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Characterization of selected plant nuclear proteins

DNA damage repair in plants with small and large genomes

Doctoral Thesis

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ABSTRACT

Preserving DNA integrity is essential for the organisms' survival and the creation of healthy progeny. To minimize the risk of changes to the genomic information eukaryotes employ a conserved DNA damage response (DDR) system to safeguard genomic stability (Britt, 1996). Plants, being immobile, have developed a highly integrated DDR to protect their genome integrity (Hays, 2002). This research explores DDR systems in plants with small and large genomes. While *Arabidopsis thaliana*, with its small genome and rosette-like interphase chromosome organization, has been extensively studied for its DDR, our understanding of DDR in plants with larger genomes, like cereals, remains limited. Given cereals' major economic importance, expanding DDR research in this field is crucial. In this work, cultivated barley (*Hordeum vulgare*) is used as a model representing plants with large genome and Rabl chromosome organization.

My first goal was to analyze uncharacterized Arabidopsis proteins potentially linked to DDR. The initial identification of four candidate proteins in Arabidopsis relied on the knowledge that DDR proteins often feature BRCT domains, and drew on homology to the BRCT5 domain in animals. Through sensitivity screenings involving DNA-damaging chemicals, one candidate, BCP1, emerged for detailed investigation. It was found that *BCP1* expression is induced by DNA damage and dependent on the DDR master regulator protein SOG1. As a culmination of my study, I provided evidence demonstrating BCP1's involvement in the process of homologous recombination.

To extend knowledge on DDR to barley, I first developed a novel method to assess the sensitivity of barley seedlings to DNA damage. Using this protocol, I examined the response of barley wild type and the *ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED* (*ATR*) mutant plants. The activation of the DNA damage response network was analyzed by conducting transcriptome analysis via RNA sequencing on both wild-type and ATR mutant plants. Additionally, a comprehensive list of potential barley homologs of known Arabidopsis DDR proteins was compiled, including SOG1 and its related transcription factors that aided transcriptomic data analysis.

Keywords: DNA damage response, genetic integrity, *Arabidopsis thaliana, Hordeum vulgare*, barley, cereals, BRCT, homologous recombination, ATR, SOG1, BCP1, transcriptomics

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ABSTRAKT

Organismy neustále čelí hrozbám pro genom, což vedlo k evoluci komplexního systému reakce na poškození DNA (DDR) u eukaryot. Rostliny, které jsou nepohyblivé, vyvinuly vysoce integrovanou DDR k ochraně své genetické integrity. Tato práce zkoumá systémy DDR u rostlin s různou velikostí genomu, včetně malých i velkých genomů. Zatímco *Arabidopsis thaliana* se svým malým genomem a Non-Rabl organizací byla ve výzkumu DDR rozsáhle studována, naše znalosti DDR u rostlin s většími genomy, jako jsou obiloviny, zůstávají omezené. Vzhledem k většímu hospodářskému významu obilovin ve srovnání s Arabidopsis je zásadní rozšířit výzkum DDR v tomto kontextu. Jako model reprezentující rostliny s velkými genomy a Rabl uspořádáním chromozomů využíváme ječmen (*Hordeum vulgare*).

Naším prvním cílem bylo objevit nové proteiny Arabidopsis potenciálně spojené s DDR. S využitím poznatku, že proteiny DDR často obsahují domény BRCT, a na základě homologie s doménou BRCT5 u živočichů jsme nejprve identifikovali čtyři kandidátní proteiny. Na základě screeningu citlivosti pomocí chemických látek poškozujících DNA se objevil jeden kandidát, BCP1, který byl podrobně prozkoumán. Zjistili jsme, že exprese *BCP1* je indukována poškozením DNA v závislosti na aktivaci SOG1. Na závěr naší studie jsme poskytli důkazy prokazující zapojení BCP1 do procesu homologní opravy.

Abychom rozšířili náš výzkum z Arabidopsis na ječmen, vyvinuli jsme nejprve novou metodu hodnocení citlivosti rostlin ječmene na poškození DNA. Pomocí tohoto protokolu jsme zkoumali reakci mutanta *ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED* (*ATR*) v ječmeni. Provedli jsme analýzu transkriptomu pomocí sekvenování RNA u divokého typu i ATR mutantních rostlin ječmene, abychom pochopili aktivaci sítě reakcí na poškození DNA. Kromě toho jsme sestavili komplexní seznam potenciálních homologů ječmene pro známé proteiny DDR z Arabidopsis, což napomohlo analýze transkriptomických dat. Nakonec jsme úspěšně identifikovali hlavní regulátor DDR ječmene, SOG1.

Klíčová slova: *Arabidopsis thaliana*, *Hordeum vulgare*, ječmen, obiloviny, BRCT, homologní rekombinace, ATR, SOG1, BCP1, transkritomika

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CONTENTS

LIST OF ABBREVIATIONS

5-meC	5-methylcytosine
6-4 PPs	6-4 pyrimidine photoproducts
8-oxo-dG	8-oxo-deoxyguanosine
AP	Abasic sites
APLF	Aprataxin-Like Forkhead-associated protein
At	*Arabidopsis thaliana*
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR-interacting protein
BARD1	BRCA1-associated RING domain protein 1
BCP	BRCT5 domain-containing protein
BER	Base excision repair
SP-BER	Short-patch base excision repair
LP-BER	Long-patch base excision repair
BLEO	Bleomycin
bp	Base pair
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BRCT	BRCA1 C-terminal domain
CDC25	Cell division control protein 25
CDK	Cyclin-dependent kinase
CenH3	Centromere histone 3
CHK1	Check-point kinase 1
CHK2	Check-point kinase 2
CKI	Cyclin kinase inhibitor
CPD	Cyclobutane dimers
CPT	Camptothecin
CYC	Cyclin
DDR	Dna damage response
DNA-PK	DNA-dependent protein kinase
DPC	DNA-protein crosslinks
DREAM	DP, RB-like, E2F and MuvB
DSB	Double-strand break
dsDNA	Double-stranded DNA
EM	Electromagnetic
ERCC1	Excision repair cross-complementing 1
Exo I	Exonuclease I
FAD	Flavin adenine dinucleotide
GGR	Global genomic repair
GSTU	Glutathione S-transferase
GUS	β-glucuronidase
H2A.X	Histone 2A variant X
HPLC	High-performance liquid chromatography
HR	Homologous recombination
Hv	*Hordeum vulgare*
IR	Ionizing radiation
KRP6	KIP-related protein 6
LIG	Ligase
MDC1	Mediator of DNA damage checkpoint protein 1
MMC	Mitomycin C
MMR	Mismatch repair
MRE11	Meiotic recombination 11
MRN	Complex MRE11-RAD50-NBS1
MS	Murashige and Skoog medium
MSH	MutS Homologue
MutL	Mutator L
MutS	Mutator S
MYB	Myeloblastosis
NAC	NAM, ATAF1/2, CUC2 domain
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
altNHEJ	Alternative NHEJ
cNHEJ	Canonical NHEJ
NSE	Non-sMC element
O^4-meT	O^4-methyltymine
O^6-meG	O^6-methylguanine
Os	*Oryza sativa*
p53	Tumor protein 53
PARP	Poly [ADP-ribose] Polymerase
PCD	Programmed cell death
PCNA	Proliferating cell nuclear antigen
PHR1	Photoreactivation repair deficient (UVR2)
POL	Polymerase
Pp	*Physcomitrium patens*
PTIP	PAX interacting protein 1
RBR	Retinoblastoma-related
RECQL3	DNA helicase Q1-like
RFC	Replication factor c
RNAP	RNA polymerase
ROS	Reactive oxygen species
RPA	Replication protein A
RT-qPCR	Reverse transcription-quantitative pcr
SDSA	Synthesis-dependent strand annealing
SGL	SOG1-like
SMR	Siamese-Related
SOG1	SUPPRESSOR OF GAMMA RESPONSE 1
Sp	*Schizosaccharomyces pombe*
SSA	Single strand annealing
SSB	Single-strand break
SSBR	Single-strand break repair
ssDNA	Single-stranded DNA
TAD	Topologically associated domain
TCR	Transcription-coupled repair

TDP1/2	Tyrosyl dna phosphodiesterase 1/2
TF	Transcription factor
TOP1/2	Topoisomerase 1/2
TOPBP1	Topoisomerase II binding protein 1
UBQ	Ubiquitin
UGT	UDP-glucosyltransferase
UV	Ultraviolet
UVR2/3	UV-resistance 2/3
XPB	*Xeroderma pigmentosum type B*
XPD	*Xeroderma pigmentosum type D*
XRCC1	X-ray repair cross-complementing protein
ZEB	Zebularine
ZEO	Zeocin
Zm	*Zea mays*

LIST OF FIGURES

LIST OF TABLES

LITERATURE OVERVIEW

Over the course of its life, an organism is exposed to a range of both internal and external factors that have the capacity to damage the DNA. DNA damage could lead to mutations that prove detrimental to the organism's survival and in some cases result in the transmission of inaccurate genetic information to the next generation. Eukaryotic organisms have developed a sophisticated network of pathways dedicated to safeguarding the genome. This intricate system is known as the DNA damage response.

1 INTRODUCTION: IT'S ALIVE

Publication of the DNA structure in 1953 by Watson and Crick, a conclusion bringing together decades of scientific discovery by Miescher, Chargaff, Wilkins, Franklin, and others, painted a picture of a double helix made out of complementary nitrogen bases and antiparallel phosphate-sugar backbones (Miescher, 1897; Chargaff, 1951; Franklin and Gosling, 1953a, 1953b; Watson and Crick, 1953; Wilkins et al., 1953). The massiveness of finally understanding the "secret of life", as Crick once described DNA in a pub, initially overshadowed the possibility of DNA being subject to damage. Interestingly the research on gene mutations was established in the 1930s (Timoféeff-Ressovsky et al., 1935), and only got more prevalent following the Second World War that saw the wide employment of chemical and physical agents. This led to a curious development where one of the ways mutations are induced and repaired was described prior to the discovery of DNA as carrier of genetic information (Dulbecco, 1949; Kelner, 1949). By the 1960s the research into gene mutations and DNA structure collided and resulted in the first descriptions of DNA damage and a mechanism to reverse it (Setlow and Carrier, 1964). With this came the understanding of DNA as a more dynamic molecule, and the rise in the study 3 R's of molecular biology – ***Replication, Recombination and Repair.***

In the following decades, many advances in DNA organization and molecular processes were made. Today we consider the genome as constantly engaged in a balancing act between preservation of the hereditary information and plasticity. Maintaining the genome integrity ensures the organism's survival, as well as the creation of healthy progeny. Meanwhile, numerous endogenous and exogenous factors can, and do, cause changes in the genome. Any change to the DNA molecule is considered damage, while only the damages that permanently change the sequence of the DNA are considered mutations. Mutations can be deleterious and may lower the organism's chance of survival or reproduction. At the same time, mutations are also a source of genetic variation, driving further molecular evolution (Tenaillon and Matic, 2020).

DNA damage occurs surprisingly frequently in every cell. The initial assessment of the average number of spontaneous damage events per human cell was 10^4-10^5 (Lindahl, 1993). Damage to the DNA molecule can be in the form of backbone breaks, chemical modifications or spatial deformation of the molecule. To deal with the damage, organisms evolved a complex network known as DNA damage response (DDR) (Britt, 1996). Activation of the DDR network

results in DNA damage repair, cell cycle halting, cell death and/or endoreduplication (occuring mostly in plants). All of these mechanisms endeavor to stop the cell with damaged DNA from dividing. Depending on the type and extent, DNA damage can either be directly reversed or repaired through one of the several repair mechanisms. It is rare that a damage is tolerated, and left unrepaired. The efficiency of the DDR system is perhaps best demonstrated by a practical example. Even with the high occurrence rate, the average mutation rate in human cells is assessed to be 0.5×10^{-9} per base pair per year (Scally and Durbin, 2012). The DNA damage repair aspect of DDR is of great interest in human research as it pertains to tumor development and aging. For plant species the mechanisms of DDR hold the answer to engineering new agricultural varieties in the face of rising population numbers, lack of resources, and the changing climate.

Plants, more so than other Eukaryotes, face distinct challenges in genome maintenance. Firstly, being sedentary, plants can't relocate in response to unfavorable environments. Secondly, most of the plant development occurs post-embryonically, as plants maintain highly-dividing cell areas that give rise to new organs. In the same line, plant gametes are developed later in the plants' lifecycle, leaving ample time for the plant to acquire mutation during the lifetime that can be transferred to the offspring. Lastly, plant metabolism relies on photosynthesis which produces a large amount of reactive oxygen species (Foyer, 2018). Due to their lifestyle, plants have developed an effective system for DDR that shows a unique integration between molecular players (Hays, 2002).

2 STUDY BACKGROUND: HERE'S LOOKING AT YOU

Most of the research on the topic of plant DDR concerns *Arabidopsis thaliana.* Arabidopsis is a model for most plant genetic research. It has a small genome (1C ≈ 134 Mbp (Hou et al., 2022)), divided into 5 chromosome pairs. However, insights into DDR of plant species with far larger genomes, like that of some crops, are very limited. Barley (*Hordeum vulgare*) is a good model species for plants with large genomes. It is a moderate climate monocot, with the genome size 1C = 5.1 Gbp and seven chromosome pairs.

Genome size differences translate into the organizational differences of the DNA packaged around proteins into chromatin fibers inside the nucleus (Doğan and Liu, 2018). Plant interphase nuclei are observed to have either Type I (also reffered to as Rabl) or Type II (e.g. Non-Rabl, Bouquet, Rosette) (Rabl, 1885; Hoencamp et al., 2021) organization. All processes a part of 3Rs happen in the confines of the chromatin, and chromatin proteins play an active role in them. The high diversity in DNA organization raises the question of whether DDR can unfold in an identical manner in plants with small and large genomes.

To address this question, a deeper understanding of DDR mechanisms in plants is essential. In the introduction of this manuscript, I will provide a comprehensive overview of DDR as the foundation for my research. In the continuation, my objective is to contribute valuable insights by examining DDR proteins and mechanisms in both Arabidopsis and barley.

3 DNA DAMAGE: WELL, NOBODY'S PERFECT

DNA damage is a frequent event in every organism, yet due to the DDR system, it rarely leads to permanent alterations to the genetic information.

3.1 ELEMENTARY: TYPES OF DNA DAMAGE

DNA damage can manifest as mismatched bases, chemical alterations to the nucleotides, physical distortion of the molecule's structure, or the breakage of phosphodiester bonds (Britt, 1996). Nucleotide modification can include covalent modifications to both the bases and sugar moieties. Alteration in the physical structure of DNA distorts the double helix and affects crucial DNA processes. Covalent bonds created between nucleotides either within the same DNA strand (intrastrand) or between opposite strands (interstrand) are both chemical alterations and physical changes. The release of the phosphodiester bond creates the cleavage in the DNA backbone that can encompass one or both strands of DNA. Single-strand breaks (SSB) represent a nick, meaning there was no loss of nucleotides, only the phosphodiester bond was interrupted. Degradation of a series of nucleotides at the place of the break creates a more toxic form of SSBs. Double-strand breaks (DSB) are the most serious damage to the DNA molecule because they disrupt the whole chromosome (van Gent et al., 2001) (**Figure 1**). Phosphodiester bonds in the parallel strands can be cleaved at identical positions, resulting in blunt DNA ends, or they might be separated by a few nucleotides to form staggered ends.

Additionally, I will emphasize here a diverse category of DNA damage known as DNA-protein crosslinks (Hacker et al., 2020). This is a frequently overlooked category that can fall

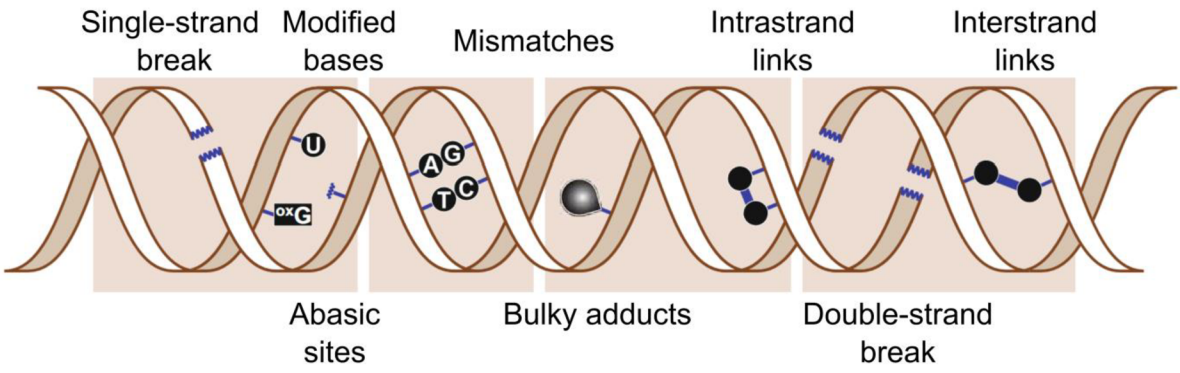

Figure 1: **Types of DNA damage.** Presented is a simplified depiction of various DNA damage types, encompassing chemical modifications (such as the presence of uracil, oxygenated guanine, or complete loss of the base), the addition of bulky adducts, intra- and interstrand DNA links, mismatched pairs, as well as double- and single-strand breaks (adapted from Dexheimer, 2013).

under covalent nucleotide alterations or an obstacle to DNA processes; these crosslinks occasionally arise from DNA breaks.

3.2 ROUND UP ALL THE USUAL SUSPECTS: DNA DAMAGE SOURCES

Sources of DNA damage can be classified as exogenous or endogenous. DNA damage resulting from external environmental factors is categorized as exogenous DNA damage, in contrast to damage originating from a normal cell metabolism, which is referred to as endogenous damage. Within eukaryotes, endogenous damage tends to be a prevailing form of DNA damage.

3.2.1 Exogenous DNA damage

Exogenous DNA damage arises from diverse physical or chemical substances that can interact with the DNA directly or indirectly through the generation of reactive molecules. Lesions can also appear as a consequence of biotic stresses, in which case the damage to the DNA is mostly indirect.

Physical agents

The most common physical source of DNA damage is electromagnetic (**EM**) radiation. Radiation can come from natural sources; the Sun emits natural radiation in the form of infrared, visible, and ultraviolet (UV) radiation, while decaying radionuclides generate gamma rays. Human-made EM radiation, such as radio waves or x-rays, is also common, i.e. as a tool in medicine. However, not all radiation carries enough energy to cause DNA damage (Goodhead, 1989).

Absorption of UV-B or UV-C* (Sutherland and Griffin, 1981) radiation damages DNA directly by creating a covalent bond between neighboring pyrimidines. The generated photoproducts induce a distortion in the double helix's structure. These photoproducts may take the form of cyclobutane dimers (**CPD**s) or two pyrimidines linked through the 6th and 4th ring positions (**6-4 PPs**) (Brash, 1988) (**Figure 2**). Less commonly, the direct impact of UV light on DNA results in DNA-protein cross-links (Peak et al., 1985) or single-strand breaks (Rosenstein and Ducore, 1983).

*UV-C radiation is contained by the ozone layer; UV-A has less energy and causes indirect DNA damage similar to that seen in IR

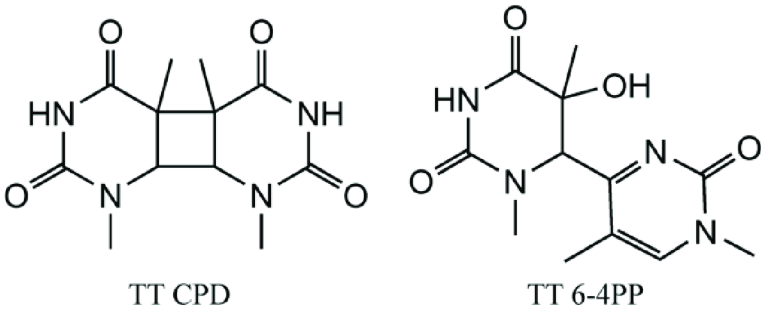

Figure 2: **UV photoproducts.** Chemical structure of CPD (cyclobutane dimers) and 6-4PP (6-4 pyrimidine) photoproducts (source: Molphy, Z., et al. (2015)

Ionizing radiation (**IR**) is a collective term for radiation with enough energy to cause a loss of electrons when interacting with molecules. Direct interaction between IR and DNA produces SSBs or DSBs (Goodhead, 1989). However, IR has a much wider effect spectrum, because it interacts with other molecules in the cell. As they are the most abundant, it is most often the water molecules that are in direct contact with radiation particles. These interactions produce a high number of reactive molecule species, mainly reactive oxygen species that cause oxidative damage to the DNA. There is a broad use of different IR for the induction of DNA breaks, for example, γ-rays are often used in research to induce DSBs.

Although radiation is by far the more prevalent type of physical DNA damage, nanoparticles are in this group as well. The last decade saw a rise in the use of nanoparticles in medicine for drug delivery. They are too large to pass through the membrane of cells but were shown to cause DNA damage indirectly. (Bhabra et al., 2009).

Chemical agents

Chemical agents causing DNA damage can be activation-dependent or independent. Activation-independent agents such as nitrosamines or alkylating agents are reactive and interact with negatively charged DNA or other molecules (Lodish et al., 2000). Activation-dependent chemical agents gain the ability to cause lesions on the DNA after metabolic reactions within cells. The ultimate result of chemical agents is often an addition of bulky products to the DNA bases distorting the DNA molecule (Turesky and Le Marchand, 2011) or DNA crosslinks.

Alkylating agents are a prevalent type of chemical agent causing covalent changes in the DNA. They are present in the environment, often used for medical purposes (Rajski and Williams, 1998; Hecht, 1999), and even exist inside cells (Taverna and Sedgwick, 1996) (see 2.2.2). Based on the mechanism of alkylation we recognize S_N1 and S_N2 types of alkylating agents. S_N1 type reacts with the oxygen atoms in the DNA bases (**Figure 3**), most often resulting in the creation of O^6-methylguanine (O^6-meG) and O^4-methyltymine (O^4-meT)

(Singer and Grunberger, 1983). O^6-meG mispairs with thymine, inducing a base transition mutation in the next replication cycle from G:C to A:T (Loechler et al., 1984). ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU), and N-methyl-N-nitrosourea (MNU).

The S_N2 alkylating agents react with nitrogen atoms of DNA bases. The reaction causes the creation of N^1 -methyladenine and N^3 –methylcytosine (Singer and Grunberger, 1983). In an intact DNA molecule, these nitrogens are protected by hydrogen bonds, but in ssDNA, they are exposed to alkylation (**Figure 3**). Such modification prevents the restoration of the double helix.

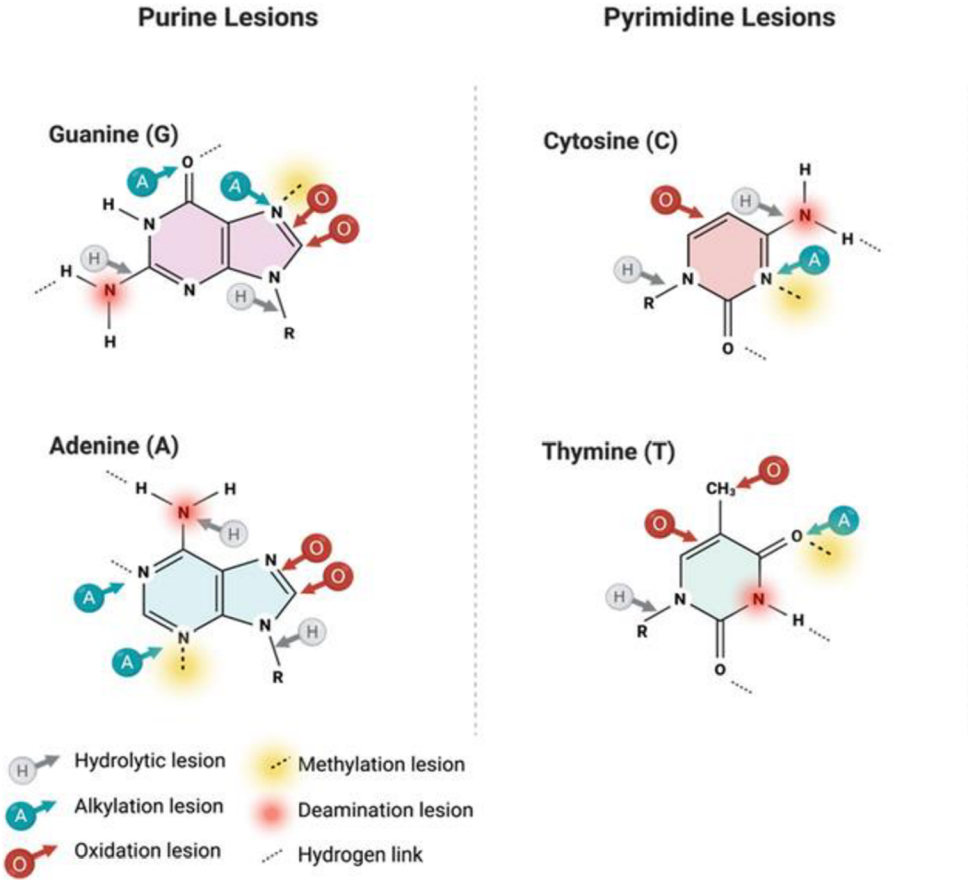

Figure 3: **Sites of DNA chemical modifications**.
Chemical modification of nucleotides caused by endogenous or exogenous agents (adapted from Hindi et al., 2021).

Because of the high mutagenic potential alkylation is swiftly removed by specialized proteins (see 5.1). Some of the better-known alkylating SN1 agents are nitrogen mustards, while SN2 often used in research are methanesulfonate (MMS) and ethyl methanesulfonate (EMS) (Lawley, 1974).

Table 1: **Examples of exogenous physical and chemical DNA-damaging agents**. Included are examples of common DNA damaging agents and the type of damage they can produce. Some of the chemical agents listed are commonly used in DDR research.

Source	Lesion
UV-A	Oxidized bases, AP, SSB
UV-B, UV-C	CPD, 6-4 PP
IR	ROS, SSB, DSB,
ROS	Oxidized bases, AP, SSB
Mustard gas	Alkylated bases
Methanesulfonate	Alkylated bases
Ethyl methanesufonate	Alkylated bases
Cisplatin	Crosslinks
Mitomycin C	Interstrand crosslinks
Bleomycin/Zeocin	DSBs
Camptothecin	DNA-protein crosslink
Zebularine	Base analogue – DNA protein crosslink

3.2.2 Endogenous DNA damage

Endogenous damage arises largely from interaction of DNA with by-products of cell metabolism (Lindahl, 1993). Most commonly DNA reacts with water molecules and/or reactive oxygen species (**ROS**) which results in hydrolysis and oxidation (Nathan, 2003).

Major sources of ROS in plants are mitochondrial respiration and the photosynthetic apparatus. ROS are also used as secondary messengers in signaling pathways responding to growth factors or stress (Foyer, 2018). Most of the metabolic processes that produce ROS take place in separate organelles (mirochondria, plastids, peroxisomes, etc.), precisely to mitigate the risk of ROS interacting with other molecules in the cell. Misregulation of these processes can lead to an overabundance of ROS. Any free radicals remaining in the cell can be removed by a series of anti-oxidative molecules and enzymes.

The most abundant ROS in cells are superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$). Both the sugar moieties and the nitrogen bases can be chemically modified in interaction with ROS (**Figure 3**). Any carbon atom in the 2-deoxyribose can be oxidized, leading to the production of peroxyl radical (Dedon, 2008). In reaction with the bases, ROS usually reduces the double bonds (Cadet et al., 2010). The danger lies in

chemical modifications to bases resulting in future base pair substitutions. It should be noted that oxidative damage is often an indirect effect of exogenous agents.

Oxidation or hydrolysis can affect the bonds in the DNA as well. The N-glycosyl bond between the nitrogen base and sugar is the most vulnerable in the DNA molecule (Lindahl, 1982). Cleavage of this bond leaves sugar moiety in the highly unstable ion state that undergoes hydrolysis creating abasic sites or apurinic/apyrimidinic (**AP**) sites. AP sites are also created as intermediates in certain DNA repair mechanisms. The consequence of AP sites not being repaired could be single-base substitutions in the next round of DNA replication (Shearman and Lawrence, 1979).

In the transient single-stranded state of DNA, the bases are exposed and can suffer hydrolytic damage (Yonekura et al., 2009). The result is a loss of an exocyclic amine in the bases that have it, meaning all bases except thymine are succeptable to this deamination. (**Figure 3**). If left unrepaired this change leads to a substitution of the base pair in the next round of replication.

Besides hydrolysis and oxidation, DNA can also undergo spontaneous methylation (**Figure 3**), when in contact with secondary metabolites such as S-adenosylmethionine (**SAM**), betaine, or choline (Rydberg and Lindahl, 1982; De Bont and van Larebeke, 2004). This type of methylation resembles the alkylation damage induced by exogenous chemical substances (see 2.2.1), and is separate from physiological methylation (Rydberg and Lindahl, 1982). Several enzymes can perform physiological DNA methylation using SAM as a donor of the methyl group during chromatin organization (Holliday and Ho, 1998), but the process described here is non-enzymatic.

Besides DNA damage spontaneously created by cells' reactive molecules, DNA can suffer from mistakes in molecular processes, namely DNA replication (Kunkel, 2009). The use of high-fidelity DNA polymerases during replication, their proofreading ability, as well as specialized repair pathways, all minimize the risk of replication errors. However, single-nucleotide insertions or deletions during replication in eukaryotes still occur at a rate of 10^{-6} to 10^{-8} (Kunkel, 2009). The sites rich in repetitive sequences are especially vulnerable to strand slippage in which DNA polymerase 'loses' its position on the strand (Viguera et al., 2001). Another potential damage can arise from proteins involved in molecular processes covalently binding to the DNA, creating a barrier for further processes (Hacker et al., 2020).

4 DNA DAMAGE RESPONSE: HOUSTON, WE HAVE A PROBLEM

DNA damage response encompasses a multilevel network of interlocked pathways containing numerous factors. Based on their function, the proteins involved in these pathways are marked as sensors, transducers, mediators, or effectors (Yoshiyama et al., 2013b). The ultimate goal of DDR activation is to prevent the cell with compromised genetic information from proliferating. This can be achieved in several ways. Upon recognition of the DNA damage, cells will temporarily halt the progression through the cell cycle. This gives the cells time for the DNA repair machinery to attempt mitigation of the damage. If the damage is too extensive and can't be repaired, DDR signaling may trigger cell death as a last resort. Plants demonstrate an additional faith of cells with damaged DNA, entering endoreduplication (Adachi et al., 2011). Such cells will not enter mitosis but may continue replicating in a process known as endoreduplication.

4.1 AND SO IT BEGINS: INITIATION OF THE DDR

The induction of the DNA damage response relies heavily on two evolutionary conserved phosphoinositide 3-kinase (PI3K)-family kinases: Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia Mutated and Rad3-related (ATR) (Tibbetts and Abraham, 2000). In animals, there is a third kinase from the same family, DNA-dependent protein kinase (DNA-PKcs), that plays a role in DDR signaling (Caron et al., 2015; Blackford and Jackson, 2017), but plants have no known homologue. ATM and ATR initiate the signaling cascade of DDR in all eukaryotes through phosphorylation of target proteins. The prevailing view of their function separates these two kinases into distinct pathways (Abraham, 2001).

4.1.1 ATM pathway activation

ATM is recruited to the DSB sites by the **MRE11-RAD50-NBS1** (**MRN**) complex (Petrini and Stracker, 2003). The MRN complex binds to the lesion site most likely through the recognition of ssDNA/dsDNA junction (**Figure 4**). It has been suggested that it may additionally act like a bridge, keeping the DNA ends in proximity to one another (de Jager et al., 2001). The **NBS1** (Nijmegen Breakage Syndrome 1) protein recruits ATM kinase onto the damaged site (Falck et al., 2005). ATM phosphorylates itself, and all of the components of the MRN complex, further securing the site. Additional research suggested that autophosphorylation of ATM may even occur before its recruitment to DSBs, as a response to damage (Bakkenist and Kastan, 2003).

The role of the MRN complex in the DSB pathway is conserved in plants (Amiard et al., 2010). However, although sensitive to DNA damage, and in the case of *mre11* and *rad50* sterile, mutants of the complex are still viable in Arabidopsis, which is not the case in mammals (Gallego et al., 2001; Bundock and Hooykaas, 2002; Waterworth et al., 2007). Additionally, repair of DSBs in *Arabidopsis* was shown to be possible in the absence of a functioning MRN complex, through the ATR pathway (Amiard et al., 2010).

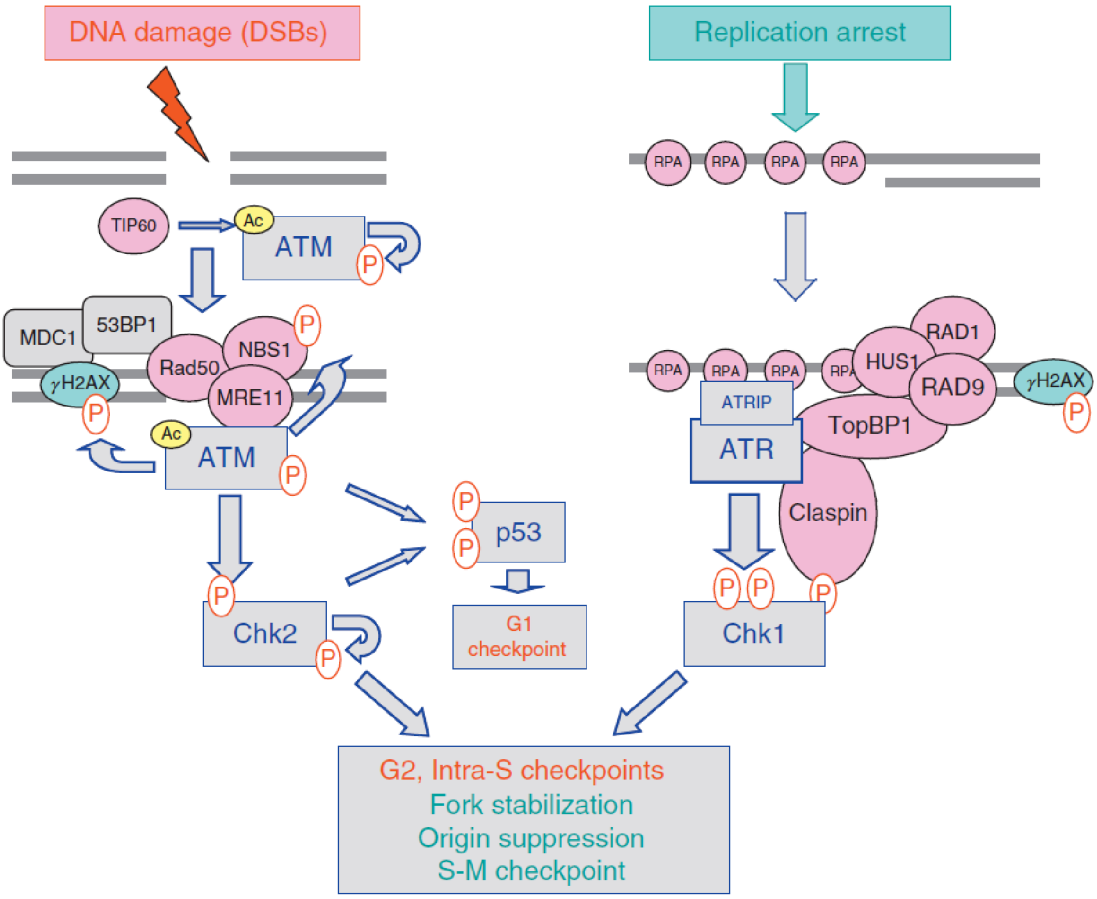

Figure 4: **The ATM and ATR pathways.** Recruitment of the ATR and ATM kinases to DNA-damaged sites. Activation of the Chk1/Chk2 pathways and cell cycle control. **53BP1** (p53 binding protein), **ATM** (Ataxia telangiectasia mutated), **ATR** (Ataxia telangiectasia mutated and Rad3-related), **ATRIP** (ATR-interacting protein), **Chk1/2** (Check-point kinase 1), **HUS1** (Hydroxyurea sensitive 1), **MDC1** (Mediator of DNA damage checkpoint protein 1), **MRE11** (Meiotic recombination 11), **NBS** (Nijmegen breakage syndrome 1), **p53** (tumor protein 53), **P** (phosphate), **RAD1/9/50** (Radiation-sensitive 1/9/50), **RPA** (Replication protein A), **TIP_{60}** (histone acetyltransferase), **TopBP1** (topoisomerase II binding protein) (adapted from Smith et al., 2010)

4.1.2 ATR pathway activation

ATR controls the repair of SSBs and stalled replication forks (**Figure 4**). Upon their formation, these sites are covered by trimeric Replication Protein A (**RPA**) (Wold, 1997). In yeast and human cells, DNA coated by RPA proteins is recognized by a protein accompanying

ATR, ATR-Interacting Protein, **Ddc2/ATRIP** (Zou and Elledge, 2003). This protein contains the same motif as NBS1, suggesting the mechanism of DNA binding by ATM and ATR is the same (Falck et al., 2005). However, while NBS1 binds to the DNA and then recruits ATM, ATR and ATRIP seem to form a complex before arriving to the damage site (Cortez et al., 2001). For ATR to be activated the **RAD9-RAD1-HUS1** (**9-1-1**) complex must co-localize to the lesion. It is loaded to the strand across from the RPA-ssDNA (Majka et al., 2006), after which the clamp encompasses both strands (Thelen et al., 1999). In yeast, the 9-1-1 complex activates ATR directly (Majka et al., 2006), while in human cells protein **TOPBP1** (Topoisomerase II binding protein 1) is needed for activation (Delacroix et al., 2007).

The RPA binding of the SSBs in plants displays greater modality because plants have multiple paralogs of the RPA subunit. In rice (*Oryza sativa*) different subunits are combined into at least two different RPA complexes (Ishibashi et al., 2005). In the case of Arabidopsis, it has been proposed that complexes incorporating various paralogs display selectivity towards ssDNA created during normal genome replication as opposed to those created by DNA damage (Aklilu et al., 2014). Similar to animals and yeast, plants feature an interacting partner for ATR, named **HUS2** (HYDROXYUREA-SENSITIVE 2), but the exact loading of ATR and/or HUS2 onto lesions was not described (Sweeney et al., 2009). Given that plants possess homologues of all the 9-1-1 complex proteins, and that they are implicated in DNA damage response, it is presumed that the activation of the ATR pathway parallels animals (Heitzeberg et al., 2004).

