 2002; Sharma et al., 2012; Farnese et al., 2016). The antioxidant enzymes, such as SOD and CAT, are crucial for ROS scavenging and maintenance of cell integrity (Asada, 2006; Xu et al., 2010). The increase in *SOD* and *CAT* gene expression and enzyme activities are usually related to enhanced stress tolerance in plants (Ding et al., 2018). In this study, *Arabidopsis* plants overexpressing *CsDDI1* displayed lower ROS levels (**Figures 7A–D**), and higher *CAT* and *SOD* gene expression and enzyme activities than the wild type plants under chilling stress condition (**Figures 7E–H**). These results indicate that *CsDDI1* overexpression lines have stronger antioxidant capacity and thereby decreased chilling-induced oxidative damage, which may help CsDDI1 play the role in repairing DNA.

It is noted that *AtCAT2* gene expression was not corrected to CAT activities (**Figures 7F, H**), as when CAT activities for both wild type and transgenic plants declines under chilling-stress condition, only did *AtCAT2* gene expression for wild type plants decline, while that for transgenic plants overexpressing *CsDDI1* demonstrated an increasing trend during the first 4 d under the same condition. Two reasons could be proposed for this. Firstly, ectopic expression of *CsDDI1* changed *AtCAT2* expression pattern, which contributed to the higher enzyme activity of CAT (**Figure 7F**). Secondly, different members of catalase gene family complement each other to form sufficient antioxidant capacity at different stages of plant growth or under different abiotic conditions. Therefore, although levels of *AtCAT2* expression was lower in the transgenic than in wild type plants during the first 2 d (**Figure 7H**), other member of CAT family could complement this and contribute to the higher enzymes activity for this period (**Figure 7F**). This implies that apart from *AtCAT2*, other *AtCAT* genes could also be activated by overexpression of *CsDDI1* in *Arabidopsis*. Work remains to be done to further investigate this.

Now the question is: how can CsDDI1 regulate the antioxidant defense system? The answer may lie in two independent lines of evidence. First, *Arabidopsis* overexpressing *CsDDI1* had significantly higher *Arabidopsis PR1* (*AtPR1*) gene expression than wild type following 6d of cold treatment (**Figure S5**). It is well documented that the onset of systemic acquired resistance (SAR) is associated with increased endogenous levels of salicylic acid (SA) (Malamy et al., 1990), and exogenous SA application also induces SAR and PR gene expression (Ward et al., 1991). Therefore, this study implies that *CsDDI1* overexpression *Arabidopsis* plants could be high in SA levels. SA significantly increases the activities of antioxidant enzymes in wheat seedlings (Agarwal et al., 2005). Exogenous SA reduces the excess H_2O_2 and enhances chilling tolerance of cucumber (*C. sativus* L.) seedings (Dong et al., 2014). Therefore, this work implies that DDI1 could activate antioxidant defense system by promoting SA biosynthesis through an as yet unknown mechanism.

In addition, expression of eight other *Arabidopsis* genes (*AtCOR47*, *AtCOR15b*, *AtHSP20*, *AtCML30*, *AtRD29A*, *AtNIA2*, *AtRH9*, and *AtPHR1*) were upregulated in transgenic *Arabidopsis* lines overexpressing *CsDDI1* under chilling stress condition (see **Figure S5**). These genes play roles in various defense pathways including cold acclimation, response to cold stress, ROS, RNA metabolism, and DNA damage repair (Wang et al., 2018b). These results suggest that *CsDDI1* positively regulates multiple defense responses which collaboratively contribute to the enhancement of chilling tolerance.

In conclusion, cold acclimation at 10°C significantly alleviated chilling injury of cucumber fruit stored in chilling stress conditions. There was little change in the expression of *CsDDI1* in the control fruit until severe chilling injury occurred, but expression was significantly upregulated right after cold acclimation. Application of NO, ABA or H_2O_2 inhibitors before exposure to cold acclimation downregulated *CsDDI1* expression and significantly aggravated chilling injury. When H_2O_2 generation was inhibited, the addition of NO or ABA restored chilling tolerance, but did not restore *CsDDI1* expression. These suggest that *CsDDI1* is involved in cold acclimation and that H_2O_2 plays a crucial role in activating transcription of *CsDDI1*. *Arabidopsis* lines overexpressing *CsDDI1* displayed faster growth in 23°C and stronger chilling tolerance in 0°C than wild-type plants. Additionally, they exhibited lower ROS levels and higher CAT and SOD expression and activity than the wild-type plants at 0 °C. Nine *Arabidopsis* genes involved in various defense responses were all upregulated in *Arabidopsis* plants overexpressing *CsDDI1*. These results strongly suggest *CsDDI1* plays a positive role in cold

tolerance induced by cold acclimation in harvested cucumber fruit and that *CsDDI1* enhances the antioxidant system to scavenge ROS when upregulated.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

SZ conceived and oversaw the work. BW performed the experiments and made the tables and figures. SZ, BW and GW wrote the manuscript. All authors have read and approved the manuscript.

ACKNOWLEDGMENTS

This work was supported in part by Joint Project of Guangdong Province Department of Science and Technology and China National Ministry of Science and Technology on Production, Education and Research (Grant number: 2011B090400580). Dr. Jeffrey Zuber from University of Rochester is thanked for critical reading of and grammatical improvement on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01723/full#supplementary-material

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