4.1.3 ATM/ATR pathway overlap

Although presented in this way the pathways of ATM and ATR seem separate, there are overlaps in their activity (**Figure 5**), and this overlap may be more pronounced in plants. The mechanism of DSB repair requires the production of single-stranded overhangs that are subsequently coated by the RPA protein. This is a structure similar to that responsible for ATR recruitment. However, while RPA slowly accumulates to this structure, NBS1 binds more quickly, prompting the idea of RPA taking over the repair, in case ATM repair is not present (Shiotani and Zou, 2009). Further, a balance between ATM and ATR at the sites of DSBs is proposed, where the ATM drives the initial response, but the process may be taken over by ATR if the single-stranded overhangs recruit enough proteins (Shiotani and Zou, 2009). The cell cycle stage influence greatly this balance, and the ATM-ATR switch seems to happen only during the S/G2 phases (Jazayeri et al., 2006).

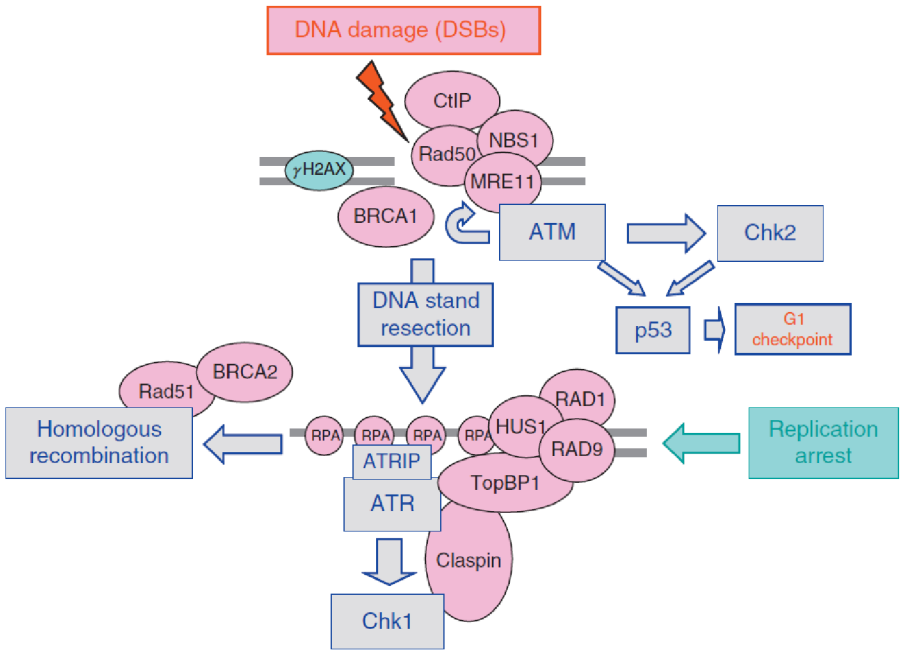

Figure 5: **Communication between ATM and ATR pathway.** Following double-strand breaks, which activate the ATM pathway, DNA ends are processed creating ssDNA overhangs which are substrates for RPA binding. ATR is loaded onto the ssDNA-RPA strand. **53BP1** (p53-binding protein), **ATM** (Ataxia telangiectasia mutated), **ATR** (Ataxia telangiectasia mutated and Rad3-related), **ATRIP** (ATR-interacting protein), **BRCA1/2** (Breast cancer 1/2), **CtIP** (CtBP interacting protein), **Chk1/2** (Check-point kinase 1), **HUS1** (Hydroxyurea sensitive 1), **MRE11** (Meiotic recombination 11), **NBS** (Nijmegen breakage syndrome 1), **p53** (tumor protein 53), **RAD1/9/51** (Radiation-sensitive 1/9/51), **RPA** (Replication protein A), **TopBP1** (Topoisomerase II binding protein) (adapted from Smith et al., 2010)

While their pathways do intersect, the lack of ATM or ATR kinase cannot be fully compensated for by the other. In mammals, mutations in these genes are fatal - *ATR* mutation is embryo-lethal (Brown and Baltimore, 2000), while a mutation in *ATM* results in serious developmental defects (Barlow et al., 1996). In Arabidopsis, aside from partial fertility issues in the *atm* mutant, both *atm* and *atr* plants are viable (Garcia et al., 2003; Culligan et al., 2004). The *atm atr* double mutant is sterile, indicating that in the *atm* mutant, ATR compensates, at least partially, for its role in meiosis (Culligan et al., 2004).

Differences between animal and plant ATM and ATR functions extend to their targets (described in more detail in the following chapters), suggesting a unique regulation of DDR in plants (Roitinger et al., 2015). Moreover, in Arabidopsis, the DDR response involves a greater focus on transcriptional changes prompted by ATM/ATR activation (Culligan et al., 2006). This regulation extends to genes in plants that are counterparts to animal genes, prompting a question of whether these genes play different roles in plants' DDR. Another possibility is that this divergence might simply indicate variations in how the shared elements are activated in

plants compared to animals. Part of this Thesis includes research on the ATR-controlled transcriptomic response to DSBs.

4.2 PASS ON WHAT YOU HAVE LEARNED: TRANSDUCERS AND MEDIATORS

Following the recruitment of ATM and ATR to the damage sites and their activation, they initiate phosphorylation of the target proteins, starting the signaling cascade. The activation of ATM and ATR is a positive feedback loop. Proteins recruited to the DNA damage sites following the start of the cascade reinforce the ATM/ATR activation or recruit additional ATM or ATR proteins (Kozlov et al., 2006). A platform for the recruitment of DDR proteins is created by the ATM/ATR phosphorylation of histone protein H2A.X, creating γH2A.X (Burma et al., 2001; Ward IM and Chen J, 2001; Stucki and Jackson, 2006). Phosphorylation of H2A.X is one of the earliest reactions in DDR and it is highly conserved within Eukaryotes (Redon et al., 2002). Arabidopsis has two copies of H2A.X that are at least partially redundant (Huefner et al., 2009). Besides its role in DDR, this histone functions in other molecular processes as well. There, its function may not be similar between different species. For example, mice deficient in H2A.X have reduced fertility, but this same phenotype is not present in Arabidopsis plants mutated for both *H2A.X* paralogs (Celeste et al., 2002; Huefner et al., 2009).

4.2.1 Cell cycle mediators

Among the first targets of activation by ATR is the **CHK1** (Check-point kinase 1) in animals (**Figures 4** and **5**). CHK1 is assumed to localize close to ATR, with the help of a mediator protein Claspin (Kumagai and Dunphy, 2000), and it disassociates from the lesion following phosphorylation (Smits et al., 2005). CHK1 moves on to phosphorylate a Cell division control protein (**CDC25A-C**). This modification inhibits the CDC25's function, which in turn leaves cyclin-dependent kinases (CDKs) inactivated (Sanchez et al., 1997). Similarly, ATM phosphorylates **CHK2** which also targets CDC25. The primary distinction lies in the specific cell cycle phases at which ATR and ATM halt cell division. ATR – CHK1 signaling prohibits entry into mitosis, while ATR – CHK2 primarily inhibits the progression through S phase. However, the interaction between ATM and ATR at the site of ssDNA overhangs during the repair of DSBs enables the activation of CHK1 during ATM signaling as well (see 3.1.3) (Shiotani and Zou, 2009). The plant homologues of CHK1/CHK2 were not identified as of yet.

4.2.2 DNA damage repair mediators

The next group of proteins activated by the ATR/ATM are the mediators of the DNA damage response. The characteristic of many proteins involved in ATR/ATM signaling pathways is the presence of BRCA1 C-terminal domain (**BRCT**) (Bork et al., 1997). BRCT domains are a group diverse in structure and organization within proteins (Wan et al., 2016). Proteins can contain a single BRCT domain, tandem BRCT domain, multiple tandem domains, or BRCT domain in combination with another functional domain (Leung and Glover, 2011). BRCT domains bind phosphorylated proteins with great specificity towards a particular target. The kinases in the DDR signaling pathway phosphorylate serine/threonine residues, which is a common post-translational modification. The accuracy of interactions between proteins in DDR is driven by the specificity of BRCT domains for the target protein (Leung and Glover, 2011).

BRCT domain was first described as a functional domain of **BRCA1** (Breast cancer gene 1), the well-known human DNA repair gene (Narod and Foulkes, 2004; Trapp et al., 2011). BRCA1 is loaded onto the DNA damage sites through the recognition of γH2A.X. From there BRCA1 is involved in the regulation of cell cycle checkpoints as well as various DNA damage repair mechanisms. Proteins from the same group, such as **BRCA2**, and BRCA1-associated RING Domain protein 1 (**BARD1**), also contain BRCT-domains and function in DDR.

Plants also possess breast cancer-related proteins, although they may not be orthologs to humans. For example, besides BRCT domains, plant BRCA1 and BARD1 also contain a plant-specific domain, the plant homeodomain (**PHD**) (Bienz, 2006). The presence of an additional functional domain indicates a potential difference in function (Trapp et al., 2011). Despite this, both BRCA1 and BARD1 were shown to function in the DDR pathways in Arabidopsis (Lafarge and Montané, 2003; Reidt et al., 2006; Block-Schmidt et al., 2011). Interestingly, most of the proteins involved in the recruitment of BRCA1 to damaged sites in human cells weren't identified in plants. Conversely, human proteins functioning in complex with BRCA1 seem to have their homologues in Arabidopsis (Trapp et al., 2011).

Apart from breast cancer-related proteins many other mediator proteins in animals were shown to contain BRCT domain. Yet, there are gaps in the knowledge of proteins transmitting or amplifying the DDR response. This is even more prominent in plants. I used the presence of a BRCT domain as an identifying characteristic to search for new proteins involved in DDR (see 7. Publication I).

4.3 ONE RING TO RULE THEM ALL: MASTER REGULATOR OF DDR

The main target of the ATM/ATR phosphorylation in animals is the effector tumor suppressor factor **p53**. The level of p53 protein is kept low in the cells under normal conditions by its interaction with **MDM2** (Mouse double minute 2) (Daujat et al., 2001). Upon DNA damage induction p53 is stabilized by phosphorylation through ATM, ATR, CHK1 and CHK2. Phosphorylated p53 guides the damaged cell into appropriate fate, mostly through transcriptional regulation (Helton and Chen, 2007). The massive control exhibited by p53 on the expression of genes involved in DNA damage repair, apoptosis and cell cycle, earned it the monocle of "guardian of the genome".

The choice of p53's target genes is thought to be influenced by its post-translational modifications. For example, additional phosphorylation of the p53's serine 46 was shown to promote the activation of proapoptotic genes (Oda et al., 2000). Besides chemical modifications, p53's interactors also play a big role in the regulation of the cell's fate. Interaction of BRCA1 protein with p53, for instance, helps activation of the DNA repair pathways (MacLachlan et al., 2002). This shows that, although p53 is the central player in the animal DDR response, there are multiple layers of its regulation guiding the process (Oren, 2003).

In plants, the protein acting at the center of DDR is the transcription factor **SOG1** (SUPPRESSOR OF GAMMA RESPONSE 1) (**Figure 6**) (Preuss and Britt, 2003; Yoshiyama et al., 2009). SOG1 coordinates the cell cycle arrest (Chen et al., 2017), DNA damage repair, apoptosis, and endoreduplication (Adachi et al., 2011) by inducing a change in the cell transcriptome. Its function resembles that of the p53, although these two proteins do not share significant sequence similarity (Yoshiyama et al., 2013b; Yoshiyama, 2016).

SOG1 is a part of the **NAC** (NAM, ATAF1/2, and CUC2) - domain family of transcription factors. It is activated via phosphorylation by ATR and/or ATM kinases (Yoshiyama et al., 2013a, 2017; Sjogren et al., 2015). Following the phosphorylation, SOG1 binds to the promoters of specific genes involved in DNA damage response (Bourbousse et al., 2018). SOG1 is responsible for the regulation of over 65% of all proteins involved in the DDR (Bourbousse et al., 2018). Apart from directly controlling the change of expression, SOG1 also regulates several transcription factors (**TF**s), which control the expression of DDR genes. For example, it induces the transcription of **WRKY25**, a TF that in turn inhibits **DEL1** (DP-E2F-

like protein 1, E2FE) transcription which enables cells to enter endocycle (Vlieghe et al., 2005; Bourbousse et al., 2018).

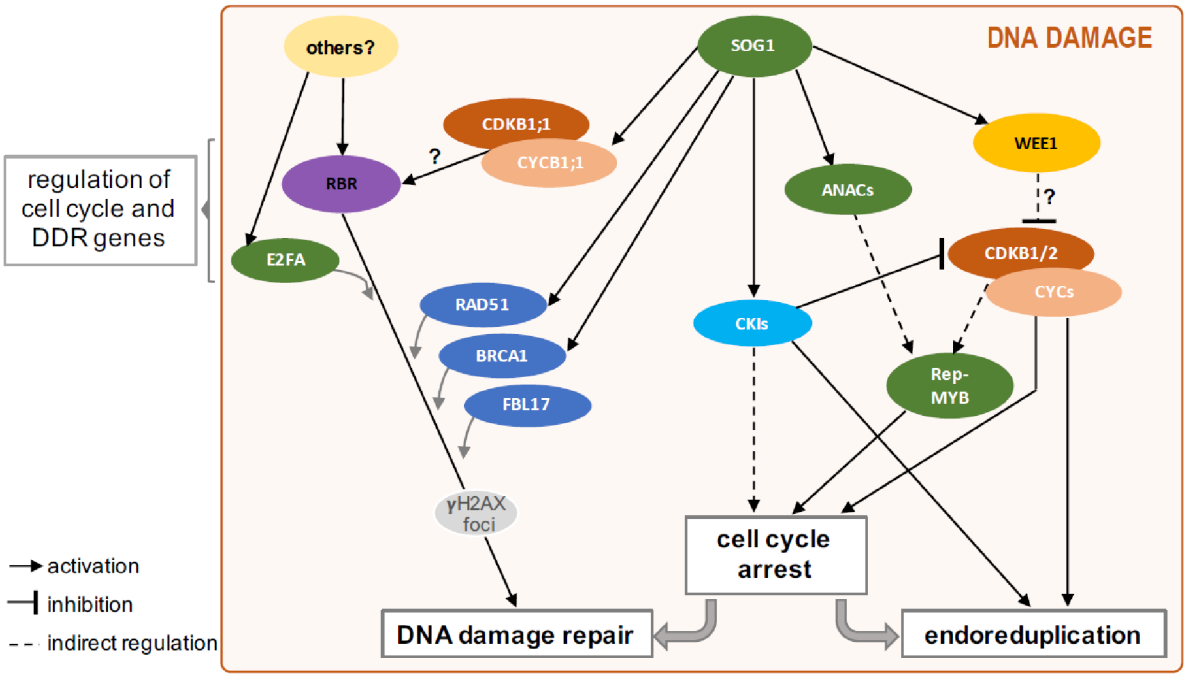

Figure 6: **Cell cycle arrest and endocycle.** Activation of genes involved in DNA damage repair, cell cycle arrest, and endoreduplication and their pathways. **ANAC** (ARABIDOPSIS NAC-DOMAIN PROTEIN), **BRCA1** (BREAST CANCER 1) **CDKB** (CYCLIN-DEPENDENT KINASE B), **CKI** (CYCLIN KINASE INHIBITOR), **CYC** (CYCLIN), E2FA (TRANSCRIPTION FACTOR E2FA), **FBL17** (F BOX-LIKE 17), **RAD51** (RADIATION SENSITIVE 51), **RBR** (RETINOBLASTOMA-RELATED), **SOG1** (SUPPRESSOR OF GAMMA RESPONSE 1) (source Gentric et al., 2021)

4.4 FINAL DESTINATION: EFFECTORS OF DDR

4.4.1 Cell cycle arrest

In animals, cell cycle is transiently inhibited soon after the initiation of DDR through CHK1 and CHK2. As described in chapter 3.2.1, CHK1 and CHK2 activate CDC25s which makes the cyclin (**CYC**)-CDK machinery - the main force behind cell cycle progression - inactive. **WEE1** kinase, which in turn inhibits entry into mitosis is also activated at this early stage of DDR (McGowan and Russell, 1995). The early pause of the cell cycle gives cells enough time to activate the p53 protein which will further down-regulate the expression of genes involved in the cell cycle progression.

Among the genes that are suppressed by p53 are cyclins A and B (Krause et al., 2000; Jackson et al., 2005), cyclin-dependent kinases (Taylor et al., 2001) and cell-division control proteins (Rother et al., 2007). However, apart from its direct impact on gene transcription, p53 also indirectly inhibits the cell cycle through the induction of the inhibitor protein **p21** (Abbas

and Dutta, 2009). Furthermore, the formation of the **DREAM** transcriptional repressor complex, comprising dimerization partner **DP**, retinoblastoma-related **RBR**, **E2F** and **MuvB,** is also triggered by the p21 protein (Quaas et al., 2012; Sadasivam and DeCaprio, 2013; Engeland, 2018).

A similar pattern of cell cycle arrest has been described in plants. However, the only characterized player at this early stage of cell cycle arrest in plants thus far is WEE1. Expression of the *WEE1* gene is induced by SOG1. However, the transcriptional activation of WEE1 happens later in the DDR progression and probably serves to re-affirm the cell cycle arrest. It has been suggested that WEE1 is also located downstream from ATM/ATR which could account for its fast activation under DNA damage (De Schutter et al., 2007).

Parallel to the cell-cycle arrest in animals by p53, SOG1-dependent arrest in plants relies on the inhibition of cyclins and cyclin-dependent kinases. Additional reinforcement of the inactivation of CYC-CDKs is accomplished through increased production of cyclin kinase inhibitors (CKIs). Besides inhibiting CDKs, some plant CKI families, like **SMR** (Siamese-related) or **KRP** (Kip-related protein) promote the entry into the endocycle (Churchman et al., 2006; Yi et al., 2014; Wang et al., 2020). At the same time, the same factors stabilize repressive-MYB TFs, leading to the depletion of the available cell cycle proteins (Kobayashi et al., 2015; Chen et al., 2017; Bourbousse et al., 2018).

Cell cycle control in plants engages also the DREAM complex proteins; however, this pathway is marked as SOG1-independent (Horvath et al., 2017; Lang et al., 2021). In plants the function of this complex in DDR has also been put in connection with the endoreduplication pathway, along with plant-specific CDKB1;1(Boudolf et al., 2004).

4.4.2 Endoreduplication

Endoreduplication is not considered a pathway used for circumventing DNA damage in animals. It was noticed however, that chemotherapeutic treatments for cancers induce a form of endoreduplication or polyploidy, reflected in the creation of giant cells with large nuclei (Storchova and Pellman, 2004) that subsequently go through cell death during division (Castedo et al., 2004). Studies suggest that some tumor cells survive endoreduplication and are the source of higher treatment resistence or even tumor re-occurence (Castedo et al., 2006; Puig et al., 2008; Kuznetsova et al., 2015). This indicates that at least aberrant animal cells are able to trigger endoreduplication as a survival strategy.

In contrast, cells of many plant species seem to easily switch to this developmental pathway during stress (Komaki and Schnittger, 2017). Instead of stopping the replication of the damaged genome, only mitosis is avoided, and cells enter another round of the cell cycle immediately following the G2 phase. It is hypothesized that plants use endoreduplication to avoid losing tissue structure, as there is no free migration of cells to replace the ones destroyed by cell death outside of meristematic tissue (Adachi et al., 2011). As demonstrated above, many of the factors involved in cell cycle arrest also promote endoreduplication (**Figure 7**).

4.4.3 Programmed cell death

The final choice a cell can make, if the damage is so overwhelming, is to go through the programmed cell death (**PCD**). Animals use apoptosis as the PCD type in DDR. Apoptosis is characterized by chromatin condensation, DNA fragmentation, and formation of apoptotic bodies. This respons is largely regulated by p53 and its activation of proapoptotic genes (Pietsch et al., 2008). Apoptosis employs several caspases (cysteinyl aspartate proteinases) to aid the destruction of cells' proteins (Kumar, 2007)

In plants, cell death follows the same morphological pattern as cell death during development. This form of PCD is classified as autolytic, autophagic, or vacuolar PCD since it entails a release of hydrolases from the vacuole and rapid degradation of cytoplasm and organelles. The full volume of the cell is filled with the vacuoles (van Doorn, 2011). The proteins involved in plant PCD have not yet been elucidated, although metacaspases have been put forward as a possible actor in the pathway (Lam and Zhang, 2012). Furthermore, an *in vitro* examination has highlighted the potential existence of a distinct pathway for stress-induced programmed cell death (PCD), supported by transcriptomic data (Olvera-Carrillo et al., 2015; Locato and De Gara, 2018).

However, before PCD, cells will activate the effectors responsible for DNA damage repair. The cells will prioritize repairing the damage first, before resorting to irreversible cell death. The primary focus of my research has largely been on the functions of various proteins in DNA damage repair. For this reason, the next chapter is dedicated to exploring the different types of DNA damage repair mechanisms.

5 DNA DAMAGE REPAIR: CONGRATULATIONS. YOU ARE BEING RESCUED

Cells have developed multiple repair mechanisms to deal with various types of lesions. Direct reversal of damage is possible for only a small number of lesions. Mismatch repair (MMR) corrects mispaired DNA bases, while base excision repair (BER) removes damaged bases. Nucleotide excision repair (NER) deals with more intricate lesions and can result in the removal of 30 base pairs containing the damaged nucleotides. Single-strand break repair (SSBR) manages single-strand breaks (SSBs), while double-strand breaks (DSBs) are processed through non-homologous end joining (NHEJ) or homologous recombination (HR). In some classifications, all the pathways not associated with DSB repair, are marked as SSB repair (Britt, 1996).

5.1 NO HARM DONE: DIRECT REVERSAL OF DNA DAMAGE

A preferred method for repairing DNA damage is direct reversal, as it's error-free and relies on specialized proteins to fix specific damage without cutting the DNA backbone. While convenient, this method is limited to a small number of lesions, such as damage from alkylation or UV radiation (Yi and He, 2013).

Photoproducts created by UV radiation are repaired by enzymes called photolyases after absorption of blue light (Sancar, 2003). Each type of UV-lesion (CPDs, and 6-4 PPs; see 2.2.1) has a designated lyase. The structure and mode of function of the two lyases are similar, the difference is in the chromophore used for the activation of the enzyme (Selby and Sancar, 2012; Kiontke et al., 2014). The photolyase recognizes the site of distorted DNA and binds it. The photoproduct is placed at the core of the enzyme where it can interact with the cofactor FADH. The process of photorepair involves the excitation of the chromatophore and transfer of energy to the FADH followed by the catalytic repair (Zhong, 2015). In Arabidopsis protein **UVR2/PHR1** (UV-RESISTANCE 2) was identified as the photolyase removing CPDs (Ahmad et al., 1997), while **UVR3** (UV-RESISTANCE 3) was suggested to repair 6-4 PPs (Jiang et al., 1997; Nakajima et al., 1998). Besides photolyases plants use NER to repair the photo damage (Molinier, 2017). Photolyases were lost during evolution from mammals' genomes, and they rely solely on NER to repair photoproducts (Lucas-Lledó and Lynch, 2009).

Alkylation of the oxygen residues of the bases like O^6-meG and O^4-meT (Singer and Grunberger, 1983) can be reversed by DNA alkyltransferases that bind an alkyl group to their

cysteine residue (Demple et al., 1985). Modified alkyltransferases are quickly degraded in the cells (Daniels et al., 2004). In contrast to other eukaryotes, plants and fission yeast have no known O^6-alkyltransferase gene (Pegg, 2011). Unlike the alkylation of oxygen that causes mispairing, alkylation of the bases' N-residues inhibits the formation of the base pairs. The mechanisms of repair either rely on transferases similar to the ones previously described, or on dioxigenases performing oxidation of the alkyl group (Mishina and He, 2006).

5.2 IN THE BEGINNING IT IS ALWAYS DARK: SINGLE-STRAND BREAK REPAIR

Single-strand breaks are among the most common lesions. They can block DNA replication machinery, and if left unrepaired potentially lead to formation of double-strand breaks (Kuzminov, 2001). Given the diverse origins of SSBs, the repair pathways can be MMR, NER, or BER.

Poly [ADP-ribose] Polymerase 1 (**PARP1**) binds to SSBs, aided by other members of the PARP family (Amé et al., 2004). At the site of the break, PARP1 initiates the addition of poly(ADP-ribose) chains to itself as well as to other proteins (PARylation). This post-translational modification serves as a mark on histones for chromatin relaxation and as a platform for recruitment of DNA repair proteins (Ray Chaudhuri and Nussenzweig, 2017). PAR tags are also removed rapidly, allowing for dynamic regulation of the process (Fisher et al., 2007). The next protein recruited to the damaged site is the XRCC1 protein (X-ray repair cross-complementing) which stabilizes the lesion site (Caldecott, 2003). The association of PARP1 and XRCC1 is conserved between plants and animals (Doucet-Chabeaud et al., 2001). Following recognition of the damaged site, the ends of the lesion will be processed by nucleases to create 3'- OH and 5'- phosphate ends. The enzymes involved in this step depend on the type of lesion that initiated the process. The size of the gap remaining is determined by the extent of this processing, which can range from a single nucleotide to multiple nucleotides. DNA polymerase fills in the resulting gap, with the specific polymerase (**POL**) employed depending on the repair pathway (Fortini et al., 2000). The final step is the ligation of the loose DNA ends. In human cells, the ligases completing the SSBR are **LIG1** and **LIG3α** (Mortusewicz et al., 2006), a plant homologue of LIG3 has not been identified yet.

5.3 YOU GOT THE WRONG GUY: MISMATCH REPAIR (MMR)

Single base mismatches created during replication, as well as small deletions and insertions, are repaired by DNA Mismatch Repair (**MMR**) (Spampinato et al., 2009). The process begins with the recognition of a mismatch by **MutS** protein dimers (**Figure 8A**). In Eukaryotes MutS Homologue proteins (**MSH**) join in heterodimers MSH2-MSH6 (MutSα), MSH2-MSH3 (MutSβ), or MSH2-MSH7 (MutSγ, found only in plants (Culligan and Hays, 2000)). The heterodimers display specificity towards different substrates (Genschel et al., 1998; Wu et al., 2003). Following their binding to the DNA, MutS dimers interact with the **MutL** complex.

The endonuclease activity of MutLα is necessary for recognition of the DNA strand containing the wrong nucleotide(s) and subsequent dissection of the mismatch. Distinguishing between the accurate nucleotide sequence strand and the erroneous one is accomplished by identifying which strand is newly replicated (Hombauer et al., 2011). Typically, the newly synthesized strand will have the Proliferating Cell Nuclear Antigen (**PCNA**) complex near its 3'-end (Modrich, 2006). As the mistake location is confirmed, a cut to the phosphodiester bond is made on either side of the nucleotide. Exonuclease I (**EXOI**) cuts while RPA guards the single-stranded portion of the molecule. DNA POLδ will fill in the gap left by the excised nucleotides and DNA ligase will re-institute the phosphodiester bond (Modrich, 2006).

In plants, the MMR system seems to be involved in the repair of UV damage as well. *MSH2* and *MSH6* have increased expression under UV-B and their mutants demonstrate higher levels of CPDs (Lario et al., 2011).

5.4 IT BECOMES AN ACT OF LETTING GO: BASE EXCISION REPAIR

The Base Excision Repair (**BER**) pathway is a highly conserved mechanism of DNA repair. It is activated by any modification of DNA bases, or by abasic (AP) sites (Lindahl and Nyberg, 1972; Loeb and Preston, 1986; Memisoglu and Samson, 2000). BER involves five main steps (**Figure 8B**). Initially, the damaged base is removed, forming an AP site. Then, the DNA strand is cleaved near the AP site, and the resulting DNA ends undergo processing. The single-strand break is repaired via DNA synthesis, and the repair is directly dependent on the strand processing extent. Finally, the DNA strand ends are joined. Different organisms may use distinct proteins for each step, but their functions remain consistent.

Damaged bases are removed by **DNA glycosylases**. Proteins in this group cut the glycosyl bond between the nitrogen base and sugar. DNA glycosylases are classified based on their substrate specificity (Jacobs and Schär, 2012). Some glycosylases also serve as lyases, aiding in the subsequent step by cleaving the phosphodiester bond next to the newly formed AP site. Otherwise, an additional enzyme, **AP endonuclease**, cuts the DNA backbone (Demple and Harrison, 1994). The result of the cut is a single-strand DNA break featuring 3'-OH and 5'-phosphate ends, with a single missing nucleotide. In mammal cells, the gap is filled by DNA polymerase β by the addition of the missing nucleotide in short-patch BER (**SP-BER**). Another possibility is the activation of long-patch BER (**LP-BER**) where the DNA ends are additionally processed, and more nucleotides are lost (Dianov et al., 1992; Levin et al., 1997). A gap of 2 to 13 nucleotides is repaired by a complex of POLδ/ε. The process leaves a displaced strand of nucleotides to be removed by **FEN1** endonuclease (Flap endonuclease 1), coupled with PCNA (Hendi et al., 2021). In the end, two single-stranded ends of the DNA are connected with a phosphodiester bond by DNA ligase 1 or 3.

Plants possess homologues of a majority of factors involved in BER found in yeast or mammals, where this pathway was first described (Memisoglu and Samson, 2000; Roldán-Arjona et al., 2019). They also have developed some unique proteins, not found in other organisms such as **DEMETER** DNA-glycosylase or **ROS1** glycosylase/lyase, indicating there are events in which BER is involved that are plant-specific (Choi et al., 2002). Plants don't

possess POLβ and ligase III, but the repair goes through LIG1 and POLλ (Córdoba-Cañero et al., 2009, 2011).

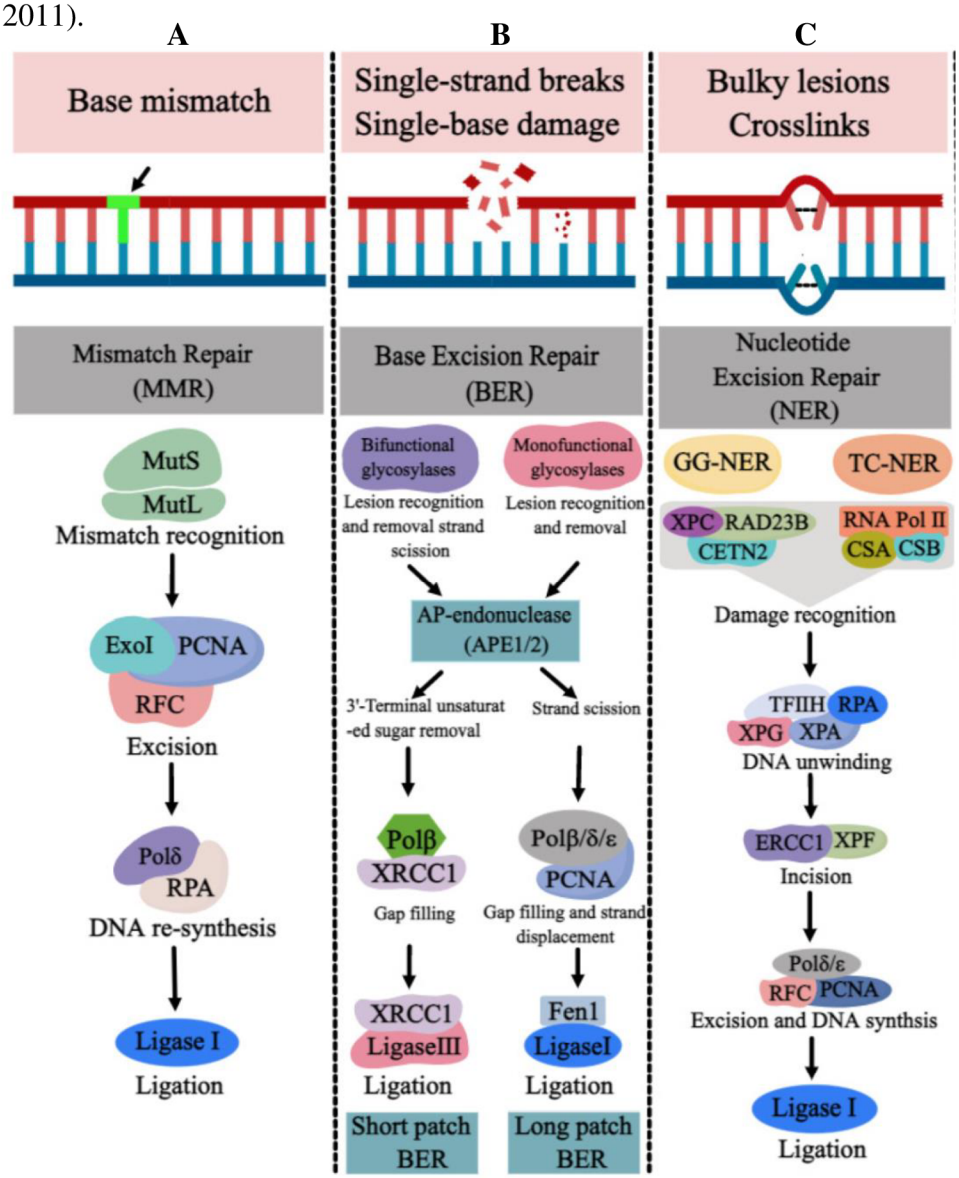

Figure 7: **MMR, BER, and NER pathways.** Proteins and stages of repair in MMR, BER, and NER. **AP** (abasic site), **CSA/B** (Cockayne syndrome A/B), **CETN2** (Centrin2), **ERCC1** (Excision repair cross-complementing 1), **ExoI** (Exonuclease I), **FenI** (Flap endonuclease 1), **MutS/L** (Mutator S/L), **RAD23B** (Radiation sensitive 23B), **RFC** (Replication factor C), **RPA** (Replication protein A), **PCNA** (Proliferating cell nuclear antigen), **Pol** (Polymerase), **TFIIH** (Transcription factor IIH), **XPA/C/G/F** (Xeroderma pigmentosum A/C/G/F), **XRCC1** (X-ray repair cross-complementing protein 1) (adapted from Wang et al., 2023).

5.5 THERE IS ALWAYS SOMETHING YOU CAN DO: NUCLEOTIDE EXCISION REPAIR

In mammalian cells, **NER** eliminates photoproducts in the absence of photolyases, along with similarly toxic lesions. There are two different NER sub-pathways: Global Genomic Repair (**GGR**) and Transcription-Coupled Repair (**TCR**) (Hanawalt, 2002) (**Figure 8C**). As the term implies, GGR occurs extensively throughout chromatin-packed DNA, whereas TCR is selectively triggered when the transcription machinery stalls. The two sub-pathways have several common components, but their difference lies in the initiation, driven by lesion recognition. In animals, GGR is activated when the **XPC** complex (Xeroderma pigmentosum C - RAD23B - Centrin2) detects a physical distortion in the DNA molecule (Lee et al., 2014). TCR initiates upon stalling of RNA polymerase II (**RNAPII**) complex during RNA transcription (Spivak, 2015).

In GGR additional factors are loaded to the damaged strand to make DNA molecule accessible. Meanwhile, additional TCR factors serve to stabilize the already exposed site and RNAPII (Beerens et al., 2005). A multi-protein complex called **TFIIH** is recruited to the site of damage. Within this complex, the ATPase/helicases **XPB** and **XPD** are responsible for unwinding the DNA, around 20 to 30 nucleotides in length. The first cut to the phosphodiester bond is made at the 5'-end by **ERCC1-XPF** (Endonuclease non-catalytic subunit 1) complex. After the first cut DNA replication machinery (with DNA polymerase δ, ε, or κ) starts adding nucleotides complementary to the undamaged strand (Ogi et al., 2010).

In TCR, as the process unfolds, DNA polymerase machinery displaces both the damaged strand and the TFII complex (Spivak, 2015). As replication reaches the end of the single-stranded DNA site, where the strands reform the double helix, the XPG nuclease severs the 3' end of the damaged chain, fully detaching it from the rest of the DNA molecule. The final step involves the formation of the bond between the newly synthesized sequence of nucleotides with the 3'-site, by ligase 1 or 3 (Paul-Konietzko et al., 2015). Again, throughout the process, ssDNA is protected from degradation by RPA proteins, and DNA polymerases and ligases are recruited onto the repair site by the PCNA complex (Ogi et al., 2010).

Many proteins in this process have counterparts in plants (Kunz et al., 2005). However, plants possess active photolyases, and photorepair interacts with NER, MMR, and homologous repair pathways (Molinier et al., 2008). XPC protein homologue in plants is **RAD4**, and as in animals, it interacts with CEN2 at the beginning of GGR (Liang et al., 2006). The components

of plant TFIIH also have different names to that of animals, but are homologues (Grice et al., 2007). Again, as written previously, plants don't have LIG3, so the final ligation is performed by a different ligase.

5.6 ONE PLUS TWO PLUS TWO PLUS ONE: DOUBLE-STRAND BREAK REPAIR

Double-stranded breaks represent the most dangerous form of DNA damage, which may cause a loss of entire chromosomal segments, if they remain unrepaired before the next cell division. Certain double-strand cuts are programmed by cells, such as meiotic crossovers (Borde and de Massy, 2013), whereas others are consequences of genome damage. Cells exercise rigorous control over the repair of double-strand breaks and the associated factors to differentiate these two scenarios. There are two different pathways for DSB repair, the first is the Homologous Recombination (**HR**); and the second is the Non-Homologous End Joining (**NHEJ**). Homologous repair requires a homologous sequence as a template, such as a homologous chromosome or a sister chromatid. This mechanism makes a homologous repair very accurate, unlike NHEJ which functions by bringing the ends of damaged DNA together, processing, and ligating them.

Although less faithful than HR, NHEJ is more frequently utilized in plants for repair (Puchta and Fauser, 2014). The amount of change at the repair site seems to differ between plants with large and small genomes (Kirik et al., 2000; Lloyd et al., 2012), leading to some discussions about the role NHEJ in the evolution of plant genomes (Puchta, 2005). On the other hand, it has also been speculated that HR in plants outside of cells' S phase could potentially be more deleterious considering a large number of repetitive sequences in the plant genome (Manova and Gruszka, 2015).

5.6.1 Homology-based repair

Homologous recombination (**HR**) is the most faithful way of repairing DSBs. However it is also used during the repair of DNA inter- and intra-strand crosslinks, as well as the repair of stalled replication forks (Li and Heyer, 2008). Broadly speaking, the process of HR can be divided into three main phases: presynaptic, synaptic, and postsynaptic. Due to its involvement in both programmed DNA double-strand breaks and DNA damage repair, the process of HR encompasses multiple sub-pathways. These sub-pathways introduce variability in specific proteins involved at each stage, beyond the essential core factors. In this context, an

overview is provided here only for the Synthesis-dependent strand annealing (**SDSA**) and Sigle strand annealing (**SSA**), which are the sub-pathways used for DSB repair in the somatic plant cells (Puchta, 2005).

Synthesis-dependent strand annealing (SDSA)

In the presynaptic stage of SDSA double-strand breaks are processed by endonucleases to create single strand ends that can be used for repair. This process engages the MRN complex recognizing the break and an exonuclease processing the ends to create ssDNA 3'-overhangs (Hartung and Puchta, 1999; Gallego and White, 2001; Akutsu et al., 2007; Krishna et al., 2007). The ssDNA is bound by the RPA proteins for the protection of an otherwise unstable molecule (Wold, 1997). The presynaptic filament is fully assembled when **RAD51** (Radiation sensitive 51) (Sung, 1994) (RecA in prokaryotes) protein binds to the strands. Additional mediator proteins like Rad55 and Rad57 (Sugiyama et al., 1997) in yeast or BRCA2 (Siaud et al., 2004) Arabidopsis cells (Yang et al., 2005), aid with stabilization of the formed DNA-protein strand.

Following the assembly of the presynaptic filament, RAD51, using ATP, catalyzes the search for homologous DNA template (Sung, 1994). Upon successfully identifying the template the ssDNA-RAD51 filament invades the double-stranded molecule causing displacement of one of the strands. The invading strand engages with the remaining template strand forming base pairs in a heteroduplex structure known as **D-loop** (**Figure 9**) (Puchta and Fauser, 2014).

The process of creation of the D-loop marks the synaptic stage of the HR. In the postsynaptic stage, DNA polymerase machinery drives the extension of the invading strand, based on the template sequence. In the resolution of D-loop the newly-synthetized strand detaches from the template strand and re-anneals to its original pair. Synthesis of the other 3'-overhang follows the same pattern as seen in SSBs, with DNA polymerase adding the nucleotides, and ligase sealing the ends (Li and Heyer, 2008). Both 3'-overhangs can invade the same template at the same time, as is the case in meiotic cells. The structure created is called a Holiday junction, and its resolution can lead to the exchange of the genetic material, creating the real recombination (Keeney, 2001).

Sigle strand annealing (SSA)

SSA can be used when the DSB is located between two homologous sites, mostly short repeat sequences. As in HR, the ends of the break are processed creating the 3'-overhangs (**Figure 8**). This resection goes until it encompasses the repeat sequence. Strand annealing is done based on the short sequence homology, guided by **RAD52** protein (Rothenberg et al.,

2008). The 3'-overhangs that don't fit into the created structures are dissected by XPF/ERCC1 endonuclease (Motycka et al., 2004). In plants this is a job of **RAD1/RAD10** heterodimer (Dubest et al., 2002). The single-stranded gaps are filled by DNA polymerase and rejoined into two intact strands (Li and Heyer, 2008).

Compared to HR, this mechanism is less precise and may result in the loss of nucleotides, yet it's significantly more efficient, and thus 5 to 10 times more frequently used in plant double-strand break repair (Orel et al., 2003).

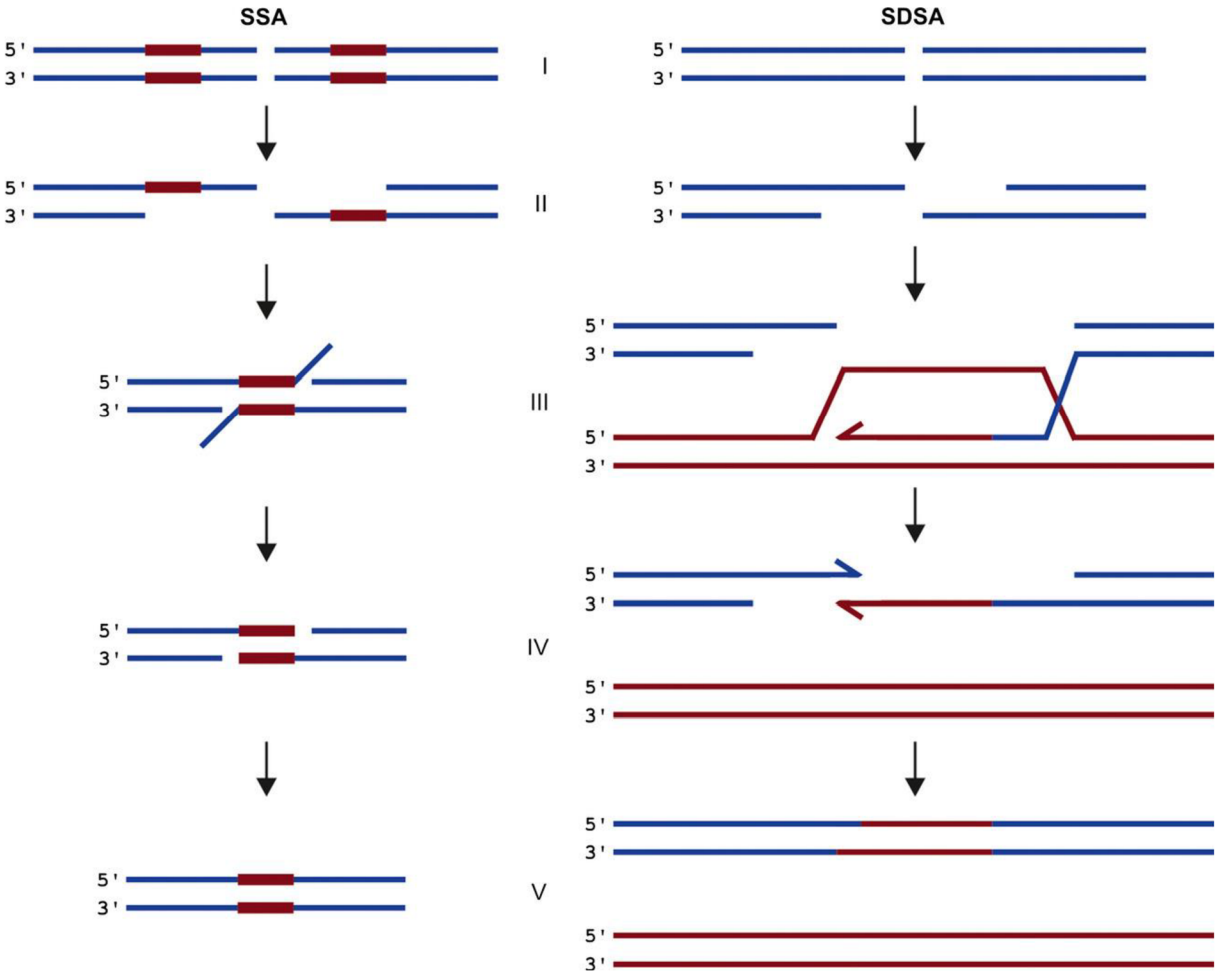

Figure 8: HR by SSA or SDSA. Scheme showing the position of DNA strands during DSB repair by SSA or SDSA. (**I**) initiation of SSA or SDSA by DSB (**II**) end resection (**III**) invasion of resected end and formation of D-loop (**IV** and **V**) resection of overhangs and ligation. SSA color map: red = short repeat sequences, blue = the rest of the DNA sequence; SDSA color map: red = homologous sequence, blue = damaged strands; (source Puchta and Fauser, 2014).

5.6.2 Non-homologous end joining

NHEJ does not need homology between sequences to instigate repair, although local microhomologies are used during the process occasionally to line up the ends of the DNA.

NHEJ is more error-prone than homology-based repair systems often resulting in nucleotide loss or insertion (Lieber, 2010). There are two NHEJ sub-pathways: the **canonical NHEJ** (**cNHEJ**), and the **alternative NHEJ** (**altNHEJ, alt-EJ**) (Kramer et al., 1994; Mladenov and Iliakis, 2011). Both sub-pathways have similar steps of end recognition, end processing and annealing.

Canonical NHEJ (cNHEJ)

cNHEJ is initiated by binding of the **KU70/KU80** heterodimer complex to DNA ends (**Figure 10**). The heterodimer creates a ring around the DNA ends, safeguarding them against additional degradation and acting as a protein kinase (Walker et al., 2001). KU70/KU80 enables recruitment of all additional proteins, including DNA-dependent protein kinase (DNA-PKcs) to the break (Smith and Jackson, 1999). Upon its recruitment DNA-PKcs autophosphorylates which seems to be the signal necessary for the other factors to access the DNA ends (Dobbs et al., 2010). The damaged ends are processed by nucleases in both directions creating 3-OH and 5'-phosphate ends. The damaged ends are brought into proximity and ligated by LIG4/XRCC4 complex (Mari et al., 2006; Wu et al., 2009), and additional factors like **XLF** (XRCC4-like factor) (Yano et al., 2008, 2011).

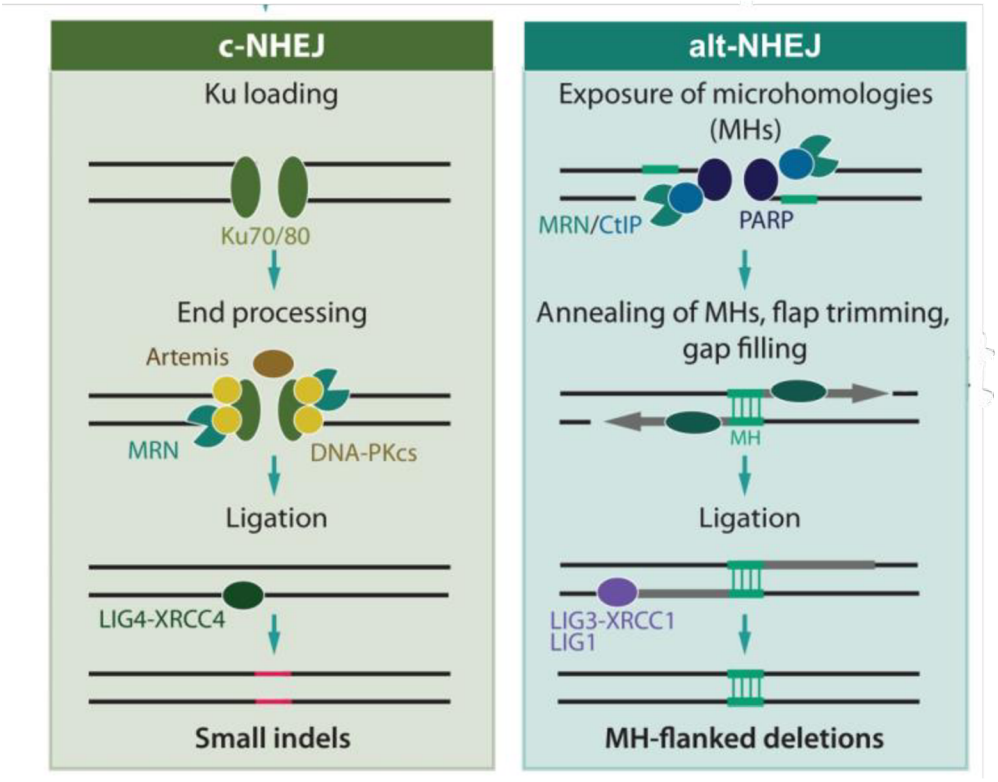

Figure 9: **cNHEJ and altNHEJ. A scheme showing the** processes and factors involved in cNHEJ and altNHEJ. **CtIP** (CtBP interacting protein), **DNA-PKcs** (DNA protein kinases), **LIG** (ligase), **MRN** (MRE11-RAD50-NBS1), **PARP** (Poly [ADP-ribose]pPolymerase), **XRCC1/4** (X-ray repair cross-complementing protein 1/4), (adapted from Schrempf et al., 2021).

Homologs of KU70, KU80, LIG4 and XRCC4 work in Arabidopsis cNHEJ (West et al., 2000, 2002; Tamura et al., 2002), with function of KU70/KU80 confirmed in rice and wheat as well (Nishizawa-Yokoi et al., 2012; Gu et al., 2014). No DNA-PKcs or a functional homologue has been identified in plants, as well as no homologue of XLF.

Alternative NHEJ (altNHEJ, alt-EJ)

altNHEJ begins with processing of the 3'-ends of the damaged DNA. In the best understood altNHEJ pathway, referred to as microhomology-mediated end joining (**MMEJ**) end resection is followed by annealing of the two processed strands based on local microhomologies (**Figure 10**). At the first glance this process resembles the previously described SSA pathway. Because of the processing to the annealing ends, deletions are a common by-product of altNHEJ (Puchta and Fauser, 2014). MMEJ in humans utilizes polymerase θ, and in plants TEBICHI to fill in the gaps left in the DNA. Other proteins involved in this process haven't been fully elucidated yet. It is considered that proteins PARP1 and LIG3α/XRCC1 play roles in recognition and ligation of DNA strands in humans (Della-Maria et al., 2011). AltNHEJ is thought to function mostly in absence of cNHEJ (Corneo et al., 2007).

AIMS OF THE STUDY

This study focused on exploring the processes of DNA damage repair in plants with different genome sizes. *Arabidopsis thaliana* and *Hordeum vulgare* (barley) were used as models for plants with small and large genomes, respectively.

6 AIMS OF THE STUDY

AIM I: CHARACTERIZATION OF A NOVEL PLANT DDR PROTEIN CONTAINING BRCT5 DOMAIN

The first objective of the work presented in this thesis is to identify novel proteins functioning as intermediaries in the DNA damage response in plants. While Arabidopsis research has produced significant components of the DDR process, some of the molecular factors involved are still unknown. The presence of a BRCT5 functional domain, a characteristic often observed in DDR proteins, serves as a method to identify a candidate proteins.

AIM II: ZEOCIN-INDUCED DNA DAMAGE RESPONSE IN BARLEY AND ITS DEPENDENCE ON ATR

The thesis' secondary aim was to describe the DNA damage response in barley from phenotypic, physiological, and transcriptomic perspectives. This encompassed a study into the role of the ATR kinase under normal conditions and during treatment with the broad-spectrum DNA damage inducer zeocin. For this genetic study to be conducted, the development of a new and rapid method of testing barley DNA damage response was crucial.

RESULTS AND DISCUSSION

Plants make the largest biomass on this planet. Even without considering their role in food production, animal feed, raw materials, or medicine, their importance as contributors to the production of planet's oxygen already makes plants invaluable. Simultaneously, the subject of DNA damage repair is an inevitable focal point in conversations surrounding aging, cancer therapies, evolution, speciation, and even space exploration. At the crossroads of plants and DNA damage response lies a discourse concerning increased demands for food production on a planet with 8 billion people, at times of impending global warming.

This section contains the summary of the results published in two first-author publications, along with discussion. Full research can be found in Appendix I and Appendix II.

7 CHARACTERIZATION OF A NOVEL PLANT DDR PROTEIN CONTAINING BRCT5 DOMAIN: THE SUMMARY

DNA damage response (DDR) factors are for the most part highly conserved between different eukaryotic species. However, plants have developed various distinctive factors, with SOG1 serving as a notable example. Examination of the DDR proteins, in plants or other species, showed a significant collection of proteins containing the BRCA1 C-Terminus (BRCT) functional domain (Bork et al., 1997). The insights into the DDR pathway show BRCT domains as the functional domain responsible for recognition and interaction among cooperative modules. The signaling cascade of the DDR starts with the ATM/ATR kinases and continues on through phosphorylation. For the accurate recognition of phosphorylated substrates, individual proteins have developed distinct specializations in binding to their intended targets. This specialization has been achieved through the evolution of BRCT domains which have diversified into multiple families and possess the ability to cooperate within a single protein (Leung and Glover, 2011). My study primarily concentrated on the characterization of proteins that specifically contain BRCT5, a relatively unexplored subset within the realm of plants.

In animals and yeast, there are several DDR factors utilizing BRCT5. For example, *Schizosaccharomyces pombe* protein Brc1 and human protein NSE5 (SLF1) both function in the recruitment of the SMC5/6 complex to the damaged site (Ohouo et al., 2010; Räschle et al., 2015). But they are functional, not structural homologues. However, the component they have in common is the BRCT5 domain. Thus far, no protein involved in plant SMC5/6 complex recruitment to the DNA has been described, nor do any of the SMC5/6 proteins contain BRCT5. Another example is the Pax2 transactivation domain interaction protein (PTIP), a mammalian protein tasked with the recruitment of 53BP1 to lesions (Gong et al., 2009). This pathway favors non-homologous end joining by competing with BRCA1. Conversely, PTIP has also been shown to promote HR (Wang et al., 2010). An entirety of this branch of DDR is yet to be uncovered in plants.

7.1 IDENTIFICATION OF CANDIDATE PROTEINS

Using the BRCT5 domain sequences of the *Sp*Brc1 and *Hs*NSE5 we searched for the potential homologues in Arabidopsis and *Physcomitrium patens.* The 3D models for the domains of the four candidates selected based on sequence homology (BCP1-4) were compared to that of the BRCT5 domain of Brc1. The superimposition model demonstrated a very high level of conservation between all the proteins (**Figure 11**). However, none of the BCP proteins had homology to the *Sp*Brc1 or *Hs*NSE5 outside of the BRCT5 domain. Additionally, although all of the proteins in question have other domains besides BRCT5, none have the same combination as seen in *Sp*Brc1 or *Hs*NSE5. One of the candidates, BCP1, has shown limited homology to the human PTIP. This suggested that the identified BCP candidates are most likely not the homologs of *Sp*Brc1, nor *Hs*NSE5, but this didn't exclude them from being involved in DDR.

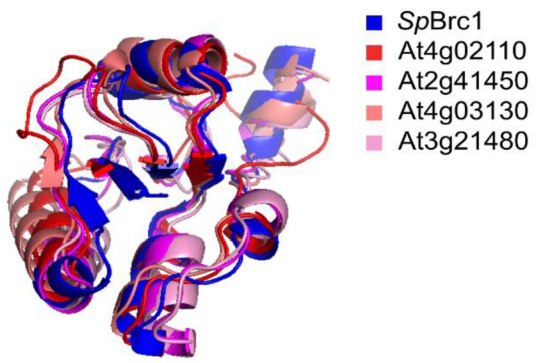

Figure 10. 3D model of BRCT5 domain. Superimposition of modeled BRCT5 protein domains of At4g02110 (BCP1), At2g41450 (BCP2), At4g03130 (BCP3), At3g21480 (BCP4), and crystal structure of *Sp*Brc1.

7.2 BCP1 HELPS IN REPAIR OF DIVERSE DNA DAMAGES

To definitively ascertain whether any of the BCPs are implicated in DNA damage response, we evaluated their reactions to DNA damage. Following the sensitivity assays BCP1 presented as the prime candidate out of the four tested proteins. Plants lacking a functional BCP1 protein exhibited heightened sensitivity to all employed DNA-damaging chemicals, displayed by root shortening (**Figure 12**). The selection of used chemicals was based on their capacity to induce various forms of DNA damage. Bleomycin causes DSBs, mitomycin C is a DNA inter-strand linker, while zebularine and camptothecin cause different types of DNA-protein crosslinks (Burger et al., 1981; Hsiang et al., 1985; Tomasz, 1995; Prochazkova et al., 2022). BCP1 having a role in the repair of such a broad spectrum of lesions pointed towards it potentially being a part of a mainstream repair pathway.

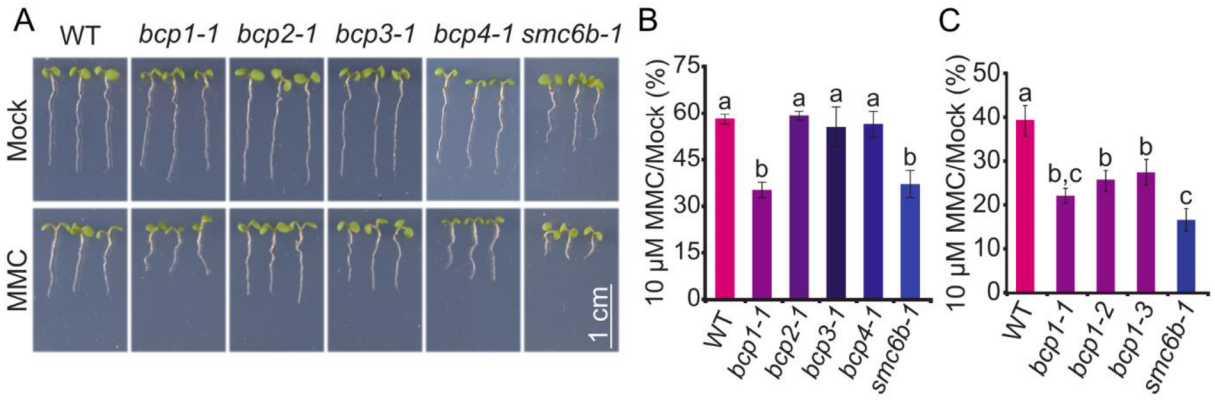

Figure 11. DNA damage assays example. (**A**) Representative phenotypes of 7-day-old wild type (WT) and mutant plants grown on media containing 10 µM mitomycin C (MMC). (**B-C**) Root length of plants under MMC relative to root length under mock conditions. The same letters represent not significantly different samples tested by one-way ANOVA with *post-hoc* Tukey's test.

7.3 *BCP1* EXPRESSION IS SOG1 DEPENDANT

The choice of BCP1 as a candidate was further cemented by its transcriptional activation following DNA damage caused by mitomycin C (Vladejić et al., 2022) and gamma-radiation (Bourbousse et al., 2018). Furthermore, the activation of *BCP1* was demonstrated to be dependent on SOG1, as there is no increase in its expression in the *sog1-1* mutant following DNA damage (**Figure 13**). SOG1 serves as the primary transcriptional regulator of the DNA damage response, and the genes under its regulation are primarily associated with the preservation of genomic stability. Interestingly, we couldn't find the canonic SOG1 binding motif (Bourbousse et al., 2018) in the promoter region of the *BCP1*, leaving the question of whether *BCP1* expression is induced by SOG1 directly, or by another TF under the control of SOG1. Besides the transcription controlled by SOG1, *BCP1* demonstrated a basal level of expression in both wild-type and *sog1* backgrounds. This could potentially stem from BCP1's

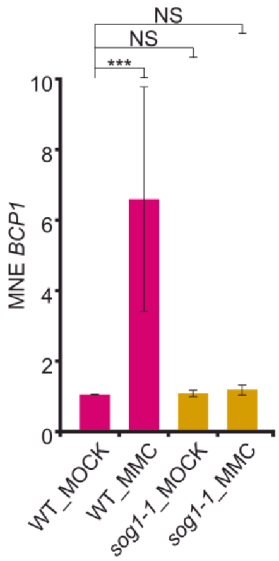

Figure 12: *BCP1* expression.
RT-qPCR analysis of *BCP1* expression in wild-type (WT) and *sog1-1* plants without (MOCK) or after 1 h treatment with 40 µM MMC.
*** - statistically significant at $p < 0.001$ in Mann-Whitney U-test

involvement in the repair of spontaneous DNA damage, or the cells may keep a low constitutive production of BCP1. However, substantiating either of these hypotheses would necessitate additional evidence.

7.4 BCP1 IS INVOLVED IN HOMOLOGOUS RECOMBINATION

Establishing BCP1's definite position within the DNA damage response pathway was accomplished by showcasing a reduction in both single-strand annealing and synthesis-dependent strand annealing events in plants missing this protein (**Figure 14**). BCP1's involvement in homologous repair confirms the significance of this protein in addressing a wide array of DNA damages, further underscored by its up-regulated expression in response to SOG1 activation. This prompts the question about the precise position of BCP1 within the homologous repair pathway. Unfortunately, that was outside of the scope of this publication, but further analysis on this topic is underway. The future analysis of BCP1 is structured around the identification of its interactors and has already begun through a series of Yeast Two-Hybrid Screens and is to be continued through a genetic analysis of their mutants (unpublished data). Any interaction would be further confirmed through the use of the classic Yeast Two-hybrid, Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation.

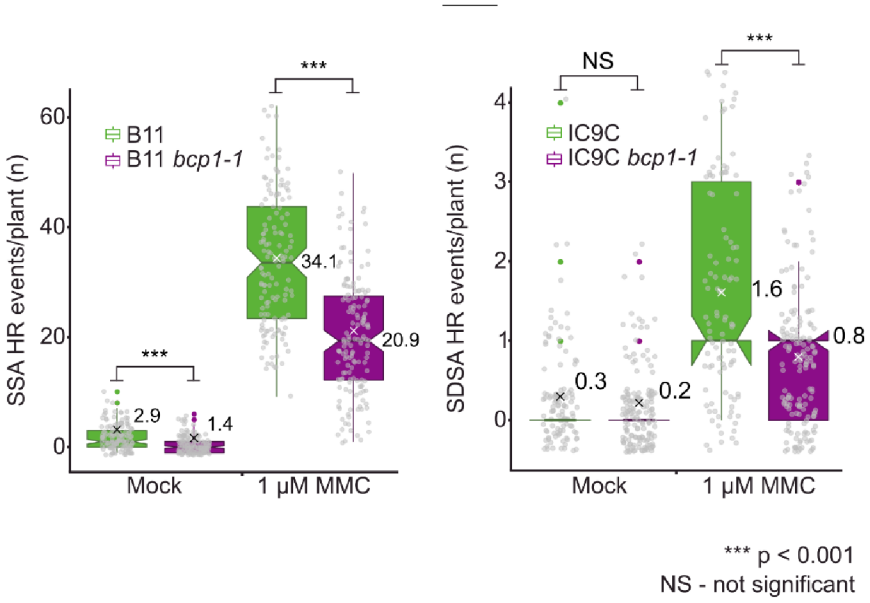

Figure 13: HR assays. Loss of BCP1 causes reduced frequency of somatic homologous recombination (HR). Wild type (WT) and *bcp1-1* plants carrying genomic substrates for SSA (*B11*) and SDSA (*IC9C*) types of HR were grown on mock and 1 µM containing MMC media for 10 days. Significant differences assessed by two sample T-tests with unequal variance, * $p < 0.05$

7.5 OUTLOOK

The role of BCP1 in DDR has been further confirmed by two independent research groups since the publication of our data in 2022. The publication by Yu et al., 2023 re-affirms many of our findings on BCP1 (DDRM2). The group identified *BCP1* in a genetic screen for mutants sensitive to camptothecin, which is in line with my data demonstrating *bcp1* mutants' sensitivity to this drug (Vladejić et al., 2022). Further, BCP1 was shown to interact directly with RAD51 through its C-terminal BRCT3 and BRCT4 domains. The interaction promotes the recruitment of RAD51 onto the DNA lesion, and BCP1 foci were detected on the DNA following DSBs. RAD51 is the protein guiding the damaged DNA into homology repair by SDSA (Sung, 1994). BCP1's interaction with it suggests it may have a role in either guiding the RAD51 onto the damaged site or during the search for homologous sequence.

In a preprint of a manuscript by Lorković et al., 2023, all of the BCPs were identified during a search for proteins possessing BRCT domains capable of interacting with γH2A.X. In their manuscript, they conclude that BCP1 does not bind to the phosphorylated histone, but instead functions downstream. They go on to suggest that BCP1 has a resemblance to human PTIP protein, based on the phylogenetic analysis of proteins with BRCT domains. I've noted the partial sequence homology of BCP1 and PTIP in my manuscript as well (Vladejić et al., 2022), but none of my experiments neither confirm nor deny this connection. Taking into account the work presented by Yu et al., 2023 and by Lorković et al., 2023, BCP1 would be activated by SOG1 and recruited onto the DNA lesions without interaction with γH2A.X. Whether it interacts with RAD51 before binding to the DNA lesion or after will have to be further analyzed.

BCP2, *BCP3* and *BCP4* were neglected for detailed analysis in my research, due to the apparent lack of sensitivity of mutants to DNA damage. However, it was noted that *bcp2-1* and *bcp3-1* alleles might not be knock-out mutants, and thus may still produce functional proteins. Therefore, further analysis on BCP2, BCP3 and BCP4 is needed. Mere two weeks following my publication the role of protein BCP3 in HR was described by Fan et al., 2022. The XIP (BCP3) protein was shown to recognize γH2A.X and interact with RAD51, making it another protein involved in the assembly of the HR machinery to the DNA lesions. Further, the aforementioned unpublished work by Lorković et al. shows BCP4 interaction with γH2A.X. The same manuscript also suggests BCP3 and BCP4 are orthologs of each other, created in a duplication event, potentially functioning as a functional homologs of the mammalian mediator protein MDC1 (Stewart et al., 2003).

8 ZEOCIN INDUCED DNA DAMAGE IN BARLEY AND ITS DEPENDENCE ON ATR: THE SUMMURY

After successfully identifying a new DDR protein in Arabidopsis, I transitioned to applying the knowledge I gained from Arabidopsis to the economically more valuable cereals. Creating resilient cereal varieties is essential for stable food production, with challenges in generating and assessing mutant populations. Success of mutagenesis depends on plants' DNA repair capacity. Understanding the DDR pathways in cereals can help with creation of more precise techniques and increase the success of mutagenesis

For my research, I selected barley as a model system. Barley is an excellent genetic cereal model due to its large, yet diploid, genome, and its Rabl chromosome organization (Hoencamp et al., 2021). Successful mutation methods in barley include TILLING method for random mutations, and precise targeting using CRISPR/Cas9 system and TALENs (Wendt et al., 2013; Szurman-Zubrzycka et al., 2018; Schreiber et al., 2019).

Curently, barley's DNA damage response (DDR) processes are poorly understood. Previous research indicates barley primarily uses non-homologous end-joining for DSB repair, including both cNHEJ and altNHEJ, while homology-based repair is less common, similar to findings in Arabidopsis (Vu et al., 2014). Only a few DDR-related genes, such as *KU80* and *PARP3*, have been explored in barley (Stolarek et al., 2015b, 2015a). Many of the proteins involved remain unexamined. I initiated my DDR studies in barley by developing DNA damage assays tailored to this species to accelerate the process of barley DDR mutant selection.

8.1 DEVELOPING *IN VITRO* DNA DAMAGE PROTOCOLS FOR BARLEY

Existing barley chemical treatment protocols involve hydroponics or seed imbibition in chemicals pre-germination (Szurman-Zubrzycka et al., 2019; Jaskowiak et al., 2020). I established the new method for barley DNA damage assays in order to circumvent issues arising in the mentioned types of *in vitro* cultivation, namely the common fungal contamination, asynchronous germination and uneven reactions to DNA-damaging chemicals. By removing the seed coat and endosperm, then cultivating plants from mature embryos, the growth and phenotypic response are uniform, with no contamination. I propose that uneven reaction to DNA damaging chemicals observed in plants germinated *in vitro* from the entire seeds is the consequence of seedlings using stored nutrients, instead of fully relying on a supplemented

medium. My protocol has barley embryos grown on ½ MS medium with 0.6% agarose for 14 days.

Zeocin, a phleomycin D1/bleomycin-type antibiotic with radiomimetic effects, was used to induce DNA damage. Radiomimetric chemicals cause a wide spectrum of damage, due to their production of ROS in cells. Although DSBs are the most toxic type of lesion they can cause, SSBs and AP sites are more prevalent (Povirk et al., 1977; Chen et al., 2008; Shimada et al., 2013). This makes radiomimetic drugs suitable for testing DDR sensitivity of various mutants. A concentration of 100 µg/ml zeocin was determined as optimal for phenotypic response measurements. The roots exhibited the most DNA damage, with severe effects seen in short length and disrupted cellular differentiation in the apical root meristem (**Figure 15**), while shoot length was less affected.

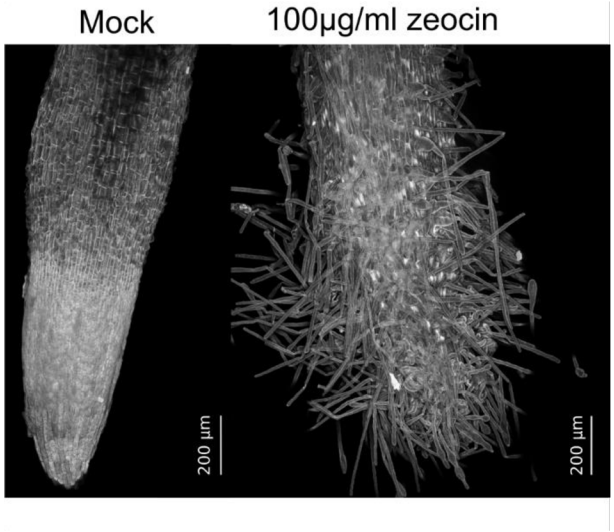

Figure 14. Root treated with zeocin. Representative confocal microscopy maximal projection images of the root surface in plants grown in mock conditions and plants treated with 100 µg/ml zeocin. Scale bar = 200 µm.

8.2 INSIGHT INTO BARLEY HOMOLOGS OF DDR GENES

In this study, I additionally provide a list of putative DDR gene candidates in barley by comparing 321 Arabidopsis genes, identified in Bouyer et al. (2018) as DDR-related, against the 83.661 genes annotated in barley (Mascher et al., 2021). Because of the incomplete annotation of the barley genome, the analysis of extensive datasets, such as those generated from RNA sequencing, is hindered. Using reciprocal BLAST and filtering based on E-value (≤0.01) and protein alignment lengths, a list of 421 putative barley DDR genes' homologs was assembled. Some genes, like *BRCA1* exhibited low sequence similarity, and posed a challenge to the identification. Sequence homology however does not equate with functional similarity, so all of these candidates will need confirmation in functional studies. Even so, this list is the most comprehensive up to date (Volkova et al., 2020).

Despite barley's genome being nearly 40 times larger than that of Arabidopsis, and containing 15,000 more genes, it doesn't seem to have significantly more DDR genes (Cheng et al., 2017; Mascher et al., 2021). My study found few duplication events among barley's DDR genes, aligning with the perspective of DDR genes as a highly conserved group. Although unique barley genes without Arabidopsis homologs might exist, generating hundreds through this method is unlikely.

The crucial target of our search was the main regulator the DDR pathway SOG1 (Yoshiyama, 2016). We found five potential candidates, and review of literature showed no consensus regarding true SOG1 ortholog. Gorbatova et al. (2020) pointed to HORVU.MOREX.r3.6HG0590960 as a putative SOG1 based on its transcriptional response to DNA damage. Meanwhile Murozuka et al. (2018) examining 167 NAC-domain proteins in barley, proposed HORVU.MOREX.r3.7HG0670800. Both of these candidates were also identified in our homology search.

Using NAC domains from SOG1 and related proteins from Arabidopsis, maize and rice I reconstructed barley SOG1 phylogeny (**Figure 16**). Because of their high conservation, functional domains are well suited for phylogeny analysis. The resulting phylogenetic tree split into two main clades. The first included *At*SOG1, *Os*SOG1, two maize homologs, and a single barley candidate. The second contained SOG1-related proteins, ANAC44 and ANAC85, rice SOG1-like, and four barley candidates. Thus, my analysis identified HORVU.MOREX.r3.7HG0670800 as barley's SOG1 homolog and four SOG1-like proteins (SGLs). The barley *SGL.B*, *C*, and *D* genomic positioning indicates they were created in two local duplications events.

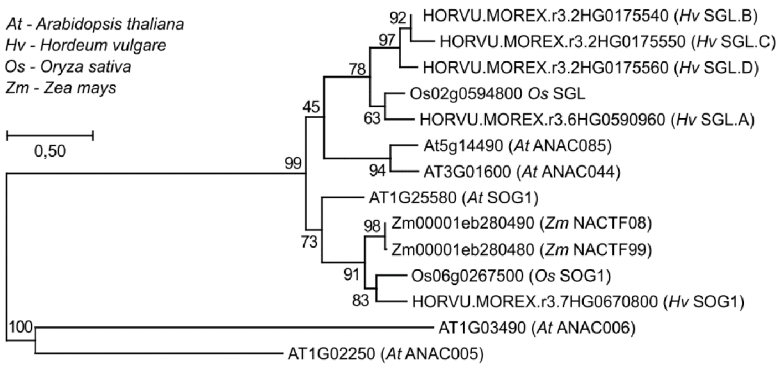

Figure 15. *Hv*SOG1 phylogenetic tree. Phylogenetic Maximum Likelihood tree based on the multiple sequence alignment of NAC domains of barley candidate proteins, Arabidopsis (*At*) SOG1, ANAC044, and ANAC085, rice (*Os*) SOG1 and SOG1-like (SGL), and maize (*Zm*) NACTF8 and NACTF99, with Arabidopsis ANAC005 and ANAC006 NAC protein domains used as an outgroup. Bootstrap values are shown next to the branches, distance scale = 0.5.

8.3 TRANSCRIPTOMIC CHANGES OF WILD-TYPE BARLEY POST-ZEOCIN TREATMENT

With the prepared protocol for treatment of barley with zeocin and identified putative DDR genes, I have gone on to analyze gene expression changes in wild type barley under DNA damage by RNA sequencing. The genes involved in oxidative metabolism were the ones showing the biggest positive change in regulation. This induction of the oxidative stress response is most likely related to the mechanism of zeocin action. As mentioned before, antibiotics from the phleomycin/bleomycin group, such as zeocin, induce the production of oxygen radicals in cells.

Transcriptomic data also showed clear induction of the DNA repair pathways in wild type plants upon zeocin treatments. The up-regulated genes included several DSB repair factors and positive HR regulators, among others. I also noted the *SGL.A* up-regulation, along with that of *SGL.D* and *SGL.C*. However, *SOG1* expression was not induced. Interestingly, the similar pattern can be seen in Arabidopsis and rice where *SOG1* expression is not elevated upon damage induction (Bourbousse et al., 2018; Nishizawa-Yokoi et al., 2023).

Besides the up-regulation of DNA damage repair genes, DDR response could be seen in the down-regulation of genes associated with cell cycle and cell division. Genes affected the most were the histone genes, which indicated a halt in DNA replication. The up-regulation of *SMR* and *KRP* genes, along with the down-regulation of *CYC* and *CDK* genes indicated cell cycle arrest. However, as previously discussed in the introduction, both SMR and KRP in plants have an additional role in driving the cell program toward endoreduplication. Factors like DEL1 and FBL17, regulated in the endocycle by SMR, were found down-regulated as well (Kumar and Larkin, 2017).

This led us to speculate that cells were preparing for the transition from mitosis to endoreduplication. I have measured the ploidy levels by flow-citometry and found a 2.5-fold increase in the endoreduplicated nuclei in the roots of zeocin-treated plants (**Figure 16**). The shift toward endoreduplication during double-strand breaks is a strategy known for Arabidopsis (Adachi et al., 2011). This implies that the transition in the cell program via the SMR-dependent pathway during DNA damage is conserved in plants.

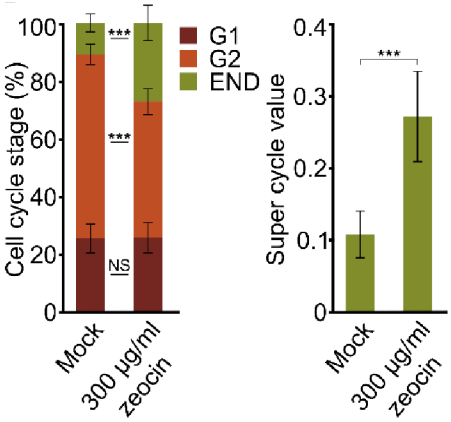

Figure 16. Endoreduplication in root nuclei under DNA damage Percentage of nuclei at specific cell cycle stages found in the root tips of plants grown on mock and medium with 300 µg/ml zeocin. Super cycle value describing the number of nuclei in endoreduplication (END). Statistical relevance was distinguished by a two-sample T-test, ($P < 0.05$). * P , 0.05, *** $P < 0.001$

8.4 ZEOCIN SENSITIVITY: TESTING THE RESPONSE OF *ATR* MUTANT

I studied the impact of DNA damage, induced by zeocin, on *ATR* mutant plants. ATR, one of the two main kinases in DDR (Culligan et al., 2006) was mutated using the TILLING method in the HorTILLUS population (Szurman-Zubrzycka et al., 2018). The loss-of-function mutant allele *hvatr.g* has a missense mutation in the UME domain, which altered its DNA repair abilities, resulting in increased aluminum tolerance but heightened sensitivity to maleic acid hydrazide, which inhibits the cell cycle progression (Szurman-Zubrzycka et al., 2019; Jaskowiak et al., 2020).

I observed a similar trend in plants grown from rescued embryos to those previously described. The *hvatr.g* mutant exhibited shorter roots than WT even in mock conditions (**Figure 17A, B**). Interestingly *hvatr.g* had significantly higher root number compared to WT plants, and while the root number of WT plants wasn't affected by zeocin treatment, *hvatr.g* root

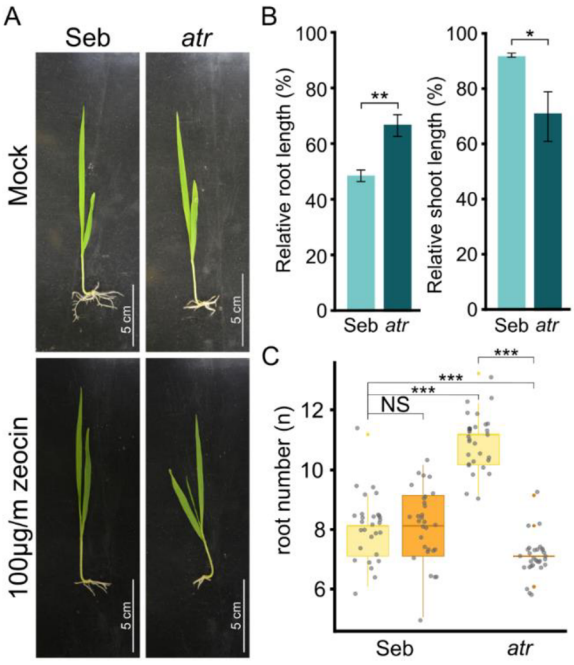

Figure 17: DNA damage assays in barley. (**A**) Phenotypic response of wild-type (Seb) and *atr* mutant barley plants cultivated in normal conditions (mock) and treated with 100 µg/ml zeocin. (**B**) Root and shoot length of barley plants under zeocine relative to length under mock conditions. Error bars indicate the standard deviation between the means of three biological replicates. The same letters represent not significantly different samples tested by one-way ANOVA with post-hoc Tukey's test *$p < 0.05$. (**C**) Number of roots in wild-type (Seb) and atr plants grown in mock and genotoxic conditions. Kruskall-Wallis H-test with post hoc Conover-Iman test of multiple comparisons was used for testing significance.

number decreased (**Figure 17 C**). The most informative characteristic for assessing the sensitivity of *hvatr.g* to zeocin was shoot legth.

8.5 EFFECTS OF *HVATR.G* MUTATION ON GENE EXPRESSION

Comparison of *hvatr.g* mutant transcriptome to that of wild-type in normal conditions showed an increase in the expression of DDR genes, pointing towards increased genomic instability even without induced DNA damage. The genes that exhibited up-regulation in *hvatr.g,* indicated a stimulation of NER and MMR pathways. In addition, there was a collective up-regulation of genes involved in the removal of cellular proteins, but also transcription and protein synthesis.Together they indicated that the cells are experiencing an increased protein turnover. The most remarkable decrease in expression was noted among the transmembrane transporter genes, particularly genes with products involved in nitrogen transport.

The underlying cause of shorter, yet numerous, roots of *hvatr.g* in normal conditions was not clear from the transcriptomic data. A couple of speculative models can be offered as a potential explanation. Potential replication-coupled defects in the *hvatr.g* mutant might alter the balance between root elongation driven by active cell division and the initiation of new roots. In wild type plants, the excessive formation of new roots could be inhibited through the interaction of ATR with factors like SOG1 and WEE1, which link the DDR with the cell cycle (Chen et al., 2023). Another potential mechanism could stem from metabolic issues in *hvatr.g* plants, as evidenced by the down-regulation of transmembrane transport genes, particularly those related to nitrate and nitrogen-compound transporters. Furthermore, the observed down-regulation of 13 *EXPANSIN* genes in *hvatr.g* plants hints at a potential effect on cell growth and root number. It's worth noting that *EXPANSINS* were linked to a root number quantitative trait locus in barley, though no specific gene has been definitively identified as the causative factor (Robinson et al., 2016).

Along with the transcriptional differences between *hvatr.g* and wild type in normal conditions, I compared their responses to zeocin as well. Despite minor differences, the transcriptional response between the genotypes was largely consistent. This could be attributed to partial functional redundancy of plant ATM and ATR kinases as described in Arabidopsis (Culligan et al., 2006). To fully understand all the genes controlled by these DDR kinases an *atm atr* double mutant would be needed. Alternatively, studying barley *sog1* mutant could provide further insights into barley's DDR (Bourbousse et al., 2018; Ogita et al., 2018).

CONCLUSION

The End.

9 CONCLUSION

The primary objective of the research presented in this manuscript was the study of DNA damage response and repair in plants with small and large genomes, with Arabidopsis and barley serving as representative models. The findings underwent evaluation by peers in the scientific community and were subsequently published as my two first author publications in peer-reviewed journals (Vladejić et al., 2022, *Frontiers in Plant Science*, Impact Factor 5.6 and Vladejic et al., 2024, *Scientific Reports*, Impact Factor 4.9).

I have successfully identified a new Arabidopsis protein called BCP1, and have provided evidence of its participation in DDR by homologous recombination. My primary focus centered on utilizing the BRCT5 domain as an identifying characteristic of the proteins involved in DDR (Vladejić et al., 2022). Subsequently, two separate investigations into BCP1 not only corroborated my findings but also extended them, revealing its interactions with proteins within the homologous repair pathway and its recruitment to sites of DNA damage.

As the second part of my research, I further explored DNA damage response, focusing on barley, a plant of significant agricultural importance. The research presented concludes with the development of a well-established protocol for conducting DNA damage assays specific to barley. Moreover, it offers a compilation of potential DDR-related genes in barley that could be of substantial value to the scientific community. Notably, the study identified the SOG1 homolog in barley and includes comprehensive transcriptomic data from both wild-type (WT) and *hvatr.g* mutant barley plants following the induction of wide-spectrum DNA damage through the use of the radiomimetic chemical zeocin (Vladejic 202).

In conclusion, this research has advanced our understanding of plant DNA damage response mechanisms by uncovering a novel DDR protein in Arabidopsis and providing insights into the DDR response in barley on phenotypic, physiological, and transcriptomic level.

AUTHOR'S CONTRIBUTION

10 AUTHOR'S CONTRIBUTION

My contributions to this study encompassed a wide range of experimental techniques and data analysis. Here is a breakdown of my roles and responsibilities:

First Author Publication I: Analysis of BRCT5 domain-containing proteins reveals a new component of DNA damage repair in Arabidopsis

Jovanka Vladejić, Fen Yang, Eva Dvořák Tomaštíková, Jaroslav Doležel, Jan J. Paleček, Ales Pecinka. *Front. Plant Sci.* (2022); DOI: doi.org/10.3389/fpls.2022.1023358

1. DNA Damage Assays:

   I conducted mutant DNA damage sensitivity assays for all tested genotypes (*bcp1-1, bcp1-2, bcp1-3, bcp2-1, bcp3-1, bcp4-1*).
   I performed measurements of phenotypic changes, and the data analysis.

2. Cell Death Assays:

   I performed the cell death assay treatments, sample coloring and data analysis.

3. RNA Sequencing Data Analysis:

   I analyzied the publically available RNA sequencing data sets.

4. RT-qPCR:

   I prepared the biological samples, extracted RNA, performed reverse transcripton of RNA to cDNA and quantifyied the *BCP1-4* genes' expression level in T-DNA insertion mutants, as well as *BCP1* expression in wild type and *SOG1* mutatnt plants by RT-qPCR

5. Genetic Engineering:

   I assembled the *promoterBCP1::GUS* fusion construct with Gateway cloning.
   I was responsible for the transformation process and the subsequent selection of transformant Arabidopsis plants.

6. GUS (β-glucuronidase) Assays and Microscopy:

   I performed treatments and GUS histochemical staining on both seedlings and flowers, along with microscopic imaging to capture the tissue localization and changes of *BCP1* expression.

7. Homologous Recombination Assays:

   I selected appropriate marker lines for homologous recombination assays (*bcp1-1 B11* and *bcp1-1 IC9C*).
   I carried out homologous recombination assays, GUS histochemical staining, data collection and subsequent analysis.

8. Fresh Weight Assays:

   I conducted fresh weight assays and measurements on the lines *bcp1-1 B11* and *bcp1-1 IC9C*

First Author Publication II: Zeocin induced DNA damage in barley and its dependence on ATR

Jovanka Vladejić, Martin Kovačik, Jana Zwyrtková, Miriam Szurman-Zubrzycka, Jaroslav Doležel, Aleš Pecinka. (2024). *Sci. Rep.*, in press.

1. DNA Damage Assays:

   I took the lead in creating and conducting DNA damage assays on barley. I measured the phenotypic response of the plants and performed the subsequent data analysis.

2. Confocal Microscopy:

   I performed treatments on barley and conducted pseudo-Schiff propidium iodide staining of the barley roots for confocal microscopy.

3. Flow-Cytometry:

   I carried out biological sample preparation and performed nuclear ploidy measurements using flow-cytometry.

4. RT-qPCR Analysis:

   I prepared the biological samples, extracted RNA, performed reverse transcripton of RNA to cDNA and quantified the expression of selected DDR genes.

5. RNA Sequencing and Data Analysis:

   I was responsible sample preparation and RNA isolation for RNA sequencing.
   I collaborated with Assoc. Prof. Aleš Pečinka in the analysis of RNA sequencing data.

6. Phylogenetic Analysis:

   I performed phylogenetic analysis of barley SOG1 candidate proteins

Additionally for both manuscripts I contributed to:

1. Figures:

   I was responsible for creating all graphical content presented in the publications.

2. Statistical Analysis:

   I conducted all statistical analyses for the studies.

3. Manuscript preparation:

   I collaborated with Assoc. Prof. Aleš Pečinka in writing the manuscripts.

REFERENCES

11 REFERENCES

Abbas, T., and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* 9, 400–414. doi: 10.1038/nrc2657

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* 15, 2177–2196. doi: 10.1101/gad.914401

Adachi, S., Minamisawa, K., Okushima, Y., Inagaki, S., Yoshiyama, K., Kondou, Y., et al. (2011). Programmed induction of endoreduplication by DNA double-strand breaks in Arabidopsis. *Proceedings of the National Academy of Sciences* 108, 10004–10009. doi: 10.1073/pnas.1103584108

Ahmad, M., Jarillo, J. A., Klimczak, L. J., Landry, L. G., Peng, T., Last, R. L., et al. (1997). An enzyme similar to animal type II photolyases mediates photoreactivation in Arabidopsis. *Plant Cell* 9, 199–207. doi: 10.1105/tpc.9.2.199

Aklilu, B. B., Soderquist, R. S., and Culligan, K. M. (2014). Genetic analysis of the Replication Protein A large subunit family in Arabidopsis reveals unique and overlapping roles in DNA repair, meiosis and DNA replication. *Nucleic Acids Research* 42, 3104–3118. doi: 10.1093/nar/gkt1292

Akutsu, N., Iijima, K., Hinata, T., and Tauchi, H. (2007). Characterization of the plant homolog of Nijmegen breakage syndrome 1: Involvement in DNA repair and recombination. *Biochem Biophys Res Commun* 353, 394–398. doi: 10.1016/j.bbrc.2006.12.030

Amé, J.-C., Spenlehauer, C., and de Murcia, G. (2004). The PARP superfamily. *Bioessays* 26, 882–893. doi: 10.1002/bies.20085

Amiard, S., Charbonnel, C., Allain, E., Depeiges, A., White, C. I., and Gallego, M. E. (2010). Distinct roles of the ATR Kinase and the Mre11-Rad50-Nbs1 complex in the maintenance of chromosomal stability in Arabidopsis. *The Plant Cell* 22, 3020–3033. doi: 10.1105/tpc.110.078527

Bakkenist, C. J., and Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499–506. doi: 10.1038/nature01368

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., et al. (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* 86, 159–171. doi: 10.1016/s0092-8674(00)80086-0

Beerens, N., Hoeijmakers, J. H. J., Kanaar, R., Vermeulen, W., and Wyman, C. (2005). The CSB protein actively wraps DNA. *J Biol Chem* 280, 4722–4729. doi: 10.1074/jbc.M409147200

Bhabra, G., Sood, A., Fisher, B., Cartwright, L., Saunders, M., Evans, W. H., et al. (2009). Nanoparticles can cause DNA damage across a cellular barrier. *Nature Nanotech* 4, 876–883. doi: 10.1038/nnano.2009.313

Bienz, M. (2006). The PHD finger, a nuclear protein-interaction domain. *Trends in Biochemical Sciences* 31, 35–40. doi: 10.1016/j.tibs.2005.11.001

Blackford, A. N., and Jackson, S. P. (2017). ATM, ATR, and DNA-PK: The trinity at the heart of the DNA damage response. *Mol Cell* 66, 801–817. doi: 10.1016/j.molcel.2017.05.015

Block-Schmidt, A. S., Dukowic-Schulze, S., Wanieck, K., Reidt, W., and Puchta, H. (2011). BRCC36A is epistatic to BRCA1 in DNA crosslink repair and homologous recombination in Arabidopsis thaliana. *Nucleic Acids Research* 39, 146–154. doi: 10.1093/nar/gkq722

Borde, V., and de Massy, B. (2013). Programmed induction of DNA double strand breaks during meiosis: setting up communication between DNA and the chromosome structure. *Current Opinion in Genetics & Development* 23, 147–155. doi: 10.1016/j.gde.2012.12.002

Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997). A superfamily of conserved domains in DNA damage- responsive cell cycle checkpoint proteins. *The FASEB Journal* 11, 68–76. doi: 10.1096/fasebj.11.1.9034168

Boudolf, V., Vlieghe, K., Beemster, G. T. S., Magyar, Z., Torres Acosta, J. A., Maes, S., et al. (2004). The plant-specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in Arabidopsis. *Plant Cell* 16, 2683–2692. doi: 10.1105/tpc.104.024398

Bourbousse, C., Vegesna, N., and Law, J. A. (2018). SOG1 activator and MYB3R repressors regulate a complex DNA damage network in Arabidopsis. *Proc Natl Acad Sci U S A* 115, E12453–E12462. doi: 10.1073/pnas.1810582115

Bouyer, D., Heese, M., Chen, P., Harashima, H., Roudier, F., Grüttner, C., et al. (2018). Genome-wide identification of RETINOBLASTOMA RELATED 1 binding sites in Arabidopsis reveals novel DNA damage regulators. *PLOS Genetics* 14, e1007797. doi: 10.1371/journal.pgen.1007797

Brash, D. E. (1988). UV mutagenetic photoproducts in Escherichia coli and human cells: A molecular genetics perspective on human skin cancer. *Photochemistry and Photobiology* 48, 59–66. doi: 10.1111/j.1751-1097.1988.tb02786.x

Britt, A. B. (1996). DNA damage and repair in plants. *annual Review of Plant Physiology*, 75–100.

Brown, E. J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev.* 14, 397–402. doi: 10.1101/gad.14.4.397

Bundock, P., and Hooykaas, P. (2002). Severe developmental defects, hypersensitivity to DNA-damaging agents, and lengthened telomeres in Arabidopsis MRE11 mutants. *Plant Cell* 14, 2451–2462. doi: 10.1105/tpc.005959

Burger, R. M., Peisach, J., and Band Horwitz, S. (1981). Mechanism of bleomycin action: In vitro studies. *Life Sciences* 28, 715–727. doi: 10.1016/0024-3205(81)90153-3

Burma, S., Chen, B. P., Murphy, M., Kurimasa, A., and Chen, D. J. (2001). ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* 276, 42462–42467. doi: 10.1074/jbc.C100466200

Caldecott, K. W. (2003). XRCC1 and DNA strand break repair. *DNA Repair (Amst)* 2, 955–969. doi: 10.1016/s1568-7864(03)00118-6

Caron, P., Choudjaye, J., Clouaire, T., Bugler, B., Daburon, V., Aguirrebengoa, M., et al. (2015). Non-redundant functions of ATM and DNA-PKcs in response to DNA double-strand breaks. *Cell Rep* 13, 1598–1609. doi: 10.1016/j.celrep.2015.10.024

Castedo, M., Coquelle, A., Vitale, I., Vivet, S., Mouhamad, S., Viaud, S., et al. (2006). Selective resistance of tetraploid cancer cells against DNA damage-induced apoptosis. *Annals of the New York Academy of Sciences* 1090, 35–49. doi: 10.1196/annals.1378.004

Castedo, M., Perfettini, J.-L., Roumier, T., Andreau, K., Medema, R., and Kroemer, G. (2004). Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 23, 2825–2837. doi: 10.1038/sj.onc.1207528

Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., et al. (2002). Genomic instability in mice lacking histone H2AX. *Science* 296, 922–927. doi: 10.1126/science.1069398

Chargaff, E. (1951). Structure and function of nucleic acids as cell constituents. *Federation Proceedings* 3, 654–9.

Chen, H., Pan, T., Zheng, X., Huang, Y., Wu, C., Yang, T., et al. (2023). The ATR–WEE1 kinase module promotes SUPPRESSOR OF GAMMA RESPONSE 1 translation to activate replication stress responses. *The Plant Cell*, koad126. doi: 10.1093/plcell/koad126

Chen, J., Ghorai, M. K., Kenney, G., and Stubbe, J. (2008). Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage. *Nucleic Acids Research* 36, 3781–3790. doi: 10.1093/nar/gkn302

Chen, P., Takatsuka, H., Takahashi, N., Kurata, R., Fukao, Y., Kobayashi, K., et al. (2017). Arabidopsis R1R2R3-Myb proteins are essential for inhibiting cell division in response to DNA damage. *Nat Commun* 8, 635. doi: 10.1038/s41467-017-00676-4

Cheng, C.-Y., Krishnakumar, V., Chan, A. P., Thibaud-Nissen, F., Schobel, S., and Town, C. D. (2017). Araport11: a complete reannotation of the Arabidopsis thaliana reference genome. *The Plant Journal* 89, 789–804. doi: 10.1111/tpj.13415

Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J. J., Goldberg, R. B., et al. (2002). DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in Arabidopsis. *Cell* 110, 33–42. doi: 10.1016/S0092-8674(02)00807-3

Churchman, M. L., Brown, M. L., Kato, N., Kirik, V., Hülskamp, M., Inzé, D., et al. (2006). SIAMESE, a plant-specific cell cycle regulator, Controls endoreplication onset in Arabidopsis thaliana. *The Plant Cell* 18, 3145–3157. doi: 10.1105/tpc.106.044834

Córdoba-Cañero, D., Morales-Ruiz, T., Roldán-Arjona, T., and Ariza, R. R. (2009). Single-nucleotide and long-patch base excision repair of DNA damage in plants. *The Plant Journal* 60, 716–728. doi: 10.1111/j.1365-313X.2009.03994.x

Córdoba-Cañero, D., Roldán-Arjona, T., and Ariza, R. R. (2011). Arabidopsis ARP endonuclease functions in a branched base excision DNA repair pathway completed by LIG1. *The Plant Journal* 68, 693–702. doi: 10.1111/j.1365-313X.2011.04720.x

Corneo, B., Wendland, R. L., Deriano, L., Cui, X., Klein, I. A., Wong, S.-Y., et al. (2007). Rag mutations reveal robust alternative end joining. *Nature* 449, 483–486. doi: 10.1038/nature06168

Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001). ATR and ATRIP: partners in checkpoint signaling. *Science* 294, 1713–1716. doi: 10.1126/science.1065521

Culligan, K. M., and Hays, J. B. (2000). Arabidopsis MutS Homologs—AtMSH2, AtMSH3, AtMSH6, and a Novel AtMSH7—form three distinct protein heterodimers with different specificities for mismatched DNA. *The Plant Cell* 12, 991–1002. doi: 10.1105/tpc.12.6.991

Culligan, K. M., Robertson, C. E., Foreman, J., Doerner, P., and Britt, A. B. (2006). ATR and ATM play both distinct and additive roles in response to ionizing radiation. *The Plant Journal* 48, 947–961. doi: 10.1111/j.1365-313X.2006.02931.x

Culligan, K., Tissier, A., and Britt, A. (2004). ATR Regulates a G2-phase cell-cycle checkpoint in Arabidopsis thaliana. *The Plant Cell* 16, 1091–1104. doi: 10.1105/tpc.018903

Daniels, D. S., Woo, T. T., Luu, K. X., Noll, D. M., Clarke, N. D., Pegg, A. E., et al. (2004). DNA binding and nucleotide flipping by the human DNA repair protein AGT. *Nat Struct Mol Biol* 11, 714–720. doi: 10.1038/nsmb791

Daujat, S., Neel, H., and Piette, J. (2001). MDM2: life without p53. *Trends Genet* 17, 459–464. doi: 10.1016/s0168-9525(01)02369-1

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis* 19, 169–185. doi: 10.1093/mutage/geh025

de Jager, M., van Noort, J., van Gent, D. C., Dekker, C., Kanaar, R., and Wyman, C. (2001). Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Mol Cell* 8, 1129–1135. doi: 10.1016/s1097-2765(01)00381-1

De Schutter, K., Joubès, J., Cools, T., Verkest, A., Corellou, F., Babiychuk, E., et al. (2007). Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. *The Plant Cell* 19, 211–225. doi: 10.1105/tpc.106.045047

Delacroix, S., Wagner, J. M., Kobayashi, M., Yamamoto, K., and Karnitz, L. M. (2007). The Rad9–Hus1–Rad1 (9–1–1) clamp activates checkpoint signaling via TopBP1. *Genes Dev.* 21, 1472–1477. doi: 10.1101/gad.1547007

Della-Maria, J., Zhou, Y., Tsai, M.-S., Kuhnlein, J., Carney, J. P., Paull, T. T., et al. (2011). Human Mre11/human Rad50/Nbs1 and DNA Ligase IIIα/XRCC1 protein complexes act together in an alternative nonhomologous end joining pathway. *Journal of Biological Chemistry* 286, 33845–33853. doi: 10.1074/jbc.M111.274159

Demple, B., and Harrison, L. (1994). Repair of oxidative damage to DNA: enzymology and biology. *Annu Rev Biochem* 63, 915–948. doi: 10.1146/annurev.bi.63.070194.004411

Demple, B., Sedgwick, B., Robins, P., Totty, N., Waterfield, M. D., and Lindahl, T. (1985). Active site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis. *Proc Natl Acad Sci U S A* 82, 2688–2692. doi: 10.1073/pnas.82.9.2688

Dianov, G., Price, A., and Lindahl, T. (1992). Generation of single-nucleotide repair patches following excision of uracil residues from DNA. *Molecular and Cellular Biology* 12, 1605–1612. doi: 10.1128/mcb.12.4.1605-1612.1992

Dobbs, T. A., Tainer, J. A., and Lees-Miller, S. P. (2010). A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation. *DNA Repair* 9, 1307–1314. doi: 10.1016/j.dnarep.2010.09.019

Doğan, E. S., and Liu, C. (2018). Three-dimensional chromatin packing and positioning of plant genomes. *Nature Plants* 4, 521–529. doi: 10.1038/s41477-018-0199-5

Doucet-Chabeaud, G., Godon, C., Brutesco, C., de Murcia, G., and Kazmaier, M. (2001). Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. *Mol Gen Genomics* 265, 954–963. doi: 10.1007/s004380100506

Dubest, S., Gallego, M. E., and White, C. I. (2002). Role of the AtRad1p endonuclease in homologous recombination in plants. *EMBO reports* 3, 1049–1054. doi: 10.1093/embo-reports/kvf211

Dulbecco, R. (1949). Reactivation of ultra-violet-inactivated bacteriophage by visible light. *Nature* 163, 949–950. doi: 10.1038/163949b0

Engeland, K. (2018). Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death Differ* 25, 114–132. doi: 10.1038/cdd.2017.172

Falck, J., Coates, J., and Jackson, S. P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* 434, 605–611. doi: 10.1038/nature03442

Fan, T., Kang, H., Wu, D., Zhu, X., Huang, L., Wu, J., et al. (2022). Arabidopsis γ-H2A.X-INTERACTING PROTEIN participates in DNA damage response and safeguards chromatin stability. *Nat Commun* 13, 7942. doi: 10.1038/s41467-022-35715-2

Fisher, A. E. O., Hochegger, H., Takeda, S., and Caldecott, K. W. (2007). Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase. *Mol Cell Biol* 27, 5597–5605. doi: 10.1128/mcb.02248-06

Fortini, P., Pascucci, B., Belisario, F., and Dogliotti, E. (2000). DNA polymerase β is required for efficient DNA strand break repair induced by methyl methanesulfonate but not by hydrogen peroxide. *Nucleic Acids Research* 28, 3040–3046. doi: 10.1093/nar/28.16.3040

Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environmental and Experimental Botany* 154, 134–142. doi: 10.1016/j.envexpbot.2018.05.003

Franklin, R. E., and Gosling, R. G. (1953a). Evidence for 2-chain helix in crystalline structure of sodium deoxyribonucleate. *Nature* 172, 156–157. doi: 10.1038/172156a0

Franklin, R., and Gosling, R. (1953b). Molecular configuration in sodium thymonucleate. *Nature*, 740–741.

Gallego, M. E., Jeanneau, M., Granier, F., Bouchez, D., Bechtold, N., and White, C. I. (2001). Disruption of the Arabidopsis RAD50 gene leads to plant sterility and MMS sensitivity. *Plant J* 25, 31–41. doi: 10.1046/j.1365-313x.2001.00928.x

Gallego, M. E., and White, C. I. (2001). RAD50 function is essential for telomere maintenance in Arabidopsis. *Proceedings of the National Academy of Sciences* 98, 1711–1716. doi: 10.1073/pnas.98.4.1711

Garcia, V., Bruchet, H., Camescasse, D., Granier, F., Bouchez, D., and Tissier, A. (2003). AtATM is essential for meiosis and the somatic response to DNA damage in plants. *The Plant Cell* 15, 119–132. doi: 10.1105/tpc.006577

Genschel, J., Littman, S. J., Drummond, J. T., and Modrich, P. (1998). Isolation of MutSbeta from human cells and comparison of the mismatch repair specificities of MutSbeta and MutSalpha. *J Biol Chem* 273, 19895–19901. doi: 10.1074/jbc.273.31.19895

Gong, Z., Cho, Y.-W., Kim, J.-E., Ge, K., and Chen, J. (2009). Accumulation of Pax2 transactivation domain interaction protein (PTIP) at sites of DNA breaks via RNF8-dependent pathway is required for cell survival after DNA damage. *Journal of Biological Chemistry* 284, 7284–7293. doi: 10.1074/jbc.M809158200

Goodhead, D. T. (1989). The initial physical damage produced by ionizing radiations. *International Journal of Radiation Biology* 56, 623–634. doi: 10.1080/09553008914551841

Gorbatova, I. V., Kazakova, E. A., Podlutskii, M. S., Pishenin, I. A., Bondarenko, V. S., Dontsova, A. A., et al. (2020). Studying gene expression in irradiated barley cultivars: PM19L-like and CML31-like expression as possible determinants of radiation hormesis effect. *Agronomy* 10, 1837. doi: 10.3390/agronomy10111837

Grice, D. M., Osmond, M. J., Stewart, M., Vonarx, E. J., and Kunz, B. A. (2007). "Roles of plant transcription factor IIH subunit homologs in nucleotide excision repair and transcription," in *New Research on DNA Repair* , ed. B. R. Landseer (New York, NY: Nova Science Publishers Inc.), 251–272.

Gu, J., Wang, Q., Cui, M., Han, B., Guo, H., Zhao, L., et al. (2014). Cloning and characterization of Ku70 and Ku80 homologues involved in DNA repair process in wheat (Triticum aestivum L.). *Plant Genetic Resources* 12, S99–S103. doi: 10.1017/S1479262114000367

Hacker, L., Dorn, A., and Puchta, H. (2020). Repair of DNA-protein crosslinks in plants. *DNA Repair* 87. Available at: https://www.sciencedirect.com/science/article/abs/pii/S1568786419303994 (Accessed August 15, 2023).

Hanawalt, P. C. (2002). Subpathways of nucleotide excision repair and their regulation. *Oncogene* 21, 8949–8956. doi: 10.1038/sj.onc.1206096

Hartung, F., and Puchta, H. (1999). Isolation of the complete cDNA of the Mre11 homologue of Arabidopsis (accession No. AJ243822) indicates conservation of DNA recombination mechanisms between plants and other eukaryotes. *Plant Physiology* 121, 312.

Hays, J. B. (2002). Arabidopsis thaliana, a versatile model system for study of eukaryotic genome-maintenance functions. *DNA Repair* 1, 579–600. doi: 10.1016/S1568-7864(02)00093-9

Hecht, S. S. (1999). DNA adduct formation from tobacco-specific N-nitrosamines. *Mutat Res* 424, 127–142. doi: 10.1016/s0027-5107(99)00014-7

Heitzeberg, F., Chen, I.-P., Hartung, F., Orel, N., Angelis, K. J., and Puchta, H. (2004). The Rad17 homologue of Arabidopsis is involved in the regulation of DNA damage repair and homologous recombination. *Plant J* 38, 954–968. doi: 10.1111/j.1365-313X.2004.02097.x

Hendi, N., Elsakrmy, N., and Ramotar, D. (2021). The base excision repair process: comparison between higher and lower eukaryotes. *Cellular and Molecular Life Sciences* 78. doi: 10.1007/s00018-021-03990-9

Hoencamp, C., Dudchenko, O., Elbatsh, A. M. O., Brahmachari, S., Raaijmakers, J. A., van Schaik, T., et al. (2021). 3D genomics across the tree of life reveals condensin II as a determinant of architecture type. *Science* 372, 984–989. doi: 10.1126/science.abe2218

Holliday, R., and Ho, T. (1998). Gene silencing and endogenous DNA methylation in mammalian cells. *Holliday, R., and T. Ho. "Gene silencing and endogenous DNA methylation in mammalian cells." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 400, 361–368.

Hombauer, H., Srivatsan, A., Putnam, C. D., and Kolodner, R. D. (2011). Mismatch repair, but not heteroduplex rejection, is temporally coupled to DNA replication. *Science* 334, 1713–1716. doi: 10.1126/science.1210770

Horvath, B., Kourova, H., Nagy, S., Nemeth, E., Magyar, Z., Papdi, C., et al. (2017). Arabidopsis RETINOBLASTOMA RELATED directly regulates DNA damage responses through functions beyond cell cycle control. *EMBO Journal*, 1261–1278.

Hou, X., Wang, D., Cheng, Z., Wang, Y., and Jiao, Y. (2022). A near-complete assembly of an Arabidopsis thaliana genome. *Mol Plant* 15, 1247–1250. doi: 10.1016/j.molp.2022.05.014

Hsiang, Y. H., Hertzberg, R., Hecht, S., and Liu, L. F. (1985). Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 260, 14873–14878.

Huefner, N. D., Britt, A. B., and Friesner, J. D. (2009). Characterization of two H2AX homologues in Arabidopsis thaliana and their response to ionizing radiation. Available at: https://www.osti.gov/etdeweb/biblio/21497057 (Accessed August 26, 2023).

Ishibashi, T., Koga, A., Yamamoto, T., Uchiyama, Y., Mori, Y., Hashimoto, J., et al. (2005). Two types of replication protein A in seed plants. *FEBS J* 272, 3270–3281. doi: 10.1111/j.1742-4658.2005.04719.x

Jackson, M. W., Agarwal, M. K., Yang, J., Bruss, P., Uchiumi, T., Agarwal, M. L., et al. (2005). p130/p107/p105Rb-dependent transcriptional repression during DNA-damage-induced cell-cycle exit at G2. *J Cell Sci* 118, 1821–1832. doi: 10.1242/jcs.02307

Jacobs, A. L., and Schär, P. (2012). DNA glycosylases: in DNA repair and beyond. *Chromosoma* 121, 1–20. doi: 10.1007/s00412-011-0347-4

Jaskowiak, J., Kwasniewska, J., Szurman-Zubrzycka, M., Rojek-Jelonek, M., Larsen, P. B., and Szarejko, I. (2020). Al-tolerant barley mutant hvatr.g shows the ATR-regulated DNA damage response to maleic acid hydrazide. *International Journal of Molecular Sciences* 21, 8500. doi: 10.3390/ijms21228500

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C. M., Lukas, J., et al. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* 8, 37–45. doi: 10.1038/ncb1337

Jiang, C.-Z., Yee, J., Mitchell, D. L., and Britt, A. B. (1997). Photorepair mutants of Arabidopsis. *Proceedings of the National Academy of Sciences* 94, 7441–7445. doi: 10.1073/pnas.94.14.7441

Keeney, S. (2001). Mechanism and control of meiotic recombination initiation. *Curr Top Dev Biol* 52, 1–53. doi: 10.1016/s0070-2153(01)52008-6

Kelner, A. (1949). Effect of visible light on the recovery of Streptomyces griseus conidia from ultra-violet irradiation injury. *Proceedings of the National Academy of Sciences* 35, 73–79. doi: 10.1073/pnas.35.2.73

Kiontke, S., Gnau, P., Haselsberger, R., Batschauer, A., and Essen, L.-O. (2014). Structural and evolutionary aspects of antenna chromophore usage by class II photolyases. *J Biol Chem* 289, 19659–19669. doi: 10.1074/jbc.m113.542431

Kirik, A., Salomon, S., and Puchta, H. (2000). Species-specific double-strand break repair and genome evolution in plants. *EMBO J* 19, 5562–5566. doi: 10.1093/emboj/19.20.5562

Kobayashi, K., Suzuki, T., Iwata, E., Magyar, Z., Bögre, L., and Ito, M. (2015). MYB3Rs, plant homologs of Myb oncoproteins, control cell cycle-regulated transcription and form DREAM-like complexes. *Transcription* 6, 106–111. doi: 10.1080/21541264.2015.1109746

Kozlov, S., Graham, M., Peng, C., Chen, P., Robinson, P., and Lavin, M. (2006). Involvement of novel autophosphorylation sites in ATM activation. *EMBO Journal* 9;25, 3504–14.

Kramer, K. M., Brock, J. A., Bloom, K., Moore, J. K., and Haber, J. E. (1994). Two different types of double-strand breaks in Saccharomyces cerevisiae are repaired by similar RAD52-independent, nonhomologous recombination events. *Mol Cell Biol* 14, 1293–1301.

Krause, K., Wasner, M., Reinhard, W., Haugwitz, U., Dohna, C. L., Mössner, J., et al. (2000). The tumour suppressor protein p53 can repress transcription of cyclin B. *Nucleic Acids Res* 28, 4410–4418. doi: 10.1093/nar/28.22.4410

Krishna, S., Wagener, B. M., Liu, H. P., Lo, Y.-C., Sterk, R., Petrini, J. H. J., et al. (2007). Mre11 and Ku regulation of double-strand break repair by gene conversion and break-induced replication. *DNA Repair* 6, 797–808. doi: 10.1016/j.dnarep.2007.01.006

Kumagai, A., and Dunphy, W. G. (2000). Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. *Mol Cell* 6, 839–849. doi: 10.1016/s1097-2765(05)00092-4

Kumar, N., and Larkin, J. C. (2017). Why do plants need so many cyclin-dependent kinase inhibitors? *Plant Signaling & Behavior* 12, e1282021. doi: 10.1080/15592324.2017.1282021

Kumar, S. (2007). Caspase function in programmed cell death. *Cell Death Differ* 14, 32–43. doi: 10.1038/sj.cdd.4402060

Kunkel, T. A. (2009). Evolving views of DNA replication (in)fidelity. *Cold Spring Harb Symp Quant Biol* 74, 91–101. doi: 10.1101/sqb.2009.74.027

Kunz, B. A., Anderson, H. J., Osmond, M. J., and Vonarx, E. J. (2005). Components of nucleotide excision repair and DNA damage tolerance in Arabidopsis thaliana. *Environmental and Molecular Mutagenesis* 45, 115–127. doi: 10.1002/em.20094

Kuzminov, A. (2001). Single-strand interruptions in replicating chromosomes cause double-strand breaks. *Proceedings of the National Academy of Sciences* 98, 8241–8246. doi: 10.1073/pnas.131009198

Kuznetsova, A. Y., Seget, K., Moeller, G. K., de Pagter, M. S., de Roos, J. A. D. M., Dürrbaum, M., et al. (2015). Chromosomal instability, tolerance of mitotic errors and multidrug resistance are promoted by tetraploidization in human cells. *Cell Cycle* 14, 2810–2820. doi: 10.1080/15384101.2015.1068482

Lafarge, S., and Montané, M. -H. (2003). Characterization of Arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1 , strongly induced by gamma rays. *Nucleic Acids Research* 31, 1148–1155. doi: 10.1093/nar/gkg202

Lam, E., and Zhang, Y. (2012). Regulating the reapers: activating metacaspases for programmed cell death. *Trends Plant Sci* 17, 487–494. doi: 10.1016/j.tplants.2012.05.003

Lang, L., Pettkó-Szandtner, A., Elbaşı, H. T., Takatsuka, H., Nomoto, Y., Zaki, A., et al. (2021). The DREAM complex represses growth in response to DNA damage in Arabidopsis. *Life Science Alliance* 4. doi: 10.26508/lsa.202101141

Lario, L. D., Ramirez-Parra, E., Gutierrez, C., Casati, P., and Spampinato, C. P. (2011). Regulation of plant MSH2 and MSH6 genes in the UV-B-induced DNA damage response. *Journal of Experimental Botany* 62, 2925–2937. doi: 10.1093/jxb/err001

Lawley, P. D. (1974). Some chemical aspects of dose-response relationships in alkylation mutagenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 23, 283–295. doi: 10.1016/0027-5107(74)90102-X

Lee, Y.-C., Cai, Y., Mu, H., Broyde, S., Amin, S., Chen, X., et al. (2014). The relationships between XPC binding to conformationally diverse DNA adducts and their excision by the human NER system: is there a correlation. *DNA Repair (Amst)* 19, 55–63. doi: 10.1016/j.dnarep.2014.03.026

Leung, C., and Glover, J. (2011). BRCT domains: easy as one, two, three. *Cell cycle* 10, 2461–70.

Levin, D. S., Bai, W., Yao, N., O'Donnell, M., and Tomkinson, A. E. (1997). An interaction between DNA ligase I and proliferating cell nuclear antigen: Implications for Okazaki fragment synthesis and joining. *Proceedings of the National Academy of Sciences* 94, 12863–12868. doi: 10.1073/pnas.94.24.12863

Li, X., and Heyer, W.-D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* 18, 99–113. doi: 10.1038/cr.2008.1

Liang, L., Flury, S., Kalck, V., Hohn, B., and Molinier, J. (2006). CENTRIN2 interacts with the Arabidopsis homolog of the human XPC protein (AtRAD4) and contributes to efficient synthesis-dependent repair of bulky DNA lesions. *Plant Mol Biol* 61, 345–356. doi: 10.1007/s11103-006-0016-9

Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 79, 181–211. doi: 10.1146/annurev.biochem.052308.093131

Lindahl, T. (1982). DNA repair enzymes. *Annu Rev Biochem* 51, 61–87. doi: 10.1146/annurev.bi.51.070182.000425

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* 362, 709–715. doi: 10.1038/362709a0

Lindahl, T., and Nyberg, B. (1972). Rate of depurination of native deoxyribonucleic acid. *Biochemistry* 11, 3610–3618. doi: 10.1021/bi00769a018

Lloyd, A. H., Wang, D., and Timmis, J. N. (2012). Single molecule PCR reveals similar patterns of non-homologous DSB repair in tobacco and Arabidopsis. *PLOS ONE* 7, e32255. doi: 10.1371/journal.pone.0032255

Locato, V., and De Gara, L. (2018). Programmed cell death in plants: An overview. *Methods Mol Biol* 1743, 1–8. doi: 10.1007/978-1-4939-7668-3_1

Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). "DNA damage and repair and their role in carcinogenesi," in *Molecular Cell Biology* (WH Freeman.).

Loeb, L. A., and Preston, B. D. (1986). Mutagenesis by apurinic/apyrimidinic sites. *Annu Rev Genet* 20, 201–230. doi: 10.1146/annurev.ge.20.120186.001221

Loechler, E. L., Green, C. L., and Essigmann, J. M. (1984). In vivo mutagenesis by O6-methylguanine built into a unique site in a viral genome. *Proceedings of the National Academy of Sciences* 81, 6271–6275. doi: 10.1073/pnas.81.20.6271

Lorković, Z. J., Klingenbrunner, M., Cho, C. H., and Berger, F. (2023). Co-evolution of functional motifs and H2A.X in the context of DNA damage response identifies the plant Mediator of DNA Damage Checkpoint 1. 2023.05.19.541430. doi: 10.1101/2023.05.19.541430

Lucas-Lledó, J. I., and Lynch, M. (2009). Evolution of mutation rates: phylogenomic analysis of the photolyase/cryptochrome family. *Mol Biol Evol* 26, 1143–1153. doi: 10.1093/molbev/msp029

MacLachlan, T. K., Takimoto, R., and El-Deiry, W. S. (2002). BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets. *Mol Cell Biol* 22, 4280–4292. doi: 10.1128/MCB.22.12.4280-4292.2002

Majka, J., Binz, S., Wold, M., and Burgers (2006). Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. *Journal of Biological Chemistry* 281, 27855–61.

Manova, V., and Gruszka, D. (2015). DNA damage and repair in plants – from models to crops. *Frontiers in Plant Science* 6. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2015.00885 (Accessed August 15, 2022).

Mari, P.-O., Florea, B. I., Persengiev, S. P., Verkaik, N. S., Brüggenwirth, H. T., Modesti, M., et al. (2006). Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proceedings of the National Academy of Sciences* 103, 18597–18602. doi: 10.1073/pnas.0609061103

Mascher, M., Wicker, T., Jenkins, J., Plott, C., Lux, T., Koh, C. S., et al. (2021). Long-read sequence assembly: a technical evaluation in barley. *The Plant Cell* 33, 1888–1906. doi: 10.1093/plcell/koab077

McGowan, C. h., and Russell, P. (1995). Cell cycle regulation of human WEE1. *The EMBO Journal* 14, 2166–2175. doi: 10.1002/j.1460-2075.1995.tb07210.x

Memisoglu, A., and Samson, L. (2000). Base excision repair in yeast and mammals. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 451, 39–51. doi: 10.1016/S0027-5107(00)00039-7

Miescher, F. (1897). *Die histochemischen und physiologischen Arbeiten von Friedrich Miescher: Arbeiten von F. Miescher. Band 2 B: Aus dem physiologischen Laboratorium von weil. Prof F. Miescher an der Universität in Basel. Untersuchungen über den Einfluss des Höhenklimas auf die Beschaffenheit des Blutes*. Vogel.

Mishina, Y., and He, C. (2006). Oxidative dealkylation DNA repair mediated by the mononuclear non-heme iron AlkB proteins. *J Inorg Biochem* 100, 670–678. doi: 10.1016/j.jinorgbio.2005.12.018

Mladenov, E., and Iliakis, G. (2011). Induction and repair of DNA double strand breaks: The increasing spectrum of non-homologous end joining pathways. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 711, 61–72. doi: 10.1016/j.mrfmmm.2011.02.005

Modrich, P. (2006). Mechanisms in eukaryotic mismatch repair. *J Biol Chem* 281, 30305–30309. doi: 10.1074/jbc.r600022200

Molinier, J. (2017). Genome and epigenome surveillance processes underlying UV exposure in plants. *Genes* 8, 316. doi: 10.3390/genes8110316

Molinier, J., Lechner, E., Dumbliauskas, E., and Genschik, P. (2008). Regulation and role of Arabidopsis CUL4-DDB1A-DDB2 in maintaining genome integrity upon UV stress. *PLoS Genet* 4, e1000093. doi: 10.1371/journal.pgen.1000093

Mortusewicz, O., Rothbauer, U., Cardoso, M. C., and Leonhardt, H. (2006). Differential recruitment of DNA Ligase I and III to DNA repair sites. *Nucleic Acids Research* 34, 3523–3532. doi: 10.1093/nar/gkl492

Motycka, T. A., Bessho, T., Post, S. M., Sung, P., and Tomkinson, A. E. (2004). Physical and functional interaction between the XPF/ERCC1 endonuclease and hRad52 *. *Journal of Biological Chemistry* 279, 13634–13639. doi: 10.1074/jbc.M313779200

Murozuka, E., Massange-Sánchez, J. A., Nielsen, K., Gregersen, P. L., and Braumann, I. (2018). Genome wide characterization of barley NAC transcription factors enables the identification of grain-specific transcription factors exclusive for the Poaceae family of monocotyledonous plants. *PLOS ONE* 13, e0209769. doi: 10.1371/journal.pone.0209769

Nakajima, S., Sugiyama, M., Iwai, S., Hitomi, K., Otoshi, E., Kim, S. T., et al. (1998). Cloning and characterization of a gene (UVR3) required for photorepair of 6-4 photoproducts in Arabidopsis thaliana. *Nucleic Acids Res* 26, 638–644. doi: 10.1093/nar/26.2.638

Narod, S. A., and Foulkes, W. D. (2004). BRCA1 and BRCA2: 1994 and beyond. *Nat Rev Cancer* 4, 665–676. doi: 10.1038/nrc1431

Nathan, C. (2003). Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signaling. *J Clin Invest* 111, 769–778. doi: 10.1172/JCI18174

Nishizawa-Yokoi, A., Motoyama, R., Tanaka, T., Mori, A., Iida, K., and Toki, S. (2023). SUPPRESSOR OF GAMMA RESPONSE 1 plays rice-specific roles in DNA damage response and repair. *Plant Physiology* 191, 1288–1304. doi: 10.1093/plphys/kiac490

Nishizawa-Yokoi, A., Nonaka, S., Saika, H., Kwon, Y.-I., Osakabe, K., and Toki, S. (2012). Suppression of Ku70/80 or Lig4 leads to decreased stable transformation and enhanced homologous recombination in rice. *New Phytologist* 196, 1048–1059. doi: 10.1111/j.1469-8137.2012.04350.x

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., et al. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 102, 849–862. doi: 10.1016/s0092-8674(00)00073-8

Ogi, T., Limsirichaikul, S., Overmeer, R. M., Volker, M., Takenaka, K., Cloney, R., et al. (2010). Three DNA polymerases, recruited by different mechanisms, carry out NER repair synthesis in human cells. *Mol Cell* 37, 714–727. doi: 10.1016/j.molcel.2010.02.009

Ogita, N., Okushima, Y., Tokizawa, M., Yamamoto, Y. Y., Tanaka, M., Seki, M., et al. (2018). Identifying the target genes of SUPPRESSOR OF GAMMA RESPONSE 1, a master transcription factor controlling DNA damage response in Arabidopsis. *The Plant Journal* 94, 439–453. doi: 10.1111/tpj.13866

Ohouo, P. Y., Bastos de Oliveira, F. M., Almeida, B. S., and Smolka, M. B. (2010). DNA damage signaling recruits the Rtt107-Slx4 scaffolds via Dpb11 to mediate replication stress response. *Mol Cell* 39, 300–306. doi: 10.1016/j.molcel.2010.06.019

Olvera-Carrillo, Y., Van Bel, M., Van Hautegem, T., Fendrych, M., Huysmans, M., Simaskova, M., et al. (2015). A conserved core of programmed cell death indicator genes discriminates

developmentally and environmentally induced programmed cell death in plants. *Plant Physiol* 169, 2684–2699. doi: 10.1104/pp.15.00769

Orel, N., Kyryk, A., and Puchta, H. (2003). Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome. *The Plant Journal* 35, 604–612. doi: 10.1046/j.1365-313X.2003.01832.x

Oren, M. (2003). Decision making by p53: life, death and cancer. *Cell Death Differ* 10, 431–442. doi: 10.1038/sj.cdd.4401183

Paul-Konietzko, K., Thomale, J., Arakawa, H., and Iliakis, G. (2015). DNA Ligases I and III support nucleotide excision repair in DT40 cells with similar efficiency. *Photochemistry and Photobiology* 91, 1173–1180. doi: 10.1111/php.12487

Peak, M. J., Peak, J. G., and Jones, C. A. (1985). Different (direct or indirect) mechanisms for the induction of DNA-protein crosslinks in human cells by far- and near-ultra-violet radiations (290 and 405 nm). *Photochemistry and Photobiology* 42, 141–146. doi: 10.1111/j.1751-1097.1985.tb01552.x

Pegg, A. E. (2011). Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. *Chem. Res. Toxicol.* 24, 618–639. doi: 10.1021/tx200031q

Petrini, J. H. J., and Stracker, T. H. (2003). The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends in Cell Biology* 13, 458–462. doi: 10.1016/S0962-8924(03)00170-3

Pietsch, E. C., Sykes, S. M., McMahon, S. B., and Murphy, M. E. (2008). The p53 family and programmed cell death. *Oncogene* 27, 6507–6521. doi: 10.1038/onc.2008.315

Povirk, L. F., Wübter, W., Köhnlein, W., and Hutchinson, F. (1977). DNA double-strand breaks and alkali-labile bonds produced by bleomycin. *Nucleic Acids Research* 4, 3573. doi: 10.1093/nar/4.10.3573

Preuss, S. B., and Britt, A. B. (2003). A DNA-damage-induced cell cycle checkpoint in Arabidopsis. *Genetics* 164, 323–334. doi: 10.1093/genetics/164.1.323

Prochazkova, K., Finke, A., Tomaštíková, E. D., Filo, J., Bente, H., Dvořák, P., et al. (2022). Zebularine induces enzymatic DNA–protein crosslinks in 45S rDNA heterochromatin of Arabidopsis nuclei. *Nucleic Acids Research* 50, 244–258. doi: 10.1093/nar/gkab1218

Puchta, H. (2005). The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J Exp Bot* 56, 1–14. doi: 10.1093/jxb/eri025

Puchta, H., and Fauser, F. (2014). Synthetic nucleases for genome engineering in plants: prospects for a bright future. *The Plant Journal* 78, 727–741. doi: 10.1111/tpj.12338

Puig, P.-E., Guilly, M.-N., Bouchot, A., Droin, N., Cathelin, D., Bouyer, F., et al. (2008). Tumor cells can escape DNA-damaging cisplatin through DNA endoreduplication and reversible polyploidy. *Cell Biol Int* 32, 1031–1043. doi: 10.1016/j.cellbi.2008.04.021

Quaas, M., Müller, G. A., and Engeland, K. (2012). p53 can repress transcription of cell cycle genes through a p21WAF1/CIP1-dependent switch from MMB to DREAM protein complex binding at CHR promoter elements. *Cell Cycle* 11, 4661–4672. doi: 10.4161/cc.22917

Rabl, C. (1885). Uber zellthilung. *Morphol Jahrb*, 214–330.

Rajski, S. R., and Williams, R. M. (1998). DNA cross-linking agents as antitumor drugs. *Chem Rev* 98, 2723–2796. doi: 10.1021/cr9800199

Räschle, M., Smeenk, G., Hansen, R. K., Temu, T., Oka, Y., Hein, M. Y., et al. (2015). Proteomics reveals dynamic assembly of repair complexes during bypass of DNA cross-links. *Science* 348, 1253671. doi: 10.1126/science.1253671

Ray Chaudhuri, A., and Nussenzweig, A. (2017). The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nat Rev Mol Cell Biol* 18, 610–621. doi: 10.1038/nrm.2017.53

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ. *Current Opinion in Genetics & Development* 12, 162–169. doi: 10.1016/S0959-437X(02)00282-4

Reidt, W., Wurz, R., Wanieck, K., Ha Chu, H., and Puchta, H. (2006). A homologue of the breast cancer-associated gene BARD1 is involved in DNA repair in plants. *The EMBO Journal* 25, 4326–4337. doi: 10.1038/sj.emboj.7601313

Robinson, H., Hickey, L., Richard, C., Mace, E., Kelly, A., Borrell, A., et al. (2016). Genomic Regions Influencing Seminal Root Traits in Barley. *The Plant Genome* 9, plantgenome2015.03.0012. doi: 10.3835/plantgenome2015.03.0012

Roitinger, E., Hofer, M., Köcher, T., Pichler, P., Novatchkova, M., Yang, J., et al. (2015). Quantitative Phosphoproteomics of the Ataxia Telangiectasia-Mutated (ATM) and Ataxia Telangiectasia-Mutated and Rad3-related (ATR) Dependent DNA Damage Response in Arabidopsis thaliana*[S]. *Molecular & Cellular Proteomics* 14, 556–571. doi: 10.1074/mcp.M114.040352

Roldán-Arjona, T., Ariza, R. R., and Córdoba-Cañero, D. (2019). DNA base excision repair in plants: An unfolding story with familiar and novel characters. *Frontiers in Plant Science* 10. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01055 (Accessed August 16, 2023).

Rosenstein, B. S., and Ducore, J. M. (1983). Induction of DNA strand breaks in normal human fibroblasts exposed to monochromatic ultraviolet and visible wavelengths in the 240–546 nm range. *Photochemistry and Photobiology* 38, 51–55. doi: 10.1111/j.1751-1097.1983.tb08365.x

Rothenberg, E., Grimme, J. M., Spies, M., and Ha, T. (2008). Human Rad52-mediated homology search and annealing occurs by continuous interactions between overlapping nucleoprotein complexes. *Proceedings of the National Academy of Sciences* 105, 20274–20279. doi: 10.1073/pnas.0810317106

Rother, K., Kirschner, R., Sänger, K., Böhlig, L., Mössner, J., and Engeland, K. (2007). p53 downregulates expression of the G1/S cell cycle phosphatase Cdc25A. *Oncogene* 26, 1949–1953. doi: 10.1038/sj.onc.1209989

Rydberg, B., and Lindahl, T. (1982). Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. *EMBO J* 1, 211–216.

Sadasivam, S., and DeCaprio, J. A. (2013). The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nat Rev Cancer* 13, 585–595. doi: 10.1038/nrc3556

Sancar, A. (2003). Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem Rev* 103, 2203–2237. doi: 10.1021/cr0204348

Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., et al. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* 277, 1497–1501. doi: 10.1126/science.277.5331.1497

Scally, A., and Durbin, R. (2012). Revising the human mutation rate: implications for understanding human evolution. *Nat Rev Genet* 13, 745–753. doi: 10.1038/nrg3295

Schreiber, M., Barakate, A., Uzrek, N., Macaulay, M., Sourdille, A., Morris, J., et al. (2019). A highly mutagenised barley (cv. Golden Promise) TILLING population coupled with strategies for screening-by-sequencing. *Plant Methods* 15, 99. doi: 10.1186/s13007-019-0486-9

Selby, C. P., and Sancar, A. (2012). The second chromophore in Drosophila photolyase/cryptochrome family photoreceptors. *Biochemistry* 51, 167–171. doi: 10.1021/bi201536w

Setlow, R. B., and Carrier, W. L. (1964). The disappearance of thymine dimers from dna: an error-correcting mechanism. *Proceedings of the National Academy of Sciences* 51, 226–231. doi: 10.1073/pnas.51.2.226

Shearman, C., and Lawrence, A. (1979). Effects of depurination on the fidelity of DNA synthesis. *Journal of Molecular Biology* 128, 197–218.

Shimada, K., Filipuzzi, I., Stahl, M., Helliwell, S. B., Studer, C., Hoepfner, D., et al. (2013). TORC2 signaling pathway guarantees genome stability in the face of DNA strand breaks. *Mol Cell* 51, 829–839. doi: 10.1016/j.molcel.2013.08.019

Shiotani, B., and Zou, L. (2009). Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Mol Cell* 33, 547–558. doi: 10.1016/j.molcel.2009.01.024

Singer, B., and Grunberger, D. (1983). "Reactions of directly acting agents with nucleic acids," in *Molecular Biology of Mutagens and Carcinogens* , eds. B. Singer and D. Grunberger (Boston, MA: Springer US), 45–96. doi: 10.1007/978-1-4613-3772-0_4

Sjogren, Bolaris, S., and Larsen, P. (2015). Aluminum-ependent terminal differentiation of the Arabidopsis root tip Is mediated through an ATR-, ALT2-, and SOG1-regulated transcriptional response. *Plant Cell* 27, 2501–15.

Smith, G. C., and Jackson, S. P. (1999). The DNA-dependent protein kinase. *Genes Dev* 13, 916–934. doi: 10.1101/gad.13.8.916

Smits, VAJ, Reaper, P., and Jackson (2005). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Current Biology* 16, 150–9.

Spampinato, C. P., Gomez, R. L., Galles, C., and Lario, L. D. (2009). From bacteria to plants: a compendium of mismatch repair assays. *Mutat Res* 682, 110–128. doi: 10.1016/j.mrrev.2009.07.001

Spivak, G. (2015). Nucleotide excision repair in humans. *DNA Repair (Amst)* 36, 13–18. doi: 10.1016/j.dnarep.2015.09.003

Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M. R., and Elledge, S. J. (2003). MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature* 421, 961–966. doi: 10.1038/nature01446

Stolarek, M., Gruszka, D., Braszewska-Zalewska, A., and Maluszynski, M. (2015a). Alleles of newly identified barley gene HvPARP3 exhibit changes in efficiency of DNA repair. *DNA Repair* 28, 116–130. doi: 10.1016/j.dnarep.2015.02.018

Stolarek, M., Gruszka, D., Braszewska-Zalewska, A., and Maluszynski, M. (2015b). Functional analysis of the new barley gene HvKu80 indicates that it plays a key role in double-strand DNA break repair and telomere length regulation. *Mutagenesis* 30, 785–797. doi: 10.1093/mutage/gev033

Storchova, Z., and Pellman, D. (2004). From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 5, 45–54. doi: 10.1038/nrm1276

Stucki, M., and Jackson, S. P. (2006). gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst)* 5, 534–543. doi: 10.1016/j.dnarep.2006.01.012

Sung, P. (1994). Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science* 265, 1241–1243. doi: 10.1126/science.8066464

Sutherland, J. C., and Griffin, K. (1981). Absorption Spectrum of DNA for Wavelengths Greater than 300 nm. *Radiation Research* 86, 399–400.

Sweeney, P. R., Britt, A. B., and Culligan, K. M. (2009). The Arabidopsis ATRIP ortholog is required for a programmed response to replication inhibitors. *Plant J* 60, 518–526. doi: 10.1111/j.1365-313X.2009.03975.x

Szurman-Zubrzycka, M. E., Zbieszczyk, J., Marzec, M., Jelonek, J., Chmielewska, B., Kurowska, M. M., et al. (2018). HorTILLUS—A rich and renewable source of induced mutations for forward/reverse genetics and pre-breeding programs in barley (Hordeum vulgare L.). *Frontiers in Plant Science* 9. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00216 (Accessed January 18, 2023).

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. *Frontiers in Plant Science* 10. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01299 (Accessed January 18, 2023).

Tamura, K., Adachi, Y., Chiba, K., Oguchi, K., and Takahashi, H. (2002). Identification of Ku70 and Ku80 homologues in Arabidopsis thaliana: evidence for a role in the repair of DNA double-strand breaks. *The Plant Journal* 29, 771–781. doi: 10.1046/j.1365-313X.2002.01258.x

Taverna, P., and Sedgwick, B. (1996). Generation of an endogenous DNA-methylating agent by nitrosation in Escherichia coli. *J Bacteriol* 178, 5105–5111. doi: 10.1128/jb.178.17.5105-5111.1996

Taylor, W. R., Schonthal, A. H., Galante, J., and Stark, G. R. (2001). p130/E2F4 binds to and represses the cdc2 promoter in response to p53. *J Biol Chem* 276, 1998–2006. doi: 10.1074/jbc.M005101200

Tenaillon, O., and Matic, I. (2020). The impact of neutral mutations on genome evolvability. *Current Biology* 30, R527–R534. doi: 10.1016/j.cub.2020.03.056

Thelen, M. P., Venclovas, Č., and Fidelis, K. (1999). A sliding clamp model for the Rad1 family of cell cycle checkpoint proteins. *Cell* 96, 769–770. doi: 10.1016/S0092-8674(00)80587-5

Tibbetts, R., and Abraham, R. (2000). "PI3K-related kinases: Roles in cell-cycle regulation and DNA damage," in *Signaling networks and cell cycle control: The molecular basis of cancer and other diseases* (Totowa, NJ: Humana Press), 267–301.

Timoféeff-Ressovsky, N. w., Zimmer, K. G., and Delbrück, M. (1935). *Über die Natur der Genmutation und der Genstruktur*. WEIDMANNSCHE BUCHHANDLUNG. Available at: https://www.elibrary.ru/item.asp?id=29889273 (Accessed August 15, 2023).

Tomasz, M. (1995). Mitomycin C: Small, fast and deadly (but very selective). *Chemistry & Biology* 2, 575–579. doi: 10.1016/1074-5521(95)90120-5

Trapp, O., Seeliger, K., and Puchta, H. (2011). Homologs of breast cancer genes in plants. *Frontiers in Plant Science* 2. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2011.00019 (Accessed August 8, 2022).

van Doorn, W. G. (2011). Classes of programmed cell death in plants, compared to those in animals. *J Exp Bot* 62, 4749–4761. doi: 10.1093/jxb/err196

van Gent, D. C., Hoeijmakers, J. H. J., and Kanaar, R. (2001). Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* 2, 196–206. doi: 10.1038/35056049

Viguera, E., Canceill, D., and Ehrlich, S. D. (2001). Replication slippage involves DNA polymerase pausing and dissociation. *EMBO J* 20, 2587–2595. doi: 10.1093/emboj/20.10.2587

Vladejić, J., Yang, F., Dvořák Tomaštíková, E., Doležel, J., Palecek, J. J., and Pecinka, A. (2022). Analysis of BRCT5 domain-containing proteins reveals a new component of DNA damage repair in Arabidopsis. *Frontiers in Plant Science* 13. Available at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1023358 (Accessed April 11, 2023).

Vlieghe, K., Boudolf, V., Beemster, G. T. S., Maes, S., Magyar, Z., Atanassova, A., et al. (2005). The DP-E2F-like gene DEL1 controls the endocycle in Arabidopsis thaliana. *Curr Biol* 15, 59–63. doi: 10.1016/j.cub.2004.12.038

Volkova, P. Y., Duarte, G. T., Soubigou-Taconnat, L., Kazakova, E. A., Pateyron, S., Bondarenko, V. S., et al. (2020). Early response of barley embryos to low- and high-dose gamma irradiation of seeds triggers changes in the transcriptional profile and an increase in hydrogen peroxide content in seedlings. *Journal of Agronomy and Crop Science* 206, 277–295. doi: 10.1111/jac.12381

Vu, G. T. H., Cao, H. X., Watanabe, K., Hensel, G., Blattner, F. R., Kumlehn, J., et al. (2014). Repair of site-specific DNA double-strand breaks in barley occurs via diverse pathways primarily involving the sister chromatid. *The Plant Cell* 26, 2156–2167. doi: 10.1105/tpc.114.126607

Walker, J. R., Corpina, R. A., and Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 412, 607–614. doi: 10.1038/35088000

Wan, B., Hang, L. E., and Zhao, X. (2016). Multi-BRCT scaffolds use distinct strategies to support genome maintenance. *Cell Cycle* 15, 2561–2570. doi: 10.1080/15384101.2016.1218102

Wang, K., Ndathe, R. W., Kumar, N., Zeringue, E. A., Kato, N., and Larkin, J. C. (2020). The CDK inhibitor SIAMESE targets both CDKA;1 and CDKB1 complexes to establish endoreplication in trichomes. *Plant Physiol* 184, 165–175. doi: 10.1104/pp.20.00271

Wang, X., Takenaka, K., and Takeda, S. (2010). PTIP promotes DNA double-strand break repair through homologous recombination. *Genes to Cells* 15, 243–254. doi: 10.1111/j.1365-2443.2009.01379.x

Ward IM and Chen J (2001). Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress - PubMed. *Journal of Biological Chemistry* 276, 47759–62.

Waterworth, W. M., Altun, C., Armstrong, S. J., Roberts, N., Dean, P. J., Young, K., et al. (2007). NBS1 is involved in DNA repair and plays a synergistic role with ATM in mediating meiotic homologous recombination in plants. *The Plant Journal* 52, 41–52. doi: 10.1111/j.1365-313X.2007.03220.x

Watson, J. D., and Crick, F. H. C. (1953). Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature* 171, 737–738. doi: 10.1038/171737a0

Wendt, T., Holm, P. B., Starker, C. G., Christian, M., Voytas, D. F., Brinch-Pedersen, H., et al. (2013). TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. *Plant Mol Biol* 83, 279–285. doi: 10.1007/s11103-013-0078-4

West, C. E., Waterworth, W. M., Jiang, Q., and Bray, C. M. (2000). Arabidopsis DNA ligase IV is induced by γ-irradiation and interacts with an Arabidopsis homologue of the double strand break repair protein XRCC4. *The Plant Journal* 24, 67–78. doi: 10.1046/j.1365-313x.2000.00856.x

West, C. E., Waterworth, W. M., Story, G. W., Sunderland, P. A., Jiang, Q., and Bray, C. M. (2002). Disruption of the Arabidopsis AtKu80 gene demonstrates an essential role for AtKu80 protein in efficient repair of DNA double-strand breaks in vivo. *The Plant Journal* 31, 517–528. doi: 10.1046/j.1365-313X.2002.01370.x

Wilkins, M., Stokes, A., and Wilson, H. (1953). Molecular structure of deoxypentose nucleic acids. *Nature*, 738.

Wold, M. S. (1997). Replication protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annual Review of Biochemistry* 66, 61–92. doi: 10.1146/annurev.biochem.66.1.61

Wu, P.-Y., Frit, P., Meesala, S., Dauvillier, S., Modesti, M., Andres, S. N., et al. (2009). Structural and functional interaction between the human DNA repair proteins DNA Ligase IV and XRCC4. *Molecular and Cellular Biology* 29, 3163–3172. doi: 10.1128/MCB.01895-08

Wu, S., Culligan, K., Lamers, M., and Hays, J. (2003). Dissimilar mispair-recognition spectra of Arabidopsis DNA-mismatch-repair proteins MSH2·MSH6 (MutSα) and MSH2·MSH7 (MutSγ). *Nucleic Acids Research* 31, 6027–6034. doi: 10.1093/nar/gkg780

Yano, K., Morotomi-Yano, K., Lee, K.-J., and Chen, D. J. (2011). Functional significance of the interaction with Ku in DNA double-strand break recognition of XLF. *FEBS Letters* 585, 841–846. doi: 10.1016/j.febslet.2011.02.020

Yano, K., Morotomi-Yano, K., Wang, S.-Y., Uematsu, N., Lee, K.-J., Asaithamby, A., et al. (2008). Ku recruits XLF to DNA double-strand breaks. *EMBO reports* 9, 91–96. doi: 10.1038/sj.embor.7401137

Yi, C., and He, C. (2013). DNA repair by reversal of DNA damage. *Cold Spring Harb Perspect Biol* 5, a012575. doi: 10.1101/cshperspect.a012575

Yi, D., Alvim Kamei, C. L., Cools, T., Vanderauwera, S., Takahashi, N., Okushima, Y., et al. (2014). The Arabidopsis SIAMESE-RELATED cyclin-dependent kinase inhibitors SMR5 and SMR7 regulate the DNA damage checkpoint in response to reactive oxygen species. *Plant Cell* 26, 296–309. doi: 10.1105/tpc.113.118943

Yonekura, S. I., Nakamura, N., Yonei, S., and Zhang-Akiyama, Q. M. (2009). Generation, biological consequences and repair mechanisms of cytosine deamination in DNA. *Journal of radiation research* 50, 19–26. doi: 10.1269/jrr.08080

Yoshiyama, K., Conklin, P. A., Huefner, N. D., and Britt, A. B. (2009). Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. *Proceedings of the National Academy of Sciences* 106, 12843–12848. doi: 10.1073/pnas.0810304106

Yoshiyama, K. O. (2016). SOG1: a master regulator of the DNA damage response in plants. *Genes Genet Syst* 90, 209–216. doi: 10.1266/ggs.15-00011

Yoshiyama, K. O., Kaminoyama, K., Sakamoto, T., and Kimura, S. (2017). Increased phosphorylation of Ser-Gln sites on SUPPRESSOR OF GAMMA RESPONSE1 strengthens the DNA damage response in Arabidopsis thaliana. *The Plant Cell* 29, 3255–3268. doi: 10.1105/tpc.17.00267

Yoshiyama, K. O., Kobayashi, J., Ogita, N., Ueda, M., Kimura, S., Maki, H., et al. (2013a). ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in Arabidopsis. *EMBO reports* 14, 817–822. doi: 10.1038/embor.2013.112

Yoshiyama, K. O., Sakaguchi, K., and Kimura, S. (2013b). DNA damage response in plants: conserved and variable response compared to animals. *Biology* 2, 1338–1356. doi: 10.3390/biology2041338

Yu, C., Hou, L., Huang, Y., Cui, X., Xu, S., Wang, L., et al. (2023). The multi-BRCT domain protein DDRM2 promotes the recruitment of RAD51 to DNA damage sites to facilitate homologous recombination. *New Phytol* 238, 1073–1084. doi: 10.1111/nph.18787

Zhong, D. (2015). Electron transfer mechanisms of DNA repair by photolyase. *Annual Review of Physical Chemistry* 66, 691–715. doi: 10.1146/annurev-physchem-040513-103631

Zou, L., and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 300, 1542–1548. doi: 10.1126/science.1083430

LIST OF APPENDICES

APPENDIX I:
FIRST AUTHOR PUBLICATION I

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Analysis of BRCT5 domain-containing proteins reveals a new component of DNA damage repair in Arabidopsis

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The integrity of plant genetic information is constantly challenged by various internal and external factors. Therefore, plants use a sophisticated molecular network to identify, signal and repair damaged DNA. Here, we report on the identification and analysis of four uncharacterized Arabidopsis BRCT5 DOMAIN CONTAINING PROTEINs (BCPs). Proteins with the BRCT5 domain are frequently involved in the maintenance of genome stability across eukaryotes. The screening for sensitivity to induced DNA damage identified BCP1 as the most interesting candidate. We show that BCP1 loss of function mutants are hypersensitive to various types of DNA damage and accumulate an increased number of dead cells in root apical meristems upon DNA damage. Analysis of publicly available *sog1* transcriptomic and SOG1 genome-wide DNA binding data revealed that *BCP1* is inducible by gamma radiation and is a direct target of this key DNA damage signaling transcription factor. Importantly, *bcp1* plants showed a reduced frequency of somatic homologous recombination in response to both endogenous and induced DNA damage. Altogether, we identified a novel plant-specific DNA repair factor that acts downstream of SOG1 in homology-based repair.

KEYWORDS

DNA damage repair, genome stability, BRCT domain, BRCT5 domain, homologous recombination, Arabidopsis

Introduction

Genome stability is constantly threatened by internally and externally-induced DNA damage (Razqallah, 2008; Chatterjee and Walker, 2017). Among others, the presence of damaged DNA negatively affects DNA replication, transcription, and cell cycle progression. Therefore, living organisms developed a sophisticated safeguarding

system that recognizes various types of DNA damage, signals their presence, and activates specific molecular effectors that repair the damaged site. This prevents the occurrence of potentially deleterious mutations. Once the repair is completed, the halted cellular processes are restarted and continued. Numerous studies demonstrated that DNA damage repair is essential for the normal growth and fertility of plants, similar to other organisms (Manova and Gruszka, 2015; Nisa et al., 2019) However, despite a generally high degree of evolutionary conservation of the eukaryotic DNA repair system, several unique DNA repair factors evolved in plants (Yoshiyama et al., 2013b; Hu et al., 2016).

Depending on the type of DNA damage, specific DNA repair pathways are activated. A common and highly toxic type of lesion is DNA double-strand break (DSB), which may be generated by external or internal factors. Its persistence in the genome may lead to a loss of genetic information, structural genome changes, and even cell death. The DSB repair begins with a recognition of the damaged site by the MRN (MRE11-RAD50-NBS1) complex and phosphorylation of histone variant H2A.X to produce gamma-H2A.X. This stimulates the binding of the transcription factor Breast cancer type 1 susceptibility protein (BRCA1), followed by signaling through Ataxia Telangiectasia Mutated (ATM) and/or ATM- and RAD3-related (ATR) kinases. The kinase activity of ATM and/or ATR activates the p53 transcription factor at the sites of DNA damage in metazoa and its functional homolog SUPPRESSOR OF GAMMA RADIATION 1 (SOG1) in plants (Preuss and Britt, 2003; Seton-Rogers, 2006; Yoshiyama et al., 2009; Hafner et al., 2019). During the following steps, these transcription factors orchestrate various responses, including pausing of the cell cycle, promotion repair by non-homologous end-joining (NHEJ) or homologous recombination (HR), or (in extreme cases) cell death. In contrast with the error-prone NHEJ, HR represents an error-free mechanism where an intact DNA molecule homologous to the damaged site is used as a template for repair (Heyer et al., 2010). Although the mechanism of HR is studied in great detail across the major branches of the tree of life, not all molecular factors taking place in this process are known.

A prominent group of proteins associated with cell cycle regulation and DNA damage repair contains the BRCA1 C-Terminus (BRCT) domain (Bork et al., 1997), which consists of approximately 100 amino acids and mediates protein-protein interactions by binding to the phosphate groups (Yu et al., 2003). Later studies in animals and yeasts suggested several structurally distinct types of BRCT domains (Wan et al., 2016) The best-studied examples of plant BRCT domain-containing proteins are the BRCA1 and its homolog BREAST CANCER ASSOCIATED RING 1 (BARD1). Both proteins are required for normal levels of somatic HR in plants, and their loss of function mutants are hypersensitive to DNA damage (Trapp et al., 2011). A conspicuous type of BRCT domain is the BRCT5 that was found in budding and fission yeast proteins Rtt107 and Brc1, respectively, and in human protein NSE5/SLF1 (Williams et al., 2010; Li et al., 2012; Räschle et al., 2015). These proteins represent species-specific cofactors involved in the loading of the evolutionary conserved DNA damage repair complex Structural maintenance of chromosomes 5/6 (SMC5/6) to chromatin (Leung et al., 2011; Räschle et al., 2015; Oravcová et al., 2019). However, none of the currently known plant SMC5/6 complex interactors contains this domain. Another example of BRCT5 domain-containing protein includes human Pax2 transactivation domain-interacting protein (PTIP) that performs ATM-dependent activation of p53 and thus promotes DSB repair in mammals (Yan et al., 2011). PTIP also lacks a functional homolog in plants. Therefore, BRCT5 domain proteins represent a little understood group in plants.

Our study demonstrates that analyzing plant proteins carrying BRCT5 domain-containing is an attractive route toward discovering new players involved in the control of plant genome stability. Thus we performed *in silico* identification of Arabidopsis BRCT5 DOMAIN CONTAINING PROTEINs (BCPs). Subsequently, loss of function mutants of four genes was characterized by the expression pattern and hypersensitivity to DNA damage. The most promising candidate BCP1 was analyzed as to its role in HR-based repair.

Materials and methods

Plant materials and growth conditions

Unless stated otherwise, all *Arabidopsis thaliana* (Arabidopsis) genotypes used in this study had Columbia (Col-0) background. T-DNA lines used in this study were: GK_301C08 (*bcp1-1*), SALK_001578C (*bcp1-2*), SALK_022790 (*bcp1-3*), GK_076D08 (*bcp2-1*), SALK_111173C (*bcp3-1*), SALK_038422 (*bcp4-1*), and SALK_123114C (*smc6b-1*). T-DNA mutant lines were obtained from the SALK institute (Alonso et al., 2003) and GABI-Kat (Kleinboelting et al., 2012) *via* the European Arabidopsis Stock Centre (NASC). Double mutants were generated by crossing homozygous single mutants and analyzing progeny in F2 generation by PCR-based genotyping for both mutations. HR reporter lines B11 in the C24 background (Swoboda et al., 1994) and IC9C (Puchta et al., 1995; Molinier et al., 2004) were crossed with *bcp1-1*. The resulting hybrids were grown into F4 generation and selected by PCR for double homozygous lines. The oligonucleotides used for genotyping are listed in Supplementary Table 1.

Plants used for phenotyping, seed generation, and crossing were grown in climate-controlled phytotron under long-day conditions (at 16 h light, 150 µmol m^{-2} s^{-1} intensity, 19°C during the day; 8 h at 18°C during the night). *In vitro* plant

cultivation was done in an air-conditioned phytochamber with a long day regime (16h light, 150 µmol m^{-2} s^{-1}, 21°C, 8h dark, 19°C).

Basic local alignment search tool and BRCT5 domain structure comparisons

New Arabidopsis BRCT5 domain-containing proteins were identified by the BLAST search (Altschul et al., 1990) using the fission yeast *Schizosaccharomyces pombe* Brc1 (*Sp*Brc1) and human *Homo sapiens* NSE5 (*Hs*NSE5) proteins against the *Arabidopsis thaliana* database (taxid: 3702). The retrieved BRCT5 domain sequences were manually aligned, and their AlphaFold structural models (Varadi et al., 2022) were compared.

Molecular cloning, plant transformation, and GUS assays

To develop the promoter-reporter line, a region 2000 bp upstream of the *BCP1* transcription start site (*ProBCP1*) was amplified by PCR and cloned by Gateway Technology (ThermoFisher Scientific; Cat. nos.: 11789100, 12538120) into *pDONR207* (Invitrogen) and then recombined into the binary vector *pKGWFS7.0*, containing *uidA* gene encoding β-glucuronidase (GUS). Plasmids carrying the *ProBCP1::GUS* fusion were transformed into *Agrobacterium tumefaciens* strain GV3101. Transformation of *Arabidopsis* Col-0 was performed using the floral dip method. (Zhang et al., 2006). The selection of transformed plants in T1 generation was carried out on a medium containing 100 µg/ml kanamycin (Sigma-Aldrich, Cat. no. 60615). Resistant plants were transferred to soil for seed production. The following generation (T2) of plants was selected based on the activation of the *GUS* reporter gene. The oligonucleotides used for genotyping or cloning are listed in Supplementary Table 1.

The expression pattern of *BCP1* in highly dividing vegetative tissues, as well as the change of expression under DNA damage stress, was examined by using a *ProBCP1::GUS* reporter line. Plants grown for seven days on a solid medium were transferred for 24 h to liquid ½ MS medium with or without 10 µM mitomycin C (MMC, Cat. no. M0503), a genotoxic agent causing intrastrand DNA crosslinks. Following the treatments, plants were stained by GUS histochemical staining. GUS solution containing 10 mM EDTA (Sigma-Aldrich, Cat. no. E5134), 2 mM potassium ferrocyanide (Lachema, Cat. no. 68 4514), 2 mM potassium ferricyanide (Lachema), 100 mM disodium phosphate (Penta, Cat. no. 15150), 100 mM monosodium phosphate (Lachema, Cat. no. 68 4639), 0.1% Triton X-100 (Sigma-Aldrich, Cat. no. T8787) and 2 mM X-Gluc (Thermo Scientific, Cat. no. R0852) was prepared as described in (Baubec et al., 2009). Seedlings were transferred to 5 ml tubes and infiltrated with GUS staining solution under a vacuum. After five to ten minutes, the vacuum was released and tubes were placed at 37°C overnight. Subsequently, the GUS staining solution was removed and plants were cleared by incubation in 70% ethanol (v/v) at 37°C. Ethanol was changed 3 times, and after the last change, plants were left overnight at 4°C. Pictures were taken under a stereo-microscope (Olympus SZX16) and fluorescent microscope (Olympus BX60).

For the analysis of *BCP1* expression in the reproductive tissues, inflorescences were fixed in 90% (v/v) acetone and incubated for 45 min at -20°C. Acetone was then removed, and samples were washed three times with 100 mM phosphate buffer, pH 7.2. After washing, the flowers were infiltrated with GUS staining solution under a vacuum for 10 min and left overnight at 37°C. The next morning the solution was removed, and samples were washed shortly with phosphate buffer and cleared in chloral hydrate solution containing eight parts chloral hydrate (Sigma-Aldrich, Cat. no. 23100) two parts water, and one part glycerol (Sigma-Aldrich, Cat. no. G516). Flowers were mounted on the microscope slide and dissected in the same solution. Pictures were taken under a stereo-microscope (Olympus SZX16) and fluorescent microscope (Olympus BX60).

Root sensitivity assays

For root sensitivity assays, surface sterilized and stratified seeds were grown on ½ MS growth medium with 0.6% agar (w/v) and 1% sucrose (w/v). Seeds were sterilized in 70% ethanol (v/v) for 5 min, followed by 8% sodium hypochlorite solution (v/v) for 6-10 min, and washed 3 times in sterile water. Seeds were stratified for 48h in 0.1% agarose solution (w/v) at 4°C in the dark. Stratified seeds were evenly distributed on Petri dishes containing ½ MS medium (mock) or ½ MS medium supplemented with 10 µM MMC (Sigma-Aldrich, Cat. no. M0503), 20 nM camptothecin (CPT; Sigma-Aldrich, Cat. no. C9911), 20 µM zebularine (Sigma-Aldrich, Cat. no. Z4775), or 50 nM bleomycin (Sigma-Aldrich, Cat. no. 203408-M). Plants grown for seven days in a horizontal position were then carefully pulled off the medium using tweezers and laid flat on a plate with agar. The length of the primary root was measured using the ImageJ plugin SmartRoot (Lobet et al., 2011). Experiments were performed in three biological replicates with typically 20 plants per replicate (minimum of 11 plants in one replicate). Statistical significance was tested with One-way ANOVA with posthoc Tukey HSD in Minitab.

Cell death assays

Sterilized and stratified seeds were grown vertically on plates with ½ MS medium with 0.8% agar (w/v) for five days and then transferred into liquid ½ MS medium for a 24 h treatment. Mock samples were grown in pure liquid ½ MS medium, while treated plants had medium supplemented with 10 µM MMC. Following the treatment, seedlings were stained with 10 $mg.mL^{-1}$ propidium

iodide solution (Sigma) on glass microscope slides. Visualization and photography were performed using Leica confocal microscope TCS SP8 (Leica, Wetzlar, Germany) and HC PL APO CS2 20x/0.75 DRY objective equipped with Leica LAS-X software with Leica Lightning module laser scanning confocal microscope (Leica). At least 13 plants for each group were analyzed. The means of the three replicates are depicted. Statistical significance was tested withKruskall-Wallis H-test with *post hoc* Conover-Iman test of multiple comparisons using rank sums with Benjamini-Hochberg procedure in R 4.2.1 (R Core Team, 2018).

Homologous recombination assays

The B11, B11 *bcp1-1*, IC9C, and IC9C *bcp1-1* plants were grown on ½ MS medium with or without (mock) 1 μM MMC under sterile conditions. Ten days-old seedlings were histochemically stained using GUS as described above. Plants were transferred to a Petri dish containing ethanol and examined using a stereo-microscope (Olympus SZX16) for HR events identified as blue-stained cells or areas. The means of the three replicates are depicted. Statistical significance was tested withMann-Whitney U-tes in Minitab (www.minitab.com).

Fresh weight measurements

Plants were grown as described in homologous recombination assays were measured on an analytical scale. Measuring was done in triplicates, and each sample was composed of 60 seedlings. Mann-Whitney U-test ($P < 0.05$) was used to assess the significance of weight differences (www.minitab.com).

RNA-seq data analysis

RNA-seq data for wild-type and *sog1-1* plants were obtained from a publicly available dataset (Bourbousse et al., 2018). The database contains gene expression values (fragment per kilobase per million reads, FPKM) in plants grown under normal and DNA damaging conditions at six-time points post gamma irradiation (20 min, 1.5, 3, 6, 12, and 24 h). From it, we acquired expression profiles of BCPs. The changes in gene expression were assessed as described in the results. For the assessment of statistical significance, we used a two-sample T-test with unequal variances.

Reverse transcription-quantitative polymerase chain reaction

T-DNA mutants lines' seeds, sterilized and stratified, were grown on ½ MS medium with 0.6% agarose. Seven days old seedlings were sampled and flash-frozen in liquid nitrogen. RNA extraction was performed by RNeasy Mini Kit (Qiagen, Cat. no. 74104). cDNA was constructed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific™, Cat. no. K1631). The qPCR was performed with the HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Cat. no. 08-24-0000S) in CFX96 Touch Real-Time PCR Detection System. Wild type and *sog1-1* plants were grown for seven days in ½ MS 0.6% agarose and then transferred to liquid ½ MS with or without 40 μM MMC for a 1 h treatment. Following the treatment samples were flash-frozen in liquid nitrogen, and RNA extraction, cDNA synthesis and RT-qPCR were performed as described above. Mann-Whitney U-test was performed in Minitab to assess statistical significance of the data.

Accession numbers

Gene information and sequences used in this article can be found in TAIR under the following accession numbers: *BCP1* (AT4G02110), *BCP2* (AT2G41450), *BCP3* (AT4G03130), *BCP4* (AT3G21480), *SMC6B* (AT5G61460).

Results

Identification of Arabidopsis BRCT5 domain-containing proteins

To identify potential Arabidopsis BRCT5 domain-containing proteins, we performed a BLAST search (Altschul et al., 1990) using the BRCT5 domains of the fission yeast *Schizosaccharomyces pombe* Brc1 (*Sp*Brc1) and human *Homo sapiens* NSE5 (*Hs*NSE5) proteins against the Arabidopsis protein database. Three candidates, At4g02110, At4g03130, and At4G21070, were found as potential genes of interest. We hypothesized that the large phylogenetic distance between Arabidopsis versus yeast and human might have reduced the efficiency of such a screen and compromised the direct identification of some candidates. Therefore, we performed an additional search for the BRCT5 domain-containing proteins in the genome of moss *Physcomitrium patens* using the same query sequences from *Sp*Brc1 and *Hs*NSE5. The BRCT5 domains were found in the moss proteins Pp3c4_1630, Pp3c7_24750, Pp3c8_2040, and Pp3c11_4990. As the next step, the moss proteins were BLASTed against the Arabidopsis genome, which revealed three additional genes At1g04020, At2g41450, and At3g21480. The sequence comparison identified a conserved pattern of amino acids (Figure 1A) with different properties typical for the BRCT5 type domain that supported all the candidates identified *via* BLAST. The candidates At4g21070 and At1g04020 were previously described as *Arabidopsis* orthologs of human *BREAST CANCER SUSCEPTIBILITY 1* (*BRCA1*) and its homolog *BREAST CANCER ASSOCIATED RING 1* (*BARD1*), whose functions in plant DNA damage repair have been already

documented (Lafarge and Montané, 2003; Reidt et al., 2006). Therefore, both *BRCA1* and *BARD1* were excluded from subsequent analyses. Based on this, we selected the remaining four candidate proteins for further analysis and named them BRCT5 DOMAIN-CONTAINING PROTEINs (BCPs): BCP1 (At4g02110), BCP2 (At2g41450), BCP3 (At4g03130) and BCP4 (At3g21480). The superimposition of computationally modeled BRCT5 domains of *Sp*Brc1 and the four selected BCPs revealed their high structural similarity (Figure 1B). Based on Araport11 gene annotation, *BCP1* is a cell cycle regulated transcriptional coactivator (Menges et al., 2002). Based on its two BRCT domains, it was also considered a possible candidate for plant homolog of the human DNA topoisomerase 2-binding protein 1 (TOPBP1), but the overall low sequence similarity did not allow for the drawing of a firm conclusion (Shultz et al., 2007). *BCP2* is described as N-acetyltransferase (Araport11) and is expressed in the female gametophyte (Wuest et al., 2010). *BCP3* and *BCP4* are both described as BRCT domain-containing DNA repair proteins (Araport11), likely based on the presence of their C-terminal BRCT domains, with no further information. Hence, we identified six BRCT5 domain-containing candidates in Arabidopsis, with four of them representing uncharacterized Arabidopsis genes.

Loss of *BCP1* causes sensitivity to DNA damage, and its transcription is SOG1 dependent

To test for the potential role of *BCPs* in DNA damage repair, we isolated their T-DNA insertional mutants (Figure 2A). All homozygous mutants were viable and did not show any obvious developmental defects during somatic development and/or sterility during the reproductive stage.

As the first step, we performed an initial screening for mutant sensitivity to different types of DNA damage. The aim was to identify if any of the genes are important for DNA damage repair. To this end, we focused on the induction of all possible types of DNA damage including DSBs, DNA inter-strand, and DNA-protein crosslinks. Seeds were germinated, plants were grown on

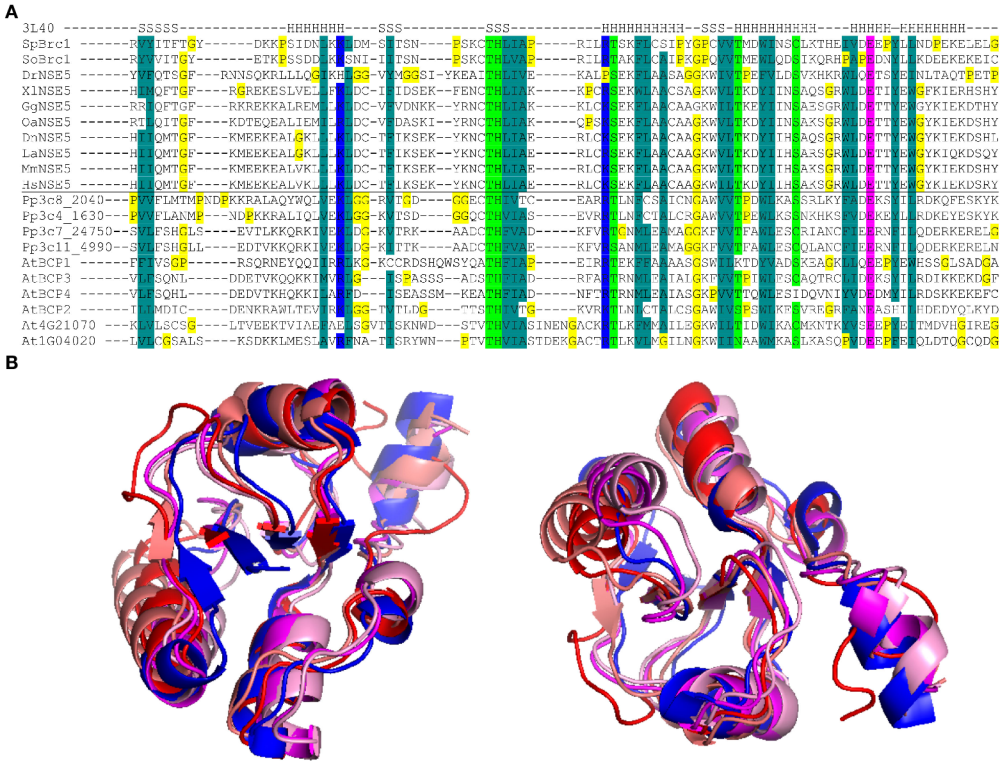

FIGURE 1
BRCT5 domain analysis. **(A)** Alignment of the core part of the BRCT5 domain with helical (H) and β-strand (S) segments above (from PDB: 3L40 structure; Williams et al., 2010). The Brc1 and NSE5 orthologs are from *Schizosaccharomyces pombe* (*Sp*), *S. octosporus* (*So*), *Physcomitrium patens* (*Pp*), *Arabidopsis thaliana* (*At*), *Danio rerio* (*Dr*), *Xenopus laevis* (*Xl*), *Gallus gallus* (*Gg*), *Ornithorhynchus anatinus* (*Oa*), *Monodelphis domestica* (*Md*), *Dasypus novemcinctus* (*Dn*), *Loxodonta africana* (*La*), *Mus musculus* (*Mm*), *Homo sapiens* (*Hs*). Coloring indicates amino acid groups conserved across the family: *dark green*, hydrophobic and aromatic; *light green*, polar; *blue*, acidic; *pink*, basic; all glycine and proline residues are highlighted in *yellow*. **(B)** Superimposition of modeled BRCT5 domains of At4g02110 (red), At2g41450 (deep pink), At4g03130 (coral), At3g21480 (pale pink), and crystal structure *Sp*Brc1 (blue) shown from two views.

the genotoxin-containing media, and their root length was measured (Figure 2B). The *smc6b-1* mutant allele of *STRUCTURAL MAINTENANCE OF CHROMOSOMES 6B* (*SMC6B*) served as a hypersensitive control. Under mock conditions, only the *bcp4-1* mutant plants had significantly shorter roots (9.4 mm ± 1.5 mm in *bcp1-4* compared to 13.23 mm ± 0.68 mm in WT control), while the root length of the remaining mutants was not significantly different from the wild type (Figure 2C). Under DNA damaging conditions, the *bcp1-1* plants were hypersensitive to 10 µM DNA inter-strand cross-linker MMC, 20 nM DNA-protein cross-linker CPT and 20 nM radiomimetic agent bleomycin causing DNA strand breaks. The *bcp1-1* plants also exhibited sensitivity to 20 µM type I DNA-protein cross-linker zebularine (Prochazkova et al., 2022). The *bcp2-1*, *bcp3-1*, and *bcp4-1* mutant plants did not show significantly increased sensitivity to any of the genotoxic treatments (Figure 2D).

Absence of sensitivity in combination with non-coding sequence location of T-DNAs stimulated us to analyze the expression of BCPs in their corresponding T-DNA insertion mutant lines by RT-qPCR (Supplementary Figure 1). The *bcp1-1* and *bcp4-1* showed a very strongly reduced amount of transcript compared to their WT variants. Surprisingly, the *bcp2-1* with T-DNA insertion in the first out of total 13 exons showed more than 90-fold over-expression of *BCP2*. This might be caused by the expression from the *Cauliflower Mosaic Virus 35S* promoter that is part of the T-DNA insertion. The *bcp3-1* showed no significant difference in the amount of transcript compared to wild type. This suggests that *bcp1-1* and *bcp4-1* are loss of function mutants, *bcp2-1* is a potential overexpressor line and *bcp3-1* might not affect BCP3 gene function.

Next, we analyzed the expression of the BCP candidates using available transcriptomic data. Surprisingly, none of the selected candidates is represented on the Arabidopsis ATH1 expression array. RNA-sequencing-based atlas of Arabidopsis developmental stages (Klepikova et al., 2016) revealed that *BCP2* and *BCP3* were only weakly expressed throughout the whole plant development and that the expression slightly increased only in some floral parts (Supplementary Figure 2). In contrast, both *BCP1* and *BCP4* showed a low to moderate expression with the highest values observed in floral organs and seeds. Surprisingly, only weak expression was found in the root tissues. To find a potential involvement of *BCPs* in DNA damage response, we analyzed their expression after gamma-irradiation in wild-type and *sog1* mutant background using a publicly available RNA-seq dataset (Bourbousse et al., 2018). Under ambient (mock) conditions, *BCP1* was expressed stably at a basal level in both WT and *sog1-1* plants (Supplementary Figure 3A). In response to gamma-irradiation, *BCP1* was upregulated 3.2-fold already 20 min post-treatment, and the amount of transcript reached its 14-fold increase maximum 1.5 h post-irradiation (Figure 3A). The amount of transcript lowered over time and returned to mock levels 24 h after the treatment. In the *sog1* plants, gamma radiation-induced expression was not observed, suggesting that transcriptional response of *BCP1* to DNA damage is SOG1-dependent, and that *BCP1* acts downstream of SOG1. This was confirmed also in RT-qPCR experiment where *BCP1* was significantly up-regulated in response to MMC treatment in wild-type but not in *sog1-1* mutant plants (Figure 3B). However, the same amount of *BCP1* expression in mock-treated wild-type and *sog1-1* plants indicates that basal *BCP1* expression is SOG1 independent. The remaining genes *BCP2*, *BCP3*, and *BCP4* showed only minor transcriptional changes that differed between wild-type and mutant plants, mostly at solitary time-points, suggesting that these genes are not gamma-irradiation inducible and their expression is not SOG1-dependent (Figure 3A).

To gain more insights into the transcriptional response of *BCP1* to DNA damage, we generated stable Arabidopsis transformants carrying *BCP1* promoter fused with the *GUS* reporter gene (*ProBCP1::GUS*). Under mock conditions, the *BCP1* promoter was active in tissues with actively dividing cells, such as the root and shoot apical meristems, lateral root meristems, and vasculature (Figure 3C_2,3,4). The signals in true leaves had a peculiar dotted pattern. After inspection at the cellular level, it was obvious that these "dots" are represented by young stomata guard cells, stomatal lineage ground cells, and guard mother cells. The older (larger) stomata guard cells and pavement cells showed little or no GUS signals (Figure 3C_5,6). As the Arabidopsis transcriptomic atlas (Klepikova et al., 2016) data suggested the highest *BCP1* transcript amount in reproduction (Supplementary Figure 2), we further examined the pattern of *BCP1* expression in inflorescences (Supplementary Figures 4A–E). We found particularly strong GUS signals in pistils throughout the entire flower development, young stamen, filaments, and perianth of closed flowers.

To visually confirm that the *BPC1* transcription is induced by DNA damage, as suggested by the transcriptomic data, we exposed seedlings of the *ProBCP1::GUS* reporter line to 10 µM MMC for 24 h and subsequently scored *BCP1* promoter activity. Intense signals appeared in almost all parts of the plant, including the true leaves (Figure 3C_7-12). This strongly supports transcriptomic data and demonstrates that BCP1 transcription is inducible by DNA damage. Based on these experiments, we considered *BCP1* as the most promising candidate for further analysis.

BCP1 is required for the repair of various types of DNA damage

To validate our initial findings based on a single mutant allele, we isolated two more *BCP1* T-DNA-insertional mutants located in the 8th exon (*bcp1-2*) and the 7th intron (*bcp1-3*) (Figure 4A). Phenotypic analysis of all three homozygous mutant lines confirmed the absence of obvious developmental defects at four and six weeks of age (Supplementary Figure 5).

Next, we extended the sensitivity assays by exposing plants of all *bcp1* mutant lines to 10 µM MMC, 20 µM zebularine, and

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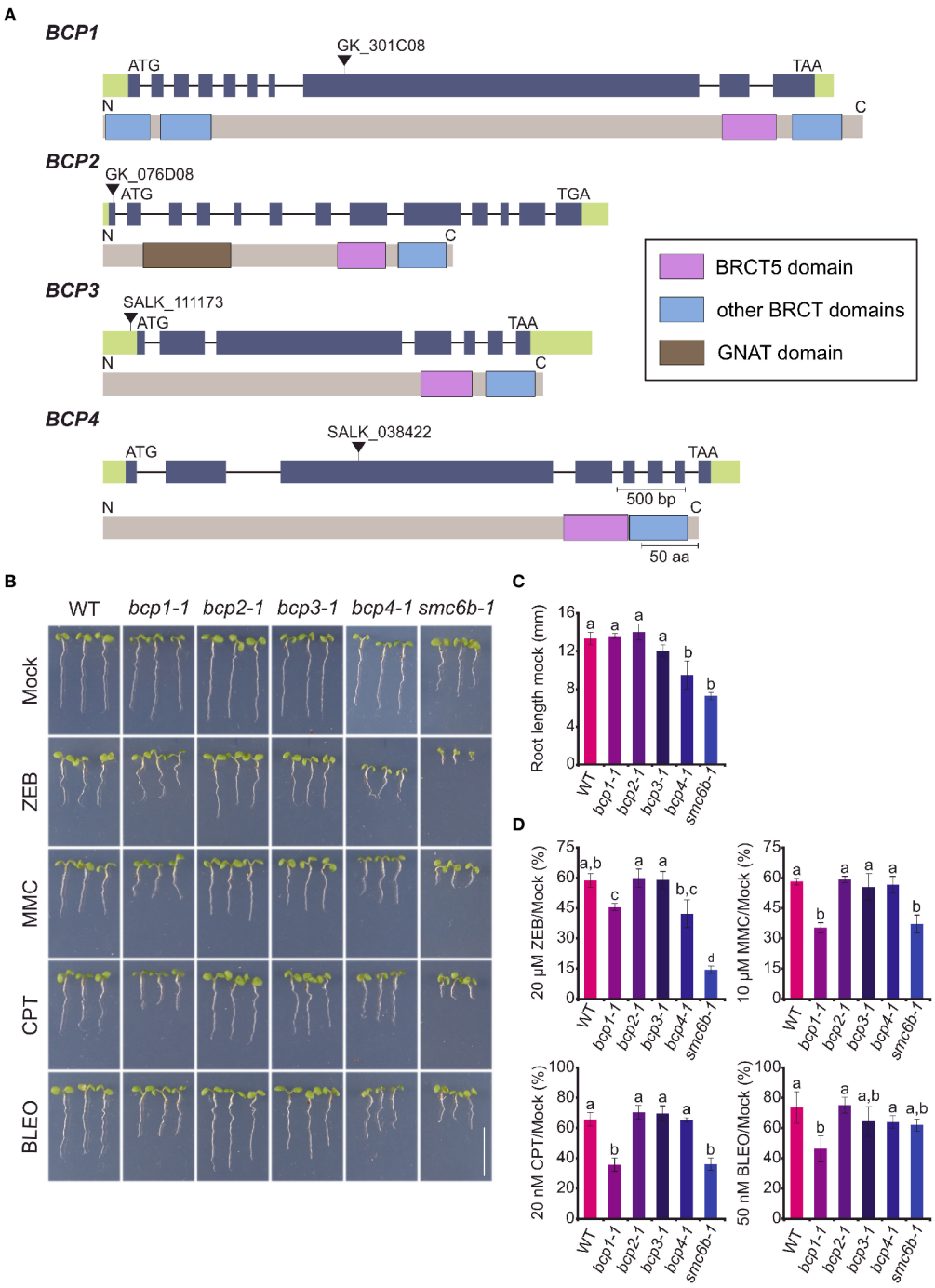

FIGURE 2
Phenotypes of mutants in uncharacterized Arabidopsis BRCT5 domain-containing genes. **(A)** Gene and protein structures of the BRCT5 CONTAINING PROTEINS (BCPs). The positions of T-DNAs in the used mutant alleles are indicated by black triangles above the gene models. Introns are indicated by a horizontal line and exons by green (untranslated regions) and purple (coding sequence) colors. Protein models under gene models (grey) show the position of known domains: BRCT - blue rectangles and GNAT (Gcn5-related N-acetyltransferase) - brown rectangle. **(B)** Representative phenotypes of seven days old wild-type (WT) and homozygous mutant plants grown on media containing 20 µM zebularine (ZEB), 10 µM mitomycin C (MMC), 20 nM camptothecin (CPT), 50nM nM bleocin (BLEO). The *smc6b-1* served as a sensitive control. Scale bar = 1 cm. **(C)** Root length of WT and mutant plants under control (mock) conditions. Error bars indicate the standard deviation between the means of three biological replicates. The letters above columns indicate similarities between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with *post hoc* Tukey's test ($P < 0.05$). **(D)** Root length of WT and mutants from B under DNA damaging treatments relative to the growth of the same genotype under mock conditions. Error bars represent the standard deviation between three biological replicates, each with at least 15 plants. Statistics were performed as in **(C)**.

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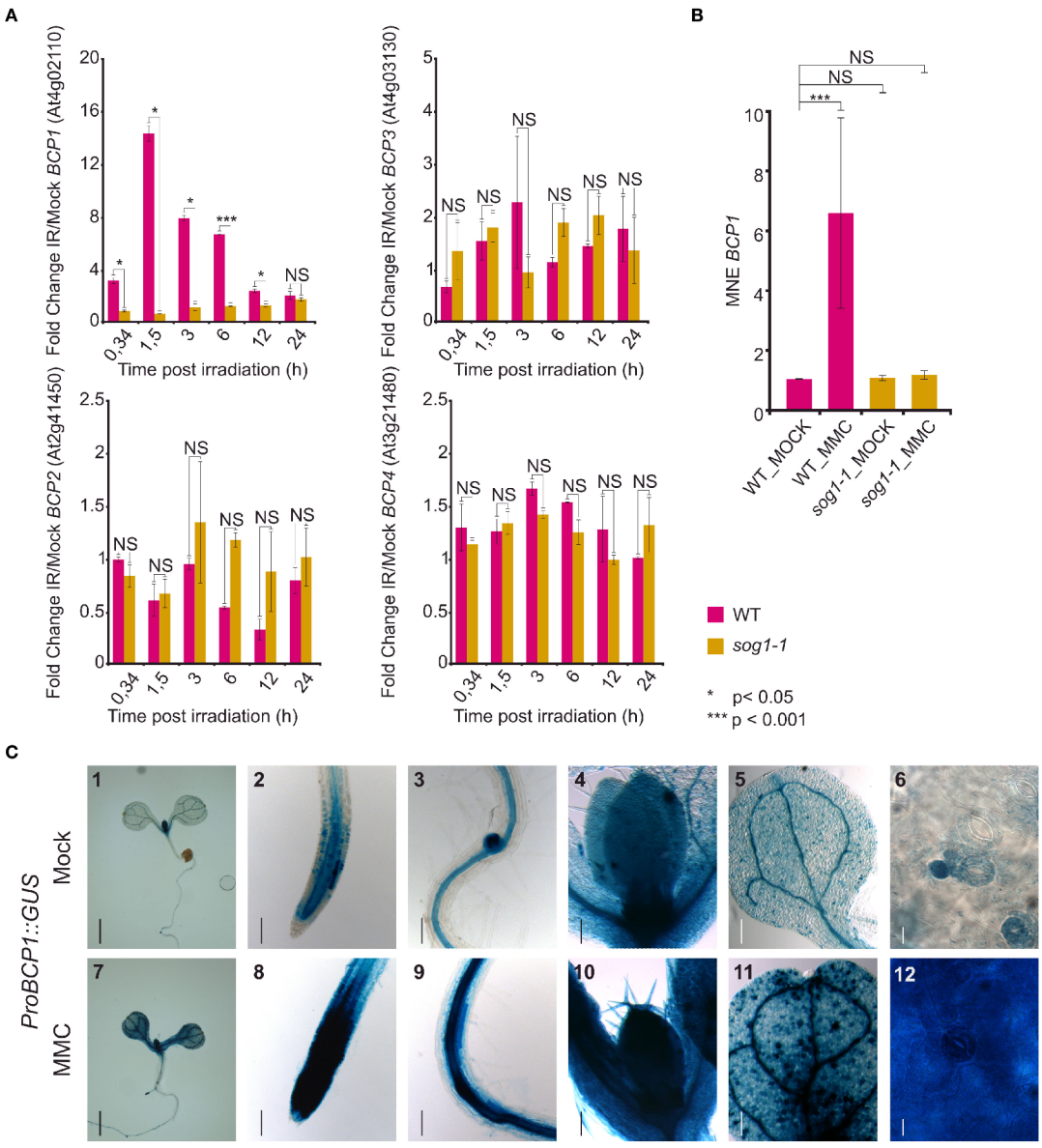

FIGURE 3
Expression of the *BCP* genes. **(A)** Relative expression of *BCP1* to *BCP4* in wild type (WT) and *sog1-1* based on RNA-sequencing experiment of Bourbousse et al. (2018). The normalized read counts from RNA-sequencing (FPKM) were used to calculate fold change in expression after gamma irradiation pulse (IR) versus mock conditions (y-axis). The x-axis indicates the harvesting time after the irradiation treatment. Error bars represent the standard error of the mean. Asterisks represent significant differences in two sample T-test with unequal variance, * $P < 0.05$, *** $P < 0.001$, NS – not significantly different. **(B)** Reverse transcription qPCR analysis of *BCP1* expression in wild-type (WT) and *sog1-1* plants without (MOCK) and after 1 h treatment with 40 µM MMC (MMC). Y-axis shows mean normalized expression relative to *PP2A*. Error bars show three biological replicates. NS = not significantly different, *** statistically significantly different in Mann-Whitney U-test at $P < 0.05$. **(C)** *In planta* analysis of *BCP1* promoter activity. Seven days old seedlings carrying *ProBCP1::GUS* were transferred to mock and DNA damaging conditions for 24h. *BCP1* promoter activity was monitored using GUS histochemical staining. Representative stereo microscope pictures of tissues showing the gene expression. (1,7) Whole seedling, (2, 8) shoot apical meristem, (3, 9) lateral root meristem, (4, 10) shoot apical meristem with first real leaves, (5, 11) cotyledon, (6, 12) leaf blade cells. Scale bars: 1,7 = 1 mm; 2-5,8-11 = 100 µm, 6,12 = 50 µm.

20 nM CPT (Figures 4B, D). In mock conditions *bcp1-1* and *bcp1-2* mutants showed no difference in root length compared to wild type, while *bcp1-3* plants had slightly longer roots (Figure 4C). Both *bcp1-1 and bcp1-2* alleles were significantly sensitive to all three drug treatments. In contrast, the intronic mutant *bcp1-3* was hypersensitive only to 10 µM MMC (Figure 4D). This is in agreement with the amount of *BCP1* transcript which was almost not detectable in *bcp1-1* and *bcp1-2*

mutants but was not significantly reduced in *bcp3-1* (Supplementary Figure 1). Specifically, MMC-treated wild-type plants reached 38.8 ± 3.6% of the standard root length compared to mock conditions, while it was only 21.8 ± 1.7%, 25.5 ± 2.5%, and 27 ± 3% of the mock-treated plant root length for *bcp1-1*, *bcp1-2*, and *bcp1-3*, respectively (all comparisons P <0.001 one-way ANOVA with *post hoc* Tukey HSD). Zebularine-treated wild-type plants reached 53.3 ± 1.7% of the mock control length. For zebularine treated *bcp1-1*, *bcp1-2* and *bcp1-3* plants it was 42.7 ± 5.6%, 41.6 ± 1.25% and 50.5. ± 4.0%, respectively (all comparisons P <0.001). Similarly, CPT-treated wild-type plants reached 43.2 ± 4% of the normal root length, but it was 25.5 ± 1.1%, 37.4 ± 1.4%, and 47.5 ± 2% for the individual *bcp1* mutant alleles, respectively (all comparisons P <0.001 one-way ANOVA with *post hoc* Tukey HSD). The sensitive control *smc6b-1* plants had massive root length reduction to 16 ± 2%, 10 ± 0.3%, and 20 ± 1% for MMC, zebularine, and CPT (all comparisons P <0.001 one-way ANOVA with *post hoc* Tukey HSD).

To assess the extent of damage at the cellular level, we performed a cell death assay based on the staining of root apices with propidium iodide (PI), where PI marks the dead cells and is excluded from the living cells (Figures 4E, F). Wild-type and *smc6b-1* were used as standard and hypersensitive controls. Mock-treated wild-type and *bcp1* plants showed no significantly different mean values of less than one dead cell per root (Figures 4E, F). After the treatment with 10 µM MMC for 24 h, the median number of dead cells per root increased to three in wild type, four in *bcp1-2* and *bcp1-3*, and five in *bcp1-1* (Figure 4F). The values in all mutant lines were significantly higher compared to wild-type plants. This shows that loss of function from *BCP1* makes Arabidopsis plants hypersensitive to diverse types of DNA damage and leads to increased cell death.

BCP1 is required for normal frequency of homologous recombination

Based on the SOG1-dependent transcriptional activation of *BCP1* upon DNA damage and hypersensitivity of *bcp1* plants to DNA damaging treatments, we hypothesized about a possible role of BCP1 in HR. To experimentally test this hypothesis, we generated double homozygous *bcp1-1 B11* and *bcp1-1 IC9C* lines (Swoboda et al., 1994; Puchta et al., 1995; Molinier et al., 2004). Owing to the organization of the reporter regions, these lines allow locus-specific monitoring of the frequency of single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA) types of HR, respectively (Orel et al., 2003). The plants were germinated and grown on media without (mock) and with 1 µM MMC for 10 days and analyzed for HR events. There were no significant differences (Mann-Whitney U test, P > 0.05) in fresh weight between all genotypes (Supplementary Figure 6), indicating similar number of cells. Under mock conditions, we found on average 2.9 ± 2.2 SSA HR events per *B11* wild type (n = 131) plant (Figure 5), while *bcp1-1 B11* plants (n = 151) showed 52% less SSA HR events per plant (1.4 ± 1.5). This difference was statistically significantly different (P < 0.001 Mann-Whitney U-test), indicating a possible role of *BCP1* in HR independent of exogenous DNA damage. In response to a mild DNA inter-strand crosslinking treatment by MMC, there were on average 34 ± 12 SSA HR events per *B11* plant (n = 137 plants) and 21 ± 10.6 per *bcp1-1 B11* plant (n = 151 plants), corresponding to a significant (P < 0.001Mann-Whitney U-test) 38% reduction in the mutant.

A similar pattern was observed in SDSA HR reporter line IC9C. Also here, we did not find significant differences (Mann-Whitney U-test, P > 0.05) in fresh weight between all genotypes (Supplementary Figure 6), indicating similar number of cells. The IC9C wild type and IC9C *bcp1-1* lines showed a similar 0.2 ± 0.46 (n = 153 plants) and 0.28 ± 0.61 (n = 109 plants) SDSA events per plant under mock conditions, respectively. After treatment with 1 µM MMC, IC9C wild type showed an average of 1.59 ± 1.31 (n = 102 plants) SDSA HR events plant, while IC9C *bcp1-1* line had 0.78 ± 0.85 (n = 158 plants) SDSA HR events per plant. This is a 50.5% decrease in the number of HR events in the *bcp1-1* mutant under mild genotoxic stress. Collectively, this shows that BCP1 is needed for normal levels of SSA and SDSA HR in Arabidopsis and suggests an involvement of BCP1 in the HR repair.

Discussion

In this work, we found a new Arabidopsis protein BCP1 which contains the BRCT5 domain and contributes to DNA damage repair by homologous recombination in a SOG1-dependent manner.

To identify Arabidopsis BRCT5 domain-containing proteins, we performed a homology search using fission yeast *Sp*Brc1 and human *Hs*NSE5. These proteins were selected because they are known to mediate interactions of the conserved SMC5/6 DNA repair complex to chromatin (Li et al., 2012; Räschle et al., 2015). While human NSE5 directly contains the BRCT5 domain, the yeast NSE5 does not, but it binds BRCT5-containing Brc1 protein which targets it to DNA damage sites. The situation in Arabidopsis resembles yeast where none of the currently known SMC5/6 complex subunits harbors a BRCT domain (Yan et al., 2013). Hence, identification of the plant BRCT5 domain-containing proteins might lead to a plant-specific SMC5/6 cofactor mediating interaction with DNA repair complexes and/or chromatin.

Via two BLASTs, first against the moss *Physcomitrium patens* and then Arabidopsis, we found in total six Arabidopsis BRCT5 domain-containing proteins, including two already known DNA damage repair factors BRCA1 and BARD1. BRCA1 is a well-known tumor suppressor in humans that is evolutionarily conserved also in plants (Trapp et al., 2011). Studies in

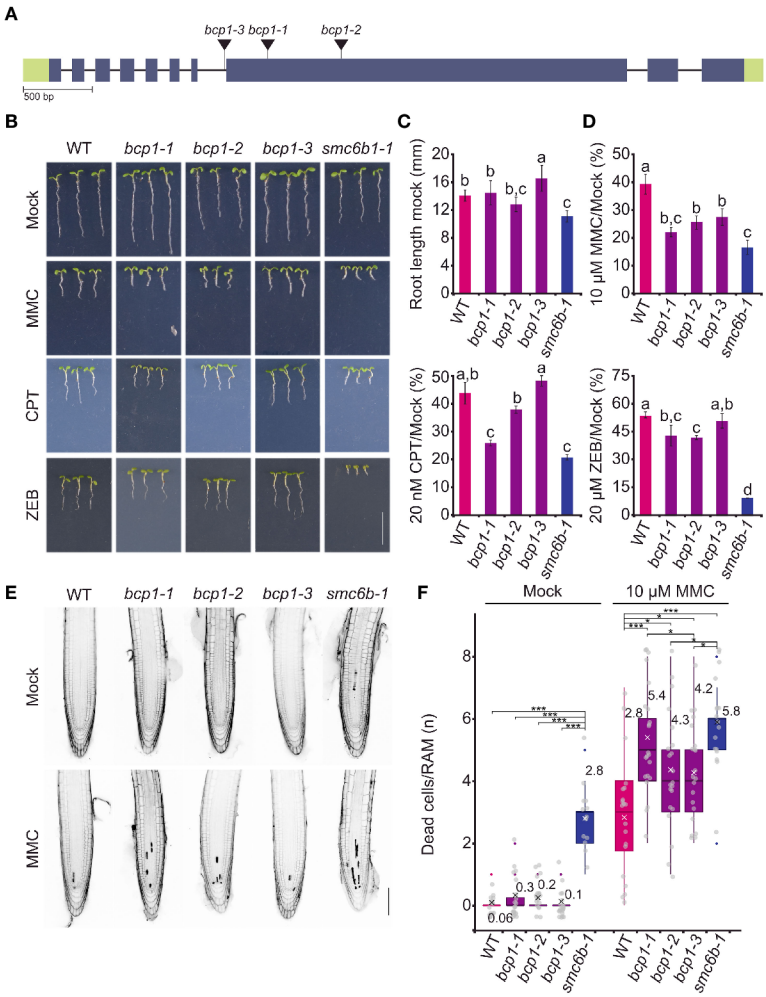

FIGURE 4
BCP1 is required for normal resistance to DNA damaging treatments. **(A)** Gene model of BCP1 with indicated positions of all used T-DNA insertional mutants. The style follows the description in Figure 2A. **(B)** DNA damage sensitivity assays of different bcp1 alleles. Representative phenotypes of seven days old wild-type (WT) and homozygous mutant plants grown on media containing 20 µM zebularine (ZEB), 10 µM mitomycin C (MMC), and 20 nM camptothecin (CPT). The smc6b-1 served as a sensitive control. Scale bar = 1 cm. **(C)** Root length of WT and mutant plants grown under control (mock) conditions. Error bars indicate the standard deviation between the means of nine biological replicates. The letters above columns indicate similarity between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with posthoc Tukey's test ($P < 0.05$). **(D)** Root length of WT and mutants under DNA damaging treatments relative to the growth of the same genotypes under mock conditions. Statistics were performed as in **(C)**. **(E)** Representative confocal microscopy images of the primary roots stained by propidium iodide in cell death assays. Five days old seedlings of WT and bcp1 mutants were exposed to mock or 10 µM MMC treatments for 24 h, stained with propidium iodide, and analyzed to reveal dead cells that appear as dark sectors inside the roots. The smc6b-1 served as a control with increased cell death. Scale bar = 100 µm. **(F)** Quantification of dead cells per root apical meristem in different genotypes and treatments (complements E). Each gray dot indicates the number of dead cells per root (n = 13-22). The boxplots' hinges are in the 1st and 3rd quartile, with a marked median. The mean is indicated by a cross with a numerical value. Whisker marks show the lowest or highest value within the 1.5 interquartile range below or above hinges. Statistical significance was tested by Kruskall-Wallis H-test with post hoc Conover-Iman test of multiple comparisons with Benjamini-Hochberg procedure ($P < ½ \alpha$, $\alpha = 0.05$). NS - not significant, * $P < 0.025$, *** $P < 0.001$.

mammals and Arabidopsis revealed that BRCA1 and BARD1 frequently act as a heterodimer (Wu et al., 1996; Reidt et al., 2006). In Arabidopsis, both BRCA1 and BARD1 are necessary for resistance to DNA damage and also for normal levels of somatic homologous recombination (Reidt et al., 2006). Furthermore, the function of BARD1 seems to go beyond the regulation of genome stability because BARD1 was found to suppress the expression of *WUSCHEL1*, a master regulator homeobox gene controlling the stem cell pool (Mayer et al., 1998), in the shoot apical meristems and thus contributing to the meristem normal growth and organization during plant development (Han et al., 2008). Besides the established role of these two proteins in plant DNA

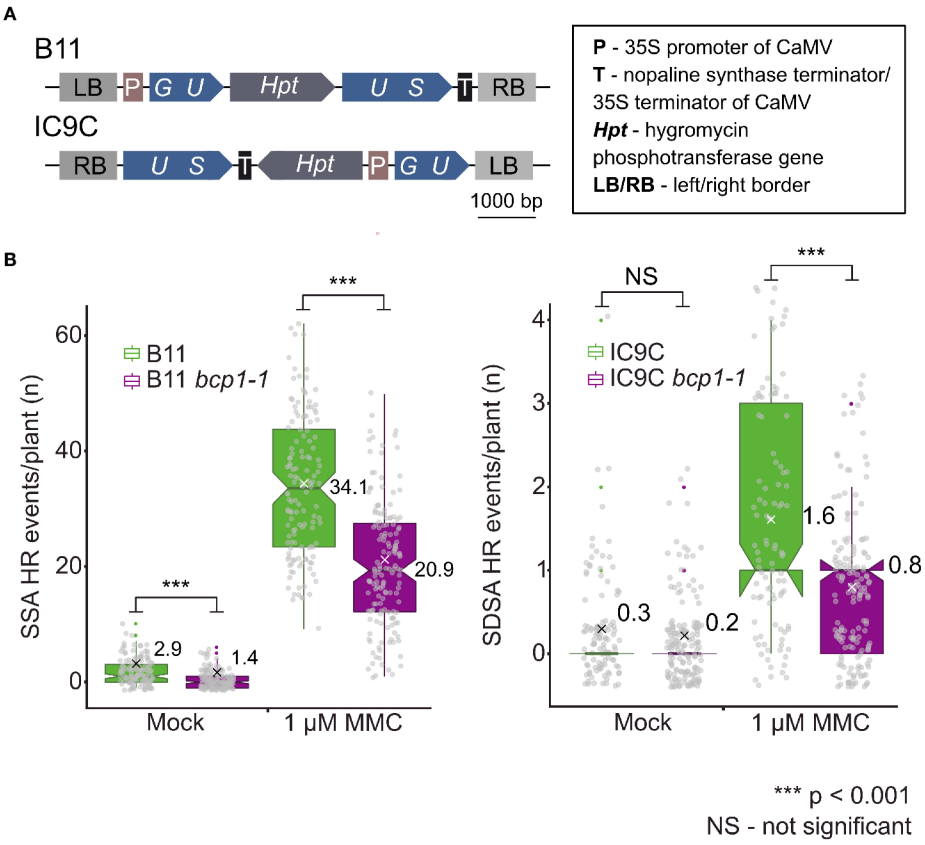

FIGURE 5
(A) Schematic model of constructs used to create *B11* and *IC9C* HR reporter lines (Swoboda et al., 1994; Molinier et al., 2004). **(B)** Loss of BCP1 causes reduced frequency of somatic homologous recombination (HR). Wild type (WT) and *bcp1-1* plants carrying genomic substrates for single-strand annealing (*B11*) and synthesis-dependent strand annealing (*IC9C*) types of HR were grown on mock and 1 µM containing MMC media for 10 days. Gray dots indicate HR events per plant. The boxplots' hinges are in the 1st and 3rd quartile, with a marked median. Mean is represented by a cross with a numerical value. Whisker marks show the lowest or highest value within the 1.5 interquartile range below or above hinges. Asterisks represent significant differences in Mann-Whitney U-test *** $P < 0.001$, NS – not significantly different.

damage repair, their exact molecular functions, including the binding targets of BRCT5 domains, remain unknown.

The BCP2, BCP3, and BCP4 proteins carry a pair of BRCT domains only at their C-termini. On contrary, BCP1 bears an additional pair of BRCT domains also at the N-terminus. The BRCT5 domain of all Arabidopsis BCPs shows a conserved pattern of specific amino acids with different properties. Furthermore, *in silico*-based modeling revealed a conserved structure of this domain in plants relative to the fission yeast Brc1. The only non-BRCT domain identified in BCPs was an N-terminally positioned Gcn5-related N-acetyltransferase domain (Uniprot) in BCP2. It implies that BCP2 might contribute to chromatin relaxation and/or transcription. However, none of the BCPs repeated the repertoire of domains in *Sp*Brc1 and/or *Hs*NSE5, suggesting that they are not direct Arabidopsis homologs, and biochemical studies will have to be conducted to explore their potential relationship at the protein-protein interaction level. BCP1 shows possible homologies to the human proteins PTIP and TOPIP1B. However, a significant homology is present only over the BRCT domain regions. Based on this, we conclude that all four identified BCPs represent novel plant-specific BRCT5 domain-containing proteins.

An important step toward the functional characterization of the BCPs was their response to DNA damage. The most promising candidate in DNA damage sensitivity assays was *BCP1*, while *BCP2, BCP3*, and *BCP4* did not differ significantly from wild-type. However, analysis of additional mutant alleles for at least *BCP2* and *BCP3* is needed because the alleles tested in this study most likely do not represent loss of function mutants. The *bcp2-1* allele may even be a *BCP2* overexpressor line. BCP1 loss-of-function mutants were hypersensitive to DNA DSBs caused by bleocin, DNA-inter-strand crosslinks induced by MMC, and two types of DNA-protein crosslinks caused by zebularine and CPT. Hence, BCP1 emerged from our analyses as an important player in DNA repair of multiple types of DNA lesions, possibly through a mainstream DNA repair pathway. The possible role of BCP2,

BCP3, and BCP4 in e.g. repair of other types of DNA damage is not excluded and should be a focus of future studies.

We made an exciting observation that BCP1 is transcriptionally upregulated in response to gamma-radiation and MMC treatments and that the activation is SOG1-dependent. SOG1 is a plant-specific transcription factor that is phosphorylated by ATM and ATR kinases and orchestrates downstream responses of the key set of genes involved in the maintenance of genome stability, including cell cycle and homologous recombination repair (Yoshiyama et al., 2013a; Yoshiyama, 2016; Ogita et al., 2018). Two recent studies defined the SOG1 consensus binding motif CTT(N)$_7$AAG and found that SOG1 is physically binding to the cis-regulatory region of *BCP1* in Arabidopsis (Bourbousse et al., 2018; Ogita et al., 2018). Surprisingly, we did not find any such a motif in the region upstream of the *BCP1* transcription start site which suggests a presence of a non-canonical SOG1 binding motif in the *BCP1* promoter.

The absence of *BCP1* transcriptional upregulation in the *sog1* mutant background also clearly places *BCP1* downstream of SOG1 in the same DNA damage repair pathway. Although *BCP1* transcription is enhanced by DNA damage, it is not fully dependent on it. This is apparent from the expression of *BCP1* promoter in both somatic and floral meristems without any stress. Our analysis suggests that *BCP1* is activated to a basal level in SOG1-independent and induced-DNA damage-independent manner. Whether this represents an activation induced by spontaneously occurring DNA damage (e.g. during DNA replication) remains to be studied. In summary, we identify *BCP1* as an Arabidopsis BRCT5 domain-containing gene directly transcriptionally controlled by SOG1 during induced DNA damage.

The critical experiment was the analysis of somatic homologous recombination using genetically engineered HR trap lines. This experiment showed a significantly reduced frequency of HR in *bcp1* mutant plants, strongly suggesting that BCP1 is needed for normal levels of HR. How BCP1 directly functions in this process is currently unknown. By its N- and C-terminal BRCT domains, it could bind two phosphorylated proteins and this way facilitate HR. Such interactors will be identified in the follow-up research.

In conclusion, out of four uncharacterized Arabidopsis BRCT5 domain-containing proteins, we identified BCP1 as a new Arabidopsis DNA damage repair factor that is directly controlled by SOG1 and ensures normal levels of homologous recombination.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE112773.

Author contributions

AP, JP, JD, and JV conceived and designed the study. JV, FY, and ET performed experiments. All authors analyzed data and interpreted the results. AP and JV wrote the paper. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1023358/full#supplementary-material

References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., et al. (2003). Genome-wide insertional mutagenesis of arabidopsis thaliana. *Science* 301, 653–657. doi: 10.1126/science.1086391

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Baubec, T., Pecinka, A., Rozhon, W., and Mittelsten Scheid, O. (2009). Effective, homogeneous and transient interference with cytosine methylation in plant genomic DNA by zebularine. *Plant J.* 57, 542–554. doi: 10.1111/j.1365-313X.2008.03699.x

Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997). A superfamily of conserved domains in DNA damage- responsive cell cycle checkpoint proteins. *FASEB J.* 11, 68–76. doi: 10.1096/fasebj.11.1.9034168

Bourbousse, C., Vegesna, N., and Law, J. A. (2018). SOG1 activator and MYB3R repressors regulate a complex DNA damage network in arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 115, E12453–E12462. doi: 10.1073/pnas.1810582115

Chatterjee, N., and Walker, G. C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environ. Mol. Mutagen.* 58, 235–263. doi: 10.1002/em.22087

Hafner, A., Bulyk, M. L., Jambhekar, A., and Lahav, G. (2019). The multiple mechanisms that regulate p53 activity and cell fate. *Nat. Rev. Mol. Cell Biol.* 20, 199–210. doi: 10.1038/s41580-019-0110-x

Han, P., Li, Q., and Zhu, Y.-X. (2008). Mutation of arabidopsis BARD1 causes meristem defects by failing to confine WUSCHEL expression to the organizing center. *Plant Cell* 20, 1482–1493. doi: 10.1105/tpc.108.058867

Heyer, W.-D., Ehmsen, K. T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. *Annu. Rev. Genet.* 44, 113–139. doi: 10.1146/annurev-genet-051710-150955

Hu, Z., Cools, T., and De Veylder, L. (2016). Mechanisms used by plants to cope with DNA damage. *Annu. Rev. Plant Biol.* 67, 439–462. doi: 10.1146/annurev-arplant-043015-111902

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., and Weisshaar, B. (2012). GABI-kat SimpleSearch: new features of the arabidopsis thaliana T-DNA mutant database. *Nucleic Acids Res.* 40, D1211–D1215. doi: 10.1093/nar/gkr1047

Klepikova, A. V., Kasianov, A. S., Gerasimov, E. S., Logacheva, M. D., and Penin, A. A. (2016). A high resolution map of the arabidopsis thaliana developmental transcriptome based on RNA-seq profiling. *Plant J.* 88, 1058–1070. doi: 10.1111/tpj.13312

Lafarge, S., and Montané, M.-H. (2003). Characterization of arabidopsis thaliana ortholog of the human breast cancer susceptibility gene 1: AtBRCA1 , strongly induced by gamma rays. *Nucleic Acids Res.* 31, 1148–1155. doi: 10.1093/nar/gkg202

Leung, G. P., Lee, L., Schmidt, T. I., Shirahige, K., and Kobor, M. S. (2011). Rtt107 is required for recruitment of the SMC5/6 complex to DNA double strand breaks. *J. Biol. Chem.* 286, 26250–26257. doi: 10.1074/jbc.M111.235200

Li, X., Liu, K., Li, F., Wang, J., Huang, H., Wu, J., et al. (2012). Structure of c-terminal tandem BRCT repeats of Rtt107 protein reveals critical role in interaction with phosphorylated histone H2A during DNA damage repair. *J. Biol. Chem.* 287, 9137–9146. doi: 10.1074/jbc.M111.311860

Lobet, G., Pagès, L., and Draye, X. (2011). A novel image-analysis toolbox enabling quantitative analysis of root system architecture. *Plant Physiol.* 157, 29–39. doi: 10.1104/pp.111.179895

Manova, V., and Gruszka, D. (2015). DNA Damage and repair in plants – from models to crops. *Front. Plant Sci.* 6. doi: 10.3389/fpls.2015.00885

Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the arabidopsis shoot meristem. *Cell* 95, 805–815. doi: 10.1016/S0092-8674(00)81703-1

Menges, M., Hennig, L., Gruissem, W., and Murray, J. A. H. (2002). Cell cycle-regulated gene expression inArabidopsis *. *J. Biol. Chem.* 277, 41987–42002. doi: 10.1074/jbc.M207570200

Molinier, J., Ries, G., Bonhoeffer, S., and Hohn, B. (2004). Interchromatid and interhomolog recombination in arabidopsis thaliana. *Plant Cell* 16, 342–352. doi: 10.1105/tpc.019042

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. doi: 10.3389/fpls.2019.00653

Ogita, N., Okushima, Y., Tokizawa, M., Yamamoto, Y. Y., Tanaka, M., Seki, M., et al. (2018). Identifying the target genes of SUPPRESSOR OF GAMMA RESPONSE 1, a master transcription factor controlling DNA damage response in arabidopsis. *Plant J.* 94, 439–453. doi: 10.1111/tpj.13866

Oravcová, M., Gadaleta, M. C., Nie, M., Reubens, M. C., Limbo, O., Russell, P., et al. (2019)Brc1 promotes the focal accumulation and SUMO ligase activity of Smc5-Smc6 during replication stress. In: *Molecular and cellular biology*. Available at: https://journals.asm.org/doi/full/10.1128/MCB.00271-18 (Accessed May 11, 2022).

Orel, N., Kyryk, A., and Puchta, H. (2003). Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome. *Plant J.* 35, 604–612. doi: 10.1046/j.1365-313X.2003.01832.x

Preuss, S. B., and Britt, A. B. (2003). A DNA-damage-induced cell cycle checkpoint in arabidopsis. *Genetics* 164, 323–334. doi: 10.1093/genetics/164.1.323

Prochazkova, K., Finke, A., Tomaštíková, E. D., Filo, J., Bente, H., Dvořák, P., et al. (2022). Zebularine induces enzymatic DNA–protein crosslinks in 45S rDNA heterochromatin of arabidopsis nuclei. *Nucleic Acids Res.* 50, 244–258. doi: 10.1093/nar/gkab1218

Puchta, H., Swoboda, P., and Hohn, B. (1995). Induction of intrachromosomal homologous recombination in whole plants. *Plant J.* 7, 203–210. doi: 10.1046/j.1365-313X.1995.7020203.x

R Core Team. (2018). *R: A Language and Environment for Statistical Computing* (Vienna:R Foundation for Statistical Computing). Available at: https://www.R-project.org.

Räschle, M., Smeenk, G., Hansen, R. K., Temu, T., Oka, Y., Hein, M. Y., et al. (2015). Proteomics reveals dynamic assembly of repair complexes during bypass of DNA cross-links. *Science* 348, 1253671. doi: 10.1126/science.1253671

Razqallah, H. (2008). DNA-Damage repair; the good, the bad, and the ugly. *EMBO J.* 27, 589–605. doi: 10.1038/emboj.2008.15

Reidt, W., Wurz, R., Wanieck, K., Ha Chu, H., and Puchta, H. (2006). A homologue of the breast cancer-associated gene BARD1 is involved in DNA repair in plants. *EMBO J.* 25, 4326–4337. doi: 10.1038/sj.emboj.7601313

Seton-Rogers, S. (2006). Putting p53 in context. *Nat. Rev. Cancer* 6, 423–423. doi: 10.1038/nrc1924

Shultz, R. W., Tatineni, V. M., Hanley-Bowdoin, L., and Thompson, W. F. (2007). Genome-wide analysis of the core DNA replication machinery in the higher plants arabidopsis and rice. *Plant Physiol.* 144, 1697–1714. doi: 10.1104/pp.107.101105

Swoboda, P., Gal, S., Hohn, B., and Puchta, H. (1994). Intrachromosomal homologous recombination in whole plants. *EMBO J.* 13, 484–489. doi: 10.1002/j.1460-2075.1994.tb06283.x

Trapp, O., Seeliger, K., and Puchta, H. (2011). Homologs of breast cancer genes in plants. *Front. Plant Sci.* 2. doi: 10.3389/fpls.2011.00019

Varadi, M., Anyango, S., Deshpande, M., Nair, S., Natassia, C., Yordanova, G., et al. (2022). AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res.* 50, D439–D444. doi: 10.1093/nar/gkab1061

Wan, B., Hang, L. E., and Zhao, X. (2016). Multi-BRCT scaffolds use distinct strategies to support genome maintenance. *Cell Cycle* 15, 2561–2570. doi: 10.1080/15384101.2016.1218102

Williams, J. S., Williams, R. S., Dovey, C. L., Guenther, G., Tainer, J. A., and Russell, P. (2010). gammaH2A binds Brc1 to maintain genome integrity during s-phase. *EMBO J.* 29, 1136–1148. doi: 10.1038/emboj.2009.413

Wuest, S. E., Vijverberg, K., Schmidt, A., Weiss, M., Gheyselinck, J., Lohr, M., et al. (2010). Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes. *Curr. Biol.* 20, 506–512. doi: 10.1016/j.cub.2010.01.051

Wu, L. C., Wang, Z. W., Tsan, J. T., Spillman, M. A., Phung, A., Xu, X. L., et al. (1996). Identification of a RING protein that can interact *in vivo* with the BRCA1 gene product. *Nat. Genet.* 14, 430–440. doi: 10.1038/ng1296-430

Yan, W., Shao, Z., Li, F., Niu, L., Shi, Y., Teng, M., et al. (2011). Structural basis of γH2AX recognition by human PTIP BRCT5-BRCT6 domains in the DNA damage response pathway. *FEBS Lett.* 585, 3874–3879. doi: 10.1016/j.febslet.2011.10.045

Yan, S., Wang, W., Marqués, J., Mohan, R., Saleh, A., Durrant, W. E., et al. (2013). Salicylic acid activates DNA damage responses to potentiate plant immunity. *Mol. Cell* 52, 602–610. doi: 10.1016/j.molcel.2013.09.019

Yoshiyama, K. O. (2016). SOG1: a master regulator of the DNA damage response in plants. *Genes Genet. Syst.* 90, 209–216. doi: 10.1266/ggs.15-00011

Yoshiyama, K., Conklin, P. A., Huefner, N. D., and Britt, A. B. (2009). Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. *Proc. Natl. Acad. Sci.* 106, 12843–12848. doi: 10.1073/pnas.0810304106

Yoshiyama, K., Kobayashi, J., Nobuo, O., Minako, U., Kimura, S., Maki, H., et al. (2013a). ATM-Mediated phosphorylation of SOG1 is essential for the DNA damage response in arabidopsis. *EMBO Rep.* 14, 817–822. doi: 10.1038/embor.2013.112

Yoshiyama, K. O., Sakaguchi, K., and Kimura, S. (2013b). DNA Damage response in plants: conserved and variable response compared to animals. *Biology* 2, 1338–1356. doi: 10.3390/biology2041338

Yu, X., Chini, C. C. S., He, M., Mer, G., and Chen, J. (2003). The BRCT domain is a phospho-protein binding domain. *Science* 302, 639–642. doi: 10.1126/science.1088753

Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., and Chua, N.-H. (2006). Agrobacterium-mediated transformation of arabidopsis thaliana using the floral dip method. *Nat. Protoc.* 1, 641–646. doi: 10.1038/nprot.2006.97

Supplementary materials

Analysis of BRCT5 domain containing proteins reveals a new component of DNA damage repair in Arabidopsis

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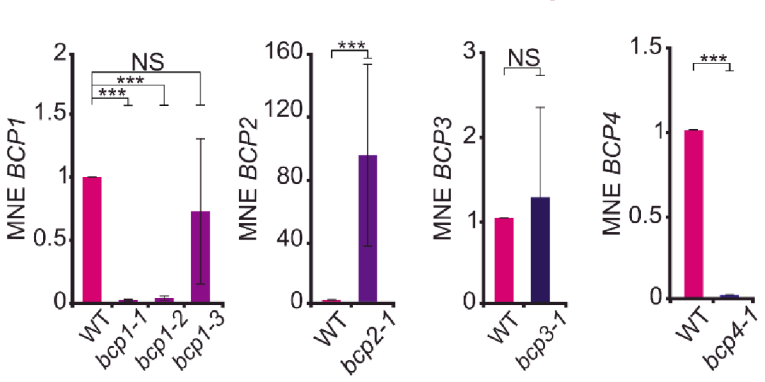
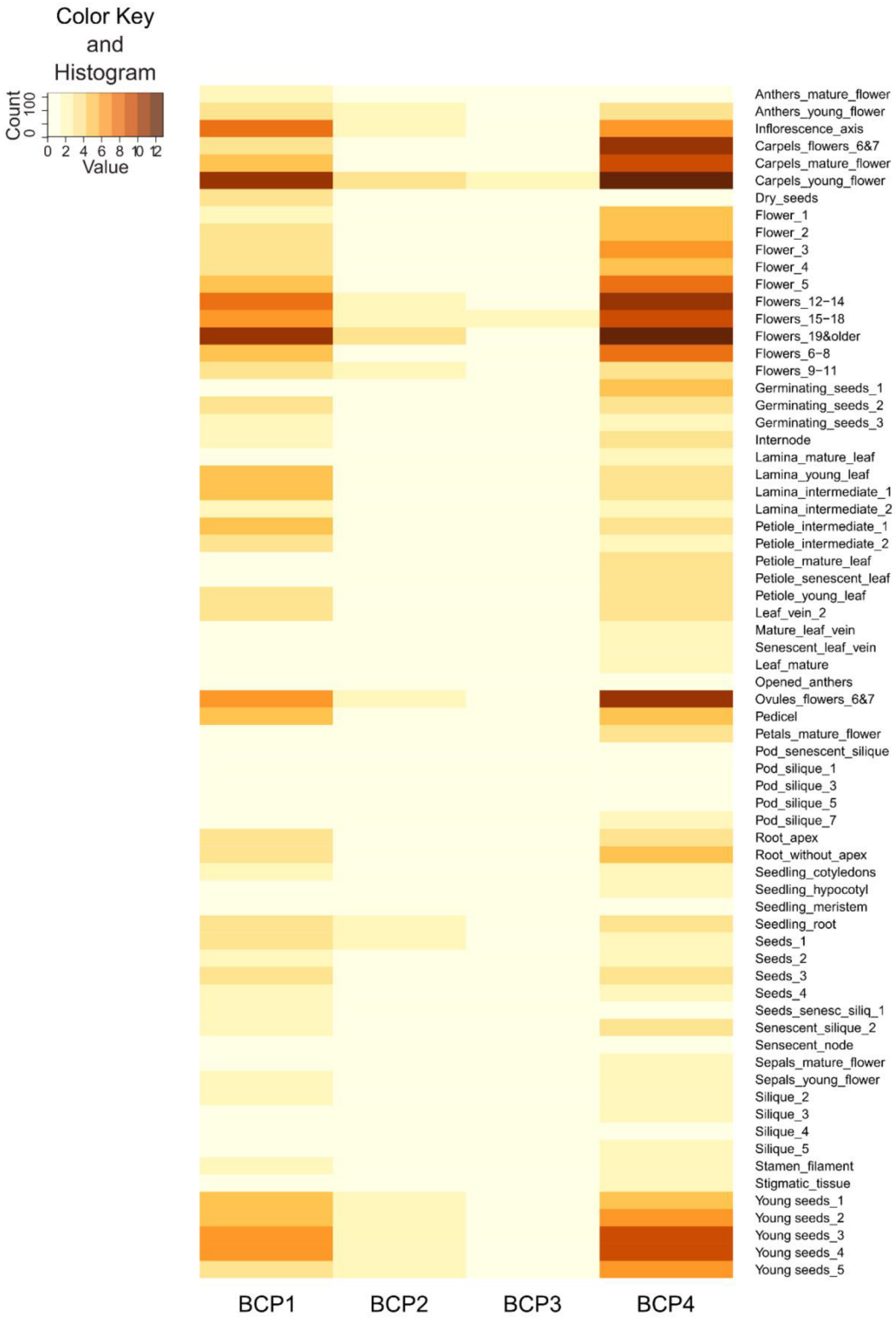
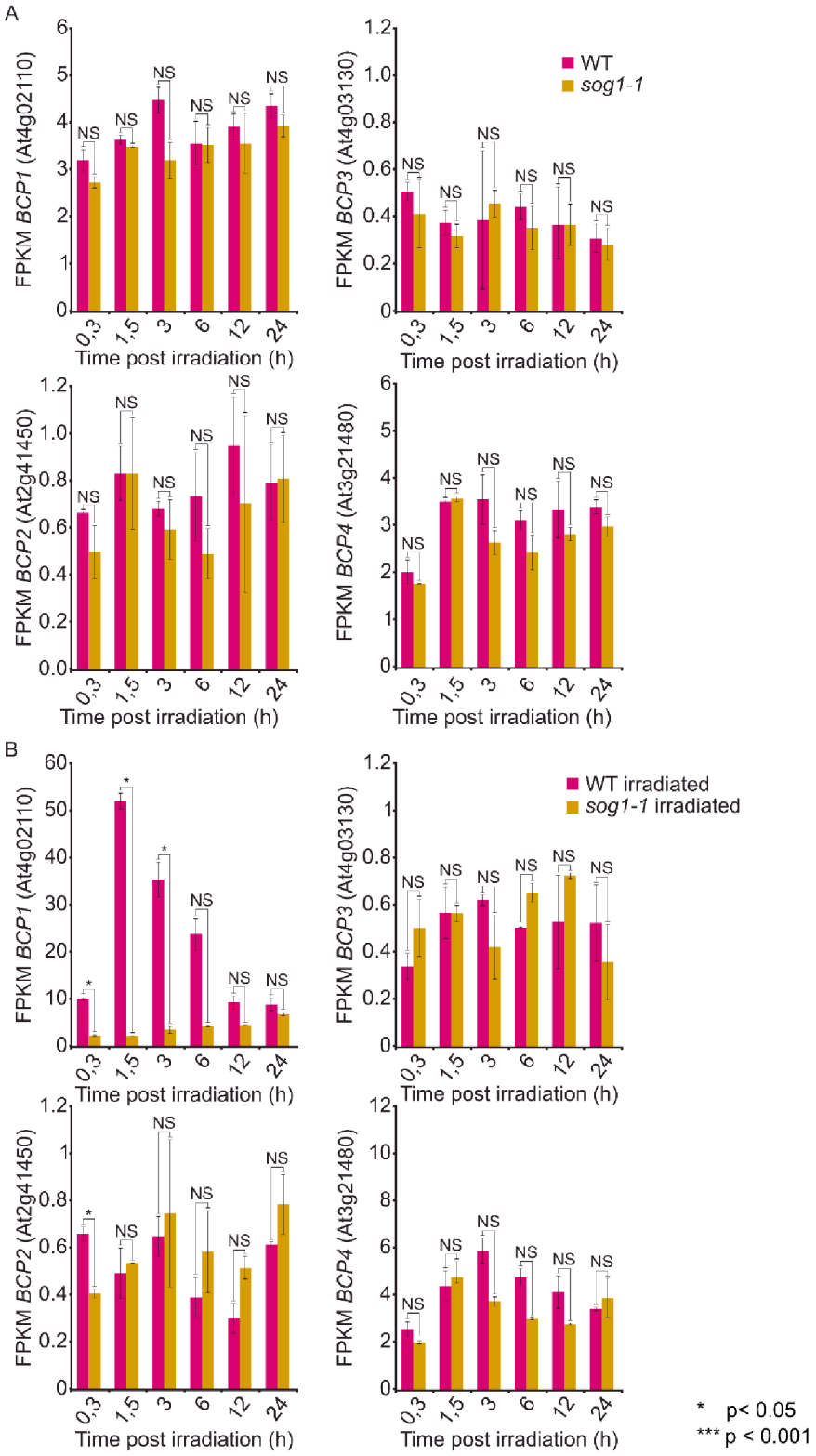


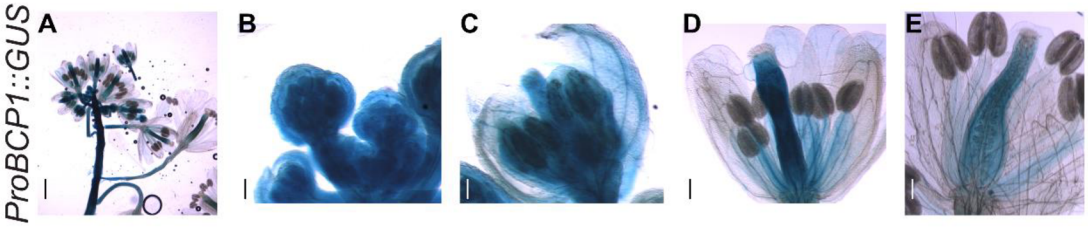
Supplementary Figure 1 | Expression of BCP genes in the respective *bcp* mutants. Reverse transcription qPCR analysis of *BCP1*, *BCP2*, *BCP3* and *BCP4* expression in wild-type (WT) and respective *bcp* mutant plants under ambient conditions. Y-axis shows mean normalized expression relative to *PP2A*. Error bars show three biological replicates. NS = not significantly different, *** $P < 0.001$ statistical difference in Mann-Whitney U-test test at $P < 0.05$.



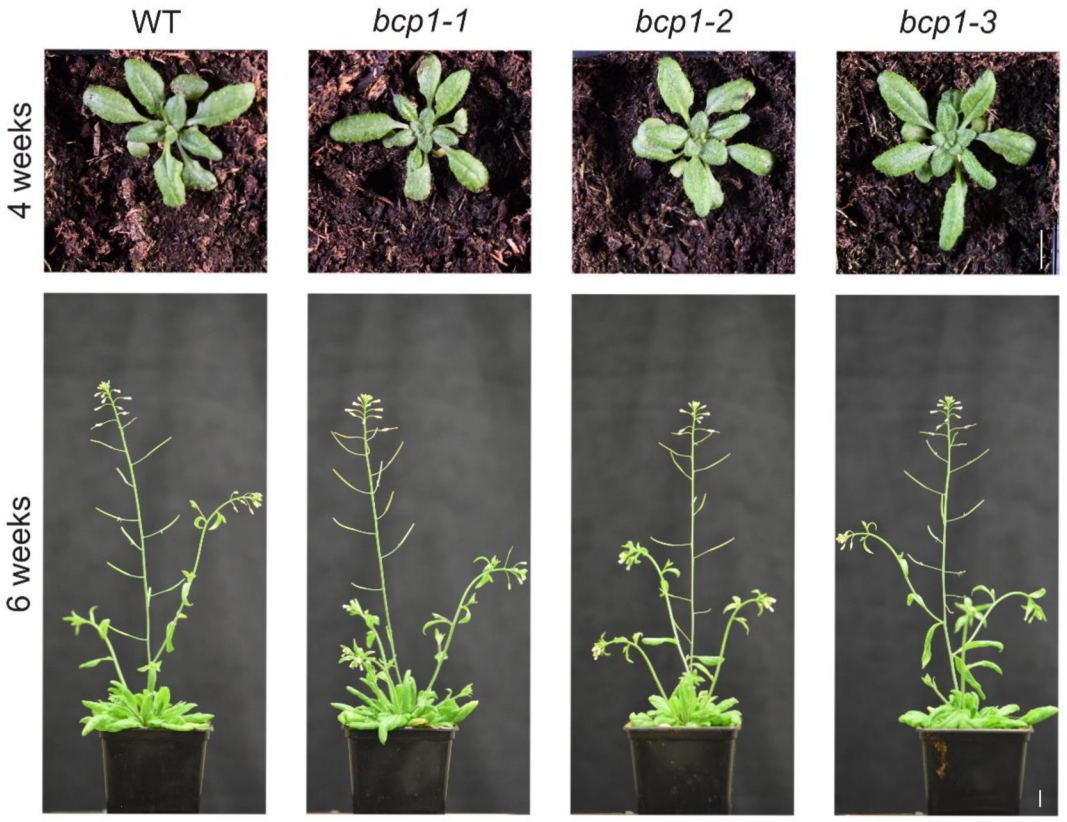
Supplementary Figure 2 | Expression of BCP genes during plant development. The expression values for individual genes on x-axis correspond to number of fragments per kilobase per million (FPKM). The y-axis shows individual tissues. The expression values were retrieved from a public dataset (Klepikova et al., 2016).



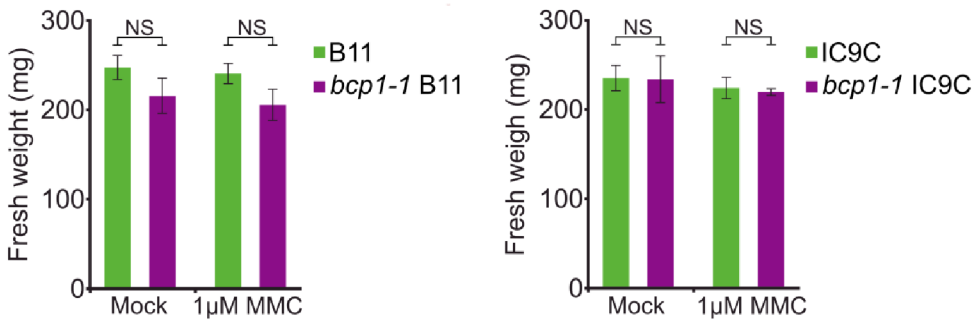
Supplementary Figure 3 | (**A**) Expression of BCP genes under mock conditions in wild-type and *sog1-1* plants. (**B**) Expression of BCP genes after gamma irradiation in wild type and *sog1-1* plants. The x-axis shows different sampling times after gamma irradiation (hours) and the y-axis shows a number of fragments per kilobase per million (FPKM). Asterisks represent significant differences in two sample T-test with unequal variance, * P < 0.05, *** P < 0.001, NS – not significantly different. The expression values were extracted from the public dataset (Bourbousse et al., 2018).



Supplementary Figure 4 | *In planta* analysis of *BCP1* expression in flower organs. Stable transformants carrying *ProBCP1::GUS* were grown in soil until flowering. Expression of *BCP1* was monitored after GUS histochemical staining of the inflorescences. Representative pictures showing the tissues with *BCP1* expression. (**A**) Full inflorescence. Scale bar = 1 mm. (**B**) Closed young flowers. Scale bar = 100 µm. (**C**) Young open flower. Scale bar = 200 µm. (**D**) Open flower with anthers and pistil. Scale bar = 100 µm. (**E**) Old flower with anthers and pistil. Scale bar = 200 µm.



Supplementary Figure 5 | Phenotypes of *BCP* mutant plants grown in soil. Homozygous BCP mutants were grown in soil and photographed at the rosette stage (4 weeks) and at seed setting (8 weeks). Scale bar = 1 cm.



Supplementary Figure 6 | Fresh weight measurements as the control for HR assays show no significant difference between control marker lines B11 and IC9C and marker lines in bcp1-1 background. B11, *bcp1-1* B11, IC9C and *bcp1-1* IC9C seedlings were grown for 10 days on ½ MS (MOCK) or ½ MS with 1 μM MMC (MMC). Three replicates, consisting of 60 plants each, were measured for each group. The statistical significance of data was assessed by Mann-Whitney U-test. NS – not significantly different.

Gene	Name	Sequence (5' to 3')	Use
At4g02110	*bcp1-1*_LP	TGTATTAGTGGACGCCTGGAATTG	genotyping
At4g02110	*bcp1-1*_RP	AGTGTTTAACTCACTCGTGGGTGA	genotyping
At4g02110	*bcp1-2*_LP	GATGGTCTTTCTCTTCTGGGG	genotyping
At4g02110	*bcp1-2*_RP	CGCCAGAGACTGATACTTTGG	genotyping
At4g02110	*bcp1-3*_LP	AGATTTGAATGGGATTCCAGG	genotyping
At4g02110	*bcp1-3*_RP	CCAAAGTATCAGTCTCTGGCG	genotyping
At2g41450	*bcp2-1*_LP	TTTGGGTCGGATTCGGGATTTTT	genotyping
At2g41450	*bcp2-1*_RP	AGTTGACAACTTGAACGTTTGTTAC	genotyping
At4g03130	*bcp3-1*_LP	CACGCATCAAATCTAGCCAAG	genotyping
At4g03130	*bcp3-1*_RP	ATCTTCAATTTCCCCACATCC	genotyping
At3g21480	*bcp4-1*_LP	CTGCCTTGCATTCTTTTCAAG	genotyping
At3g21480	*bcp4-1*_RP	TGTAAGACAACTCGCCTCACC	genotyping
o8474		ATAATAACGCTGCGGACATCTACATTTT	genotyping
LBb1.3		ATTTTGCCGATTTCGGAAC	genotyping
At4g02110	*proBCP1*_FWD	AAAAAGCAGGCTTATTAAAAATTTGTAAGTAA AACCATTTGCTATAACAAGAATTTATAGCT	cloning
At4g02110	*proBCP1*_REV	AGAAAGCTGGGTTTTTTTTTTTTGAAAAATTAG GGTTTTATTAGGGTGGAGG	cloning
At4g02110	attB1 adapter	GGGGACAAGTTTGTACAAAAAAGCAGGCT	cloning
At4g02110	attB2 adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT	cloning
At4g02110	*BCP1_FWD*	TGCAGAGGTGGAAATTACGGTGCTAG	RT-qPCR
At4g02110	*BCP1_REV*	TTTACCTACACCAGCCTCCCTTTTGC	RT-qPCR
At2g41450	*BCP2_FWD*	TTCATTGGTTTTGAAGTCCACGCTTG	RT-qPCR
At2g41450	*BCP2_REV*	GTGTGTATATGTTACAGCAGCAAGAGGT	RT-qPCR
At4g03130	*BCP3_FWD*	CCCCATTTCAAGTGCTCTACGACGAT	RT-qPCR
At4g03130	*BCP3_REV*	AGCAGCGACACTTCCATCTTCATCAT	RT-qPCR
At3g21480	*BCP4_FWD*	TGGGCTCGTTCTGATTCCAAACTGTT	RT-qPCR
At3g21480	*BCP4_REV*	ATCATTCCCTAAAACTGCCGCTTCAC	RT-qPCR
At1g69960	*PP2A_FWD*	TAACGTGGCCAAAATGATGC	RT-qPCR
At1g69960	*PP2A_REV*	GTTCTCCACAACCGCTTGGT	RT-qPCR

Supplementary Table 1 | Oligonucleotides used in this study.

APPENDIX II:
FIRST AUTHOR PUBLICATION II

Vladejić, J., Kovacik, M., Zwyrtková, J., Szurman-Zubrzycka, M., Doležel, J., & Pecinka, A. (2024) Zeocin-induced DNA damage response in barley and its dependence on ATR. *Sci Rep* **14**, 3119 (2024). https://doi.org/10.1038/s41598-024-53264-0

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Zeocin-induced DNA damage response in barley and its dependence on ATR

Jovanka Vladejić[1,2], Martin Kovacik[1,2], Jana Zwyrtková[1], Miriam Szurman-Zubrzycka[3], Jaroslav Doležel[1] & Ales Pecinka[1]✉

DNA damage response (DDR) is an essential mechanism by which living organisms maintain their genomic stability. In plants, DDR is important also for normal growth and yield. Here, we explored the DDR of a temperate model crop barley (*Hordeum vulgare*) at the phenotypic, physiological, and transcriptomic levels. By a series of in vitro DNA damage assays using the DNA strand break (DNA-SB) inducing agent zeocin, we showed reduced root growth and expansion of the differentiated zone to the root tip. Genome-wide transcriptional profiling of barley wild-type and plants mutated in DDR signaling kinase *ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED* (*hvatr.g*) revealed zeocin-dependent, ATR-dependent, and zeocin-dependent/ATR-independent transcriptional responses. Transcriptional changes were scored also using the newly developed catalog of 421 barley DDR genes with the phylogenetically-resolved relationships of barley *SUPRESSOR OF GAMMA 1* (*SOG1*) and *SOG1-LIKE* (*SGL*) genes. Zeocin caused up-regulation of specific DDR factors and down-regulation of cell cycle and histone genes, mostly in an ATR-independent manner. The ATR dependency was obvious for some factors associated with DDR during DNA replication and for many genes without an obvious connection to DDR. This provided molecular insight into the response to DNA-SB induction in the large and complex barley genome.

Cells combat DNA damage caused by various factors such as ultraviolet radiation, pathogens, transposable elements, and replication errors. This is known as the DNA damage response (DDR) and involves a complex network of sensors, transducers, mediators, and effectors[1]. Preservation of the cellular DNA is imperative for the normal progression of the cell cycle and growth. DNA single and double-strand breaks (SSBs and DSBs) are highly toxic forms of damage. SSBs induced by oxidative stress are processed by Poly(ADP-ribose) polymerases (PARPs) and Poly(ADP-ribose) glycohydrolases, while those resulting from TOPOISOMERASE I activity are first processed by Tyrosyl-DNA phosphodiesterase 1 (TDP1). Ligation is carried out by single-strand DNA ligase I, and X-ray repair cross-complementing protein 1 (XRCC1) in plants. DSB repair is more complex, involving canonical non-homologous end joining (cNHEJ) for blunt-ended breaks, and alternative end joining (alt-EJ), single-strand annealing (SSA), or homologous recombination (HR) for staggered ends. Individual pathways are not equal in their repair fidelity. The alt-EJ is error-prone, SSA leads to DNA loss, and only HR enables error-free repair. The alt-EJ pathway is promoted by POLYMERASE Q (TEBICHI), while SSA and HR depend on the damage recognition by the MRE 11, RAD50 and NIJMEGEN BREAKAGE SYNDROME (MRN) complex, which activates ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) kinases[2]. ATM is activated by DSBs, while ATR has preference for single-stranded DNA. The Arabidopsis *atm* and *atr* single mutants develop normally, but *atm* mutants are partially, and *atm atr* double mutants are fully sterile[3,4]. The *atm* plants are sensitive preferentially to DSB inducers[3], while *atr* plants are sensitive to agents interfering with replication[4]. Both ATM and ATR phosphorylate key transcription factor SUPPRESSOR OF GAMMA 1 (SOG1) that orchestrates downstream DDR responses in Arabidopsis[5,6]. SOG1 activation leads to transcriptional changes in two-thirds of DDR-responsive genes, including cell cycle regulators and DDR effector proteins[7,8].

Knowledge on DDR in cereals, including cultivated barley (*Hordeum vulgare* L. subsp. *vulgare*), is limited, and the functional understanding of DDR factors in barley is just beginning. Barley is a genetic model for temperate cereals with a large diploid genome (1C = 5.1 Gbp) and Rabl chromosome organization. Early studies examined DNA repair in barley seeds and embryos using *N*-methyl-*N*-nitrosourea and methyl methane

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sulfonate treatments[9–12]. Recent research assessed barley DSB repair capacity and pathway choice, revealing the involvement of NHEJ, alt-EJ, and HR pathways and a high (> 81%) coincidence of sister chromatid exchanges indicating a frequent use of sister chromatids as a template for repair[13]. In addition, 148 barley genes related to DNA damage repair response and replication were identified and served as the first dedicated resource for DDR analysis in barley[14]. Also, delayed transcriptomic response to gamma-radiation was analyzed using microarrays[15]. In this study, dry seeds were irradiated, then imbibed for 2, 24 or 48 h and subsequently the expression changes were analyzed. The low irradiation dose affected expression of phytohormones, late embryogenesis abundant proteins and cell wall components, while the high dose caused expression changes indicating cell cycle arrest, activation of DNA damage repair, and antioxidants. A barley ATR mutant (*hvatr.g*) was identified and characterized in the HorTILLUS tilling population[16]. The *hvatr.g* plants resemble wild-type (WT) but exhibit increased DNA damage levels even under normal conditions. They also have a deregulated cell cycle and continue dividing in the presence of toxic concentrations of aluminum[16]. The *hvatr.g* plants show reduced efficiency of DNA repair during DNA-replication stress, confirming the involvement of the barley ATR homolog in DNA damage repair[17].

Here, we describe the effects of radiomimetic agent zeocin on the growth of barley WT and *hvatr.g* mutant plants. We devised a methodology that allows robust testing of barley sensitivity to genotoxic stress. Subsequently, we performed genome-wide transcriptomic analysis after DNA damage induction in WT and *hvatr.g* plants and identified sets of genes that are misregulated by zeocin treatments in ATR dependent or independent manner. Our analyses were supported by the newly developed list of 421 barley DDR genes.

Results

Establishing barley in vitro DNA damage treatment

We developed a protocol to assess barley plant sensitivity to DNA damaging treatments. Approximately 20 dissected mature barley embryos were cultured on 100 ml of regular ½ MS medium in plastic cultivation boxes, resulting in synchronized germination approximately 24 h after initiation of cultivation.

We established an effective DNA damage treatment for WT barley cultivar (*cv.*) Golden Promise) using zeocin dilution series (100, 300, and 500 µg/ml). Zeocin is an antibiotic that causes DNA single and double strand breaks in a ratio 9:1, respectively, in experiments using Phage DNA[18]. Golden Promise strain embryos (n = 18–20 per experimental point) were cultured on mock and zeocin-containing media for 14 days (Fig. 1A). Phenotypic parameters were analyzed to identify informative traits. No significant differences were observed in root number between mock and zeocin treatments (Fig. 1B). However, the average root length showed a significant reduction, from 27 ± 2 mm (mock) to 16 ± 0.6, 11.9 ± 0.3, and 11.1 ± 0.5 mm (42%, 60%, and 60% reduction) with increasing zeocin concentrations (Fig. 1B). Similar trends were observed for the longest root length and cumulative root length parameters (Fig. 1B). The longest root length decreased from 36 ± 2.5 mm (mock) to 20 ± 0.9, 14 ± 0.3, and 14 ± 0.4 mm in individual zeocin treatments (Fig. 1B). Cumulative root length decreased significantly from 181 ± 15.2 mm (mock) to 110.5 ± 14.5, 76 ± 1, and 69.1 ± 0.5 mm with increasing zeocin concentrations. Additionally, two shoot parameters were assessed (Fig. 1B). The total shoot length (from the base of hypocotyl to the tip of the longest leaf) decreased from 153.2 ± 11.2 mm (mock) to 131.1 ± 6.6, 119.5 ± 2.0, and 68.8 ± 3.4 mm after zeocin treatments, corresponding to 14.2%, 22%, and 55.1% reduction, respectively (Fig. 1B). The length of hypocotyl relative to the whole stem length did not show significant differences between treatments (Fig. 1B).

Based on reduced root growth, we examined root apices and observed elongated root hairs and the absence of meristematic zone and the typical conically shaped root tip in 100 µg/ml zeocin-treated plants (n = 3) (Fig. 1C). This suggests premature differentiation of barley root apical meristem tissues upon DNA damage. DNA damage response often affects cell cycle dynamics[19]. Flow-cytometric DNA content measurements in 7-day-old mock-treated plants (n = 20) showed approximately 25.8 ± 5.2% 2C (G0/G1), 63.3 ± 3.7% 4C (G2), and 10.9 ± 3.3% 8C (endoreduplicated) nuclei in root apices (Fig. 1D). After 300 µg/ml zeocin treatment (n = 20), there was a 27% reduction in G2 nuclei (46.7 ± 4.6%), and endoreduplicated nuclei (8C) were 2.5 times more frequent (27.1 ± 6.3%). The super cycle value[20], indicating the percentage of endoreduplicated nuclei, showed a significant 2.5-fold increase after zeocin treatment (Fig. 1D).

Collectively, we observed reduced root growth and shorter stems after zeocin treatment, with 100 µg/ml zeocin being sufficient for significant changes. Thus, we successfully established zeocin-induced in vitro DNA damage conditions in barley.

Hvatr.g plants are sensitive to zeocin treatment

To explore barley's DNA damage response (DDR) in the context of DNA damage signaling, we utilized a loss-of-function mutant allele of ATR kinase (*hvatr.g*) in the Sebastian background[21]. Previous study on hydroponically grown *hvatr.g* plants demonstrated shorter roots compared to *cv.* Sebastian (WT)[17,21]. We observed a similar trend in plants grown from rescued embryos (n = 9–10) on solid media in vitro (Fig. 2A). Cultivar Sebastian was used as wild-type, and its phenotypic responses were comparable to those of *cv.* Golden Promise. Under mock conditions, *hvatr.g* plants exhibited a 60% reduction in root length compared to WT plants (13.4 ± 0.3 mm and 32.8 ± 1.4 mm, respectively) (Fig. 2B). Following 100 µg/ml zeocin treatment, the average root length was 15.8 ± 0.8 mm for WT plants and 8.9 ± 0.1 mm for *hvatr.g* plants (Fig. 2B). This corresponded to a 51.5% reduction for zeocin-treated WT plants and a 33.8% reduction for zeocin-treated *hvatr.g* plants (Fig. 2B). The greater reduction in WT plants likely indicates their higher potential for root shortening compared to *hvatr.g* plants, which already had significantly reduced root length. Similar results were obtained for cumulative root length and the longest root (Supplemental Fig. 1). The median root number was significantly higher in *hvatr.g* plants (median root number = 11) compared to WT plants (median root number = 8) (Fig. 2C). After zeocin treatment, WT plants maintained the median number of roots (median root number = 8), while the number decreased (median root number = 7; 36% reduction) in *hvatr.g* plants. Additionally, we analyzed plant height (Fig. 2D).

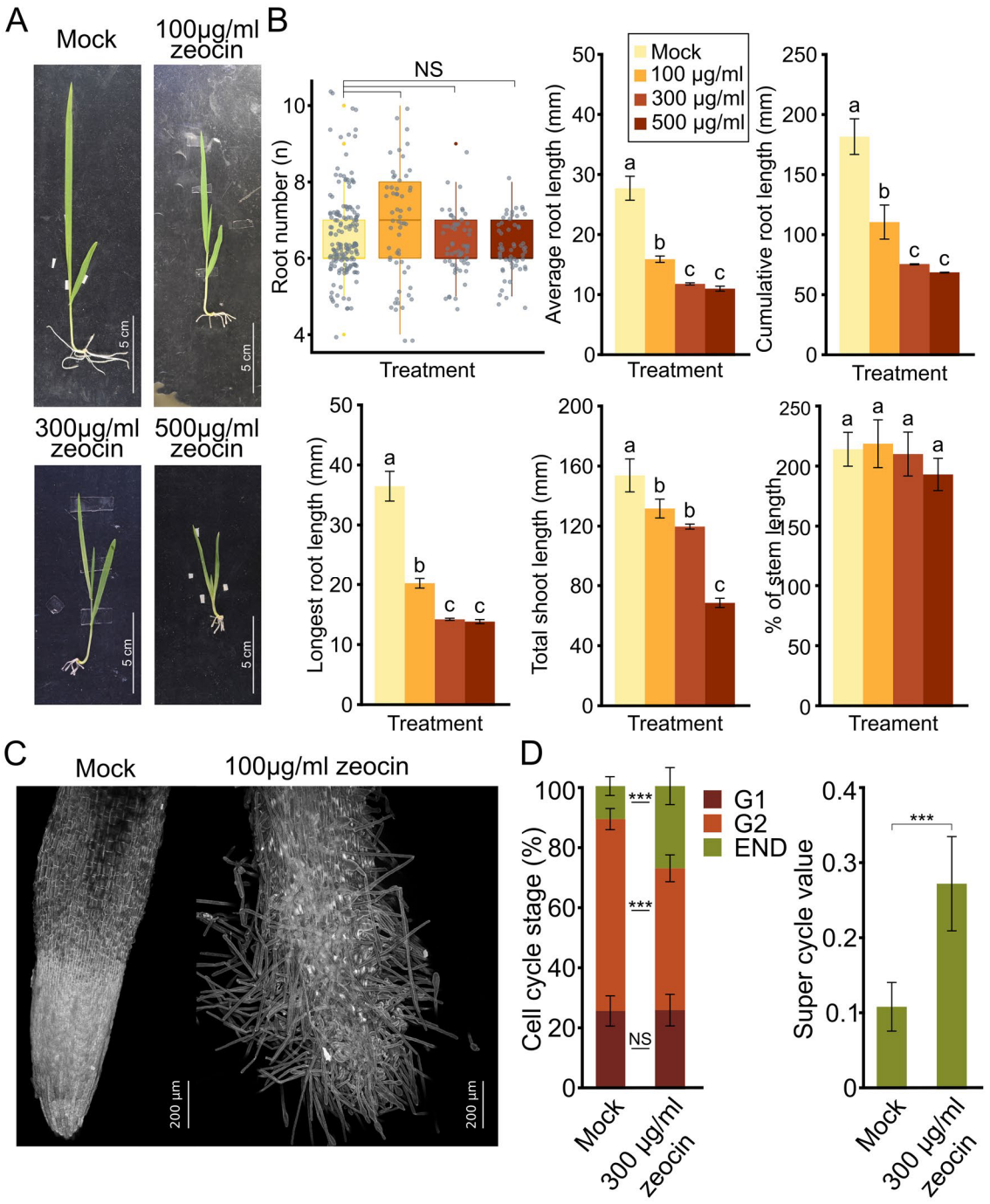

Figure 1. Wild-type barley (*cv.* Golden Promise) parameters in response to zeocin treatment. (**A**) Representative phenotypes of barley seedlings grown for 7 days on ½ MS (mock) and zeocin concentrations (100, 300, 500 µg/ml) containing media. Scale bars = 5 cm. (**B**) Effect of zeocin treatments on barley seedling growth. Error bars indicate the standard deviation between the means of three biological replicates. The letters above columns indicate similarities between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with post hoc Tukey's test ($P < 0.05$). Kruskall-Wallis H-test with post hoc Conover-Iman test of multiple comparisons with Benjamini–Hochberg procedure ($P < ½\ \alpha$, $\alpha = 0.05$) were used to analyze differences in root numbers. NS, not significant. (**C**) Representative confocal microscopy maximal projection images of the root surface in plants grown in mock conditions and plants treated with 100 µg/ml zeocin. Scale bar = 200 µm. (**D**) Percentage of nuclei at specific cell cycle stages found in the root tips of plants grown on mock and medium with 300 µg/ml zeocin. Super cycle value describing the amount of nuclei in endoreduplication (END). Statistical relevance distinguished by two-sample T-test, ($P < 0.05$). *$P < 0.05$, ***$P < 0.001$.

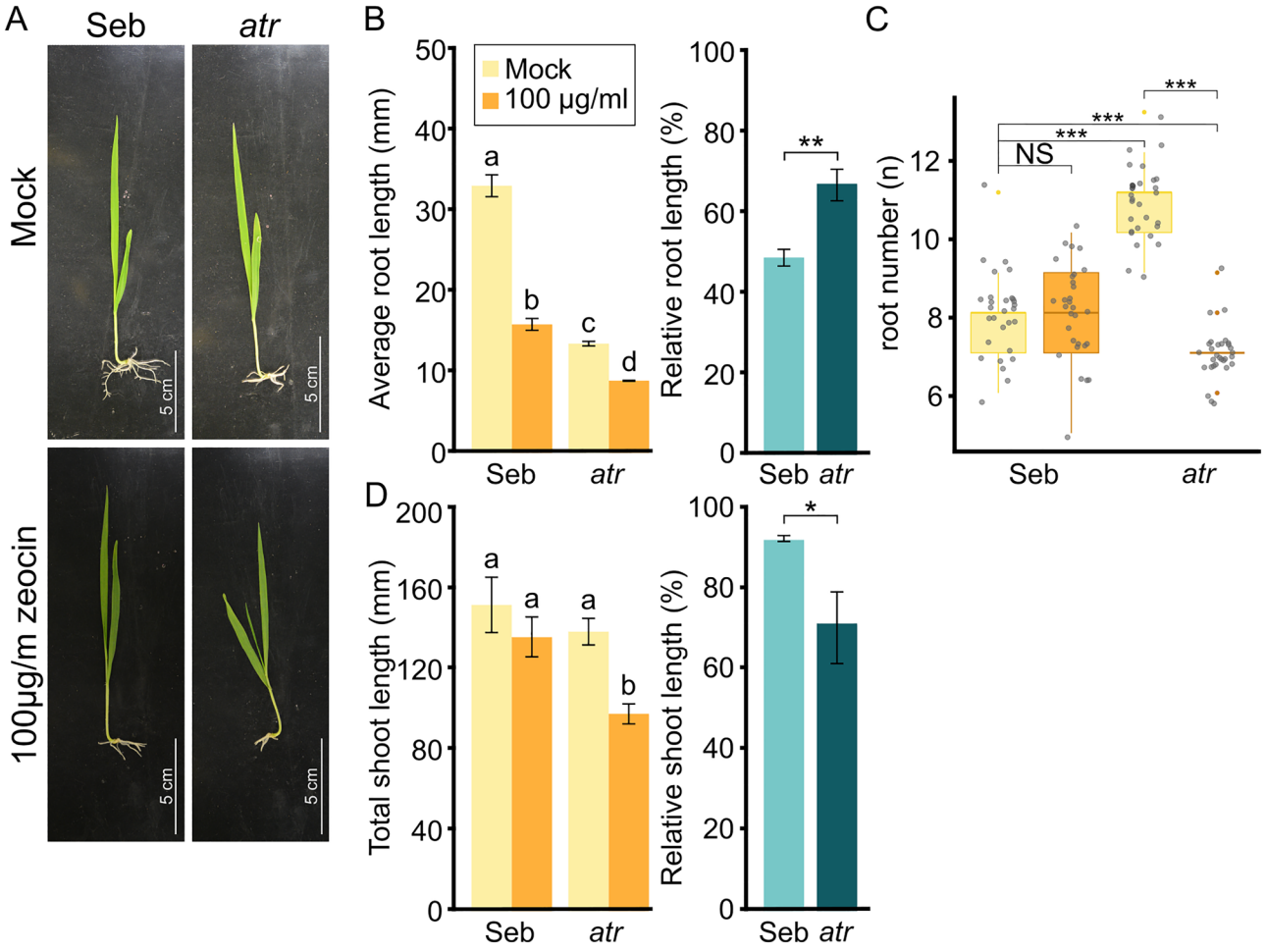

Figure 2. Barley *atr* mutant plants are sensitive to zeocin treatment. (**A**) Representative images of wild-type cultivar Sebastian (Seb) and *atr* mutant plants treated with 100 µg/ml zeocin, or untreated (mock). Scale bars = 5 cm. (**B**) Effect of zeocin treatment on Seb and *atr* plants' root growth. Error bars indicate the standard deviation between the means of three biological replicates. For the absolute values represented the letters above columns indicate similarities between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with post hoc Tukey's test ($P < 0.05$). For the assessment of the statistical significance of the relative values the two-sample T-test was used, $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Number of roots in wild-type (Seb) and *atr* plants grown in mock and genotoxic conditions. Kruskall–Wallis H-test with post hoc Conover-Iman test of multiple comparisons with Benjamini–Hochberg procedure ($P < ½\ \alpha$, $\alpha = 0.05$) was used to analyze differences in root numbers. NS, not significant, $^{***}P < 0.001$. (**D**) Effect of zeocin treatment on Seb and *atr* plants' shoot growth. Error bars indicate the standard deviation between the means of three biological replicates. For the absolute values represented the letters above columns indicate similarities between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with post hoc Tukey's test ($P < 0.05$). For the assessment of the statistical significance of the relative values the two-sample T-test was used, $^{*}P < 0.05$.

Zeocin-treated WT plants exhibited a non-significant reduction of 10.4% ($P > 0.05$) in height (135.0 ± 10.3 mm) compared to mock-treated plants (150.8 ± 14 mm), whereas zeocin-treated *hvatr.g* plants experienced a 29.4% reduction (97.1 ± 5.3 mm) compared to mock-treated *hvatr.g* plants (137.6 ± 6.9 mm).

These findings suggest that total shoot length is the most informative characteristic for assessing the DNA damaging effects of zeocin on barley *hvatr.g* plants.

Identification of barley homologs of DDR genes

To prepare for the transcriptomic analysis, we identified barley homologs of known DNA damage response (DDR) genes. Using the published list of 321 Arabidopsis DDR genes[22], we performed a series of BLASTs and selection steps, resulting in the discovery of 421 barley homologs (Supplemental Dataset 1). Visual inspection revealed the absence of *BRCA1* due to a lower homology. This gene was added manually and made the final set of 421 barley homologs. Approximately 50% of the identified proteins (n = 204) were coded by single-copy homologs, such as *ATM*, *ATR*, *WEE1*, *BRCA1*, *BARD*, and *RAD51*. We also observed an expansion of *PCNA2*ed family to 11 members in barley. Arabidopsis *PCNA2* interacts with the translesion synthesis polymerase POLH and is involved in the repair of UV-induced damage[23].

Of special interest was the master regulator of plant DDR SOG1 and its potential homologs. SOG1 is a member of a large NAC (NAM, ATAF1/2, and CUC2) family of transcription factors in plants and a list of 167 barley NAC domain containing proteins was published, highlighting HORVU.MOREX.r3.7HG0670800 (alias HORVU7Hr1G042420) as the most likely homolog of Arabidopsis ANAC008 (*At*SOG1)[24]. On contrary, another gene HORVU.MOREX.r3.6HG0590960 (alias HORVU6Hr1G053540) was suggested as *HvSOG1* based on upregulation upon DNA damage treatment[25]. Therefore, we analyzed SOG1 in barley in more detail. Via BLAST we found five barley genes that shared a significant homology with the *AtSOG1* (Supplemental Dataset 1). Three were analyzed previously[24]. To assess their relationships, we performed a phylogenetic study using five SOG1 and SOG1-LIKE (SGL) factors from barley, two from rice, two from maize and three from Arabidopsis (Fig. 3). The Arabidopsis factors included *At*SOG1/*AtANAC8* and two closely related transcription factors *At*ANAC44 and *At*ANAC85 that were also shown to play a role in DNA damage response[26].

Multiple sequence alignment and phylogenetic tree revealed three main clades, (i) the *At*ANAC005 and *At*ANAC006 outgroup, (ii) the SOG1 and (iii) the SGL clades. The SOG1 clade contained Arabidopsis SOG1, rice *Os*SOG1, both maize SOG1 candidates (*Zm*NACTF99 and *Zm*NACTF08) and HORVU.MOREX.r3.7HG0670800, suggesting it as the most likely barley SOG1 ortholog (Fig. 3). The SGL clade, defined based on a lower 45% confidence at its root but high confidence at the branches, contained *At*ANAC44 and *At*ANAC85, rice *Os*SGL, and the four remaining barley candidates. The barley protein HORVU.MOREX.r3.6HG0590960 was most closely related (63%) to the *Os*SGL; therefore, we named it as *Hv*SGL.A. The remaining three candidates HORVU.MOREX.r3.2HG0175540 (SGL.B), HORVU.MOREX.r3.2HG0175550 (SGL.C), and HORVU.MOREX.r3.2HG0175560

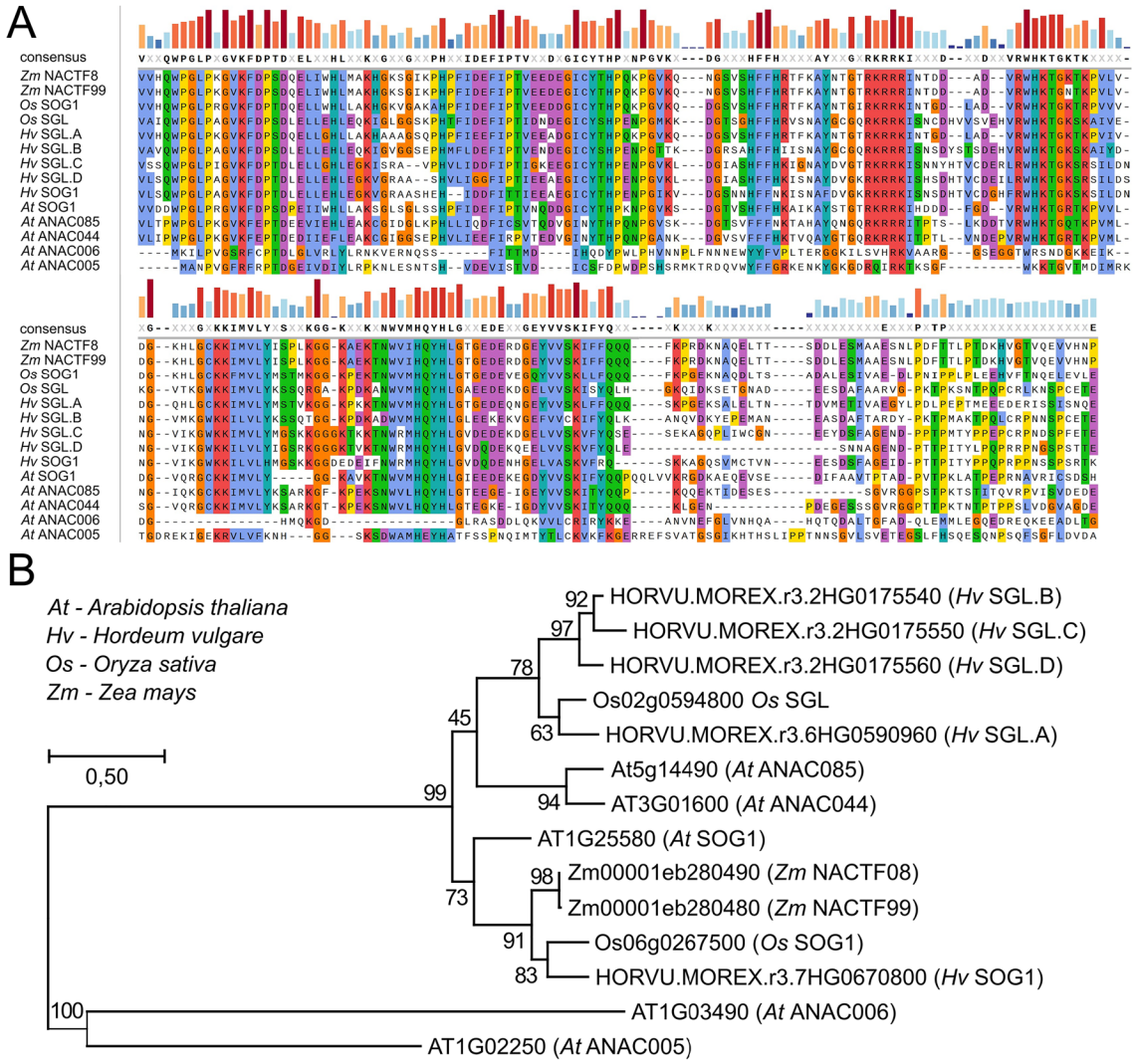

Figure 3. Phylogenetic analysis of barley SOG1 and SGLs. (**A**) Multiple sequence alignment of NAC protein domains present in five barley SOG1 protein candidates with the NAC domains of *Arabidopsis thaliana* (*At*) SOG1, ANAC044, and ANAC085, *Oryza sativa* (*Os*) SOG1 and SOG1-like (SGL), and *Zea mays* (*Zm*) NACTF8 and NACTF99 proteins by MUSCLE. Amino acids are highlighted based on consensus sequence and their physicochemical properties. (**B**) Phylogenetic Maximum Likelihood tree built based of the multiple sequence alignment, with *Arabiodopsis* ANAC005 and ANAC006 NAC protein domains used as an outgroup. Bootstrap values are shown next to the branches, distance scale = 0.5.

(SGL.D) showed 58 to 66% protein identity and their consequent gene identifiers suggest that they are inparalogs that arose by a local tandem triplication.

Hence, we developed a list of 421 barley homologs of known DDR genes and found a single *SOG1* ortholog and four *SGL* genes (*SGL.A*, *SGL.B*, *SGL.C* and *SGL.D*) in barley.

Transcriptomic responses of WT barley to zeocin treatments

We analyzed the transcriptional response of barley plants to zeocin treatments. Initially, we examined the expression of barley *BRCA1* and *RAD51* genes in seven-day-old WT (*cv.* Golden Promise) plants treated with 300 µg/ml zeocin for 0, 0.5, 1, and 6 h using RT-qPCR. Both *BRCA1* and *RAD51* showed significant up-regulation, with BRCA1 exhibiting a six-fold increase and RAD51 showing an almost 11-fold up-regulation at the 6-h time point (Fig. 4). Subsequently, we conducted RNA-seq analysis of root apices isolated from *cv.* Sebastian WT and *hvatr.g* plants treated with 0 (mock) and 500 µg/ml zeocin for 6 h, aiming to identify differentially expressed genes (DEGs).

In zeocin-treated versus mock-treated WT plants, we identified 719 DEGs, including 404 significantly up-regulated genes and 315 down-regulated genes (Supplemental Dataset 2). Gene ontology analysis of the up-regulated DEGs revealed their involvement in biological processes such as glutathione metabolic process (GO:0006749), response to stimulus (GO:0050896), response to stress (GO:0006950), and defense response (GO:0006952) (Fig. 5A; Supplemental Dataset 3). Notably, many up-regulated DEGs were associated with oxidative stress signaling (Fig. 5B), including 14 *GLUTATHIONE S-TRANSFERASEs* (*GSTU*) and 14 *UDP-GLUCOSYLTRANSFERASEs* (*UGT*) involved in reactive oxygen species detoxification. Other oxidative stress-related genes, such as *OXIDATIVE STRESS 3*, reductases, and a homolog of *CATION EXCHANGER 5*, were also identified. The observed oxidative stress response can be attributed to the radiomimetic activity of zeocin, which generates hydrogen peroxide upon interaction with a metal ion, leading to oxidative damage to DNA and other cellular components[27].

We found 7.6 and 2.8-fold up-regulation for *RAD51* and *BRCA1* in zeocin-treated WT plants, respectively, indicating activation of DDR also in our RNA-seq experiment. Subsequently, we inspected the full lists of significantly down-regulated and up-regulated DDR candidates. In accordance with the phenotypic data, zeocin treatment had a profound inhibitory effect on the cell cycle, including DNA replication and mitotic cell division. We found significant down-regulation for six *CYCLINs* (*CYC*) (*CYCB1;2.A-C*, *CYCB1;3.A-B*; *CYCB2;3.B*) and two *CYCLIN DEPENDENT KINANSES* (*CDKs*) *CDKB1;2* and *CDKB2;2*, whose Arabidopsis homologs promote G1/S and G2/M transitions, respectively.

Although the GO term analysis indicated genes related to DNA replication are both up- and down-regulated, a more detailed analysis revealed repressive effects of zeocin treatment on DNA replication. Seven genes marked by the GO term analysis as involved in DNA replication were up-regulated by zeocin treatment: *RPA1*, *RPA2*, *RAD51*, *DNA POLYMERASE ZETA SUBUNIT REV3*, and *RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE*. We argue that the up-regulation of these genes should not be taken as indication for active replication but rather as a sign of its repression and ongoing DDR. The RPA subunits play roles in both DNA replication and repair, DNA POLYMERASE ZETA and RAD51 are primarily associated with DDR, and RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE is involved in nucleotide synthesis. Next, we found down-regulation of almost all genes coding subunits of the Minichromosome maintenance complex (MCM), DNA primase and REPLICATION PROTEIN A complex. The reduced replication was recognizable also by looking at the expression of histones and replication-coupled chromatin modifiers. In total 63 *HISTONE* genes were down-regulated, including 15 copies of *H2A* in several variants, 9 copies of *H2B*, 23 copies of *H3.1* and 16 copies of *H4*. The downregulated

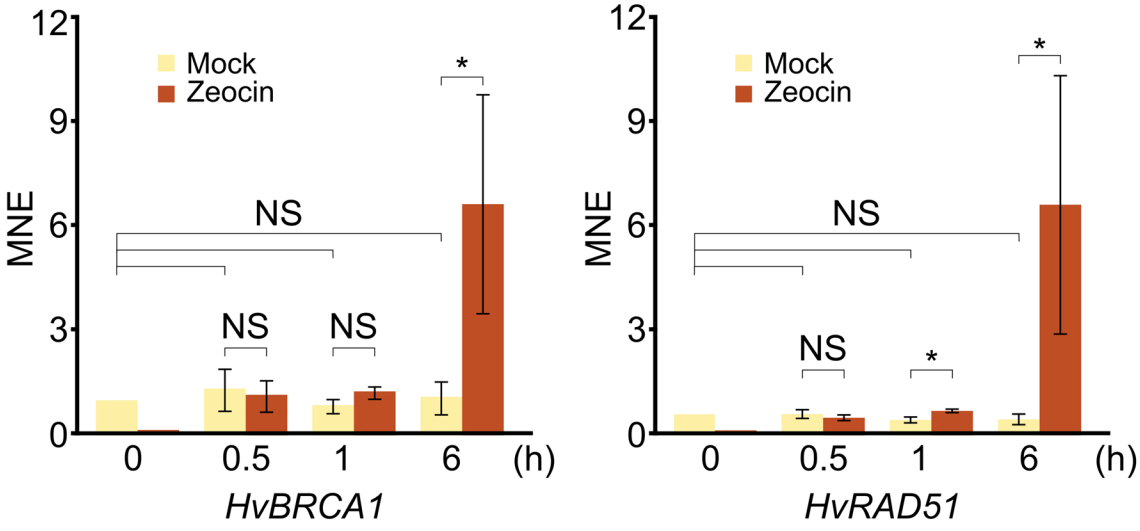

Figure 4. Barley *BRCA1* and *RAD51* are transcriptionally upregulated after zeocin treatment in Golden Promise wild-type. Reverse transcription-quantitative PCR values were normalized to the expression of *UBC2* as mean normalized expression (MNE). Error bars indicate standard deviation of three biological replicates. Statistical significance of the difference presented was tested by two-sample T-test, ($P<0.05$). *$P<0.05$.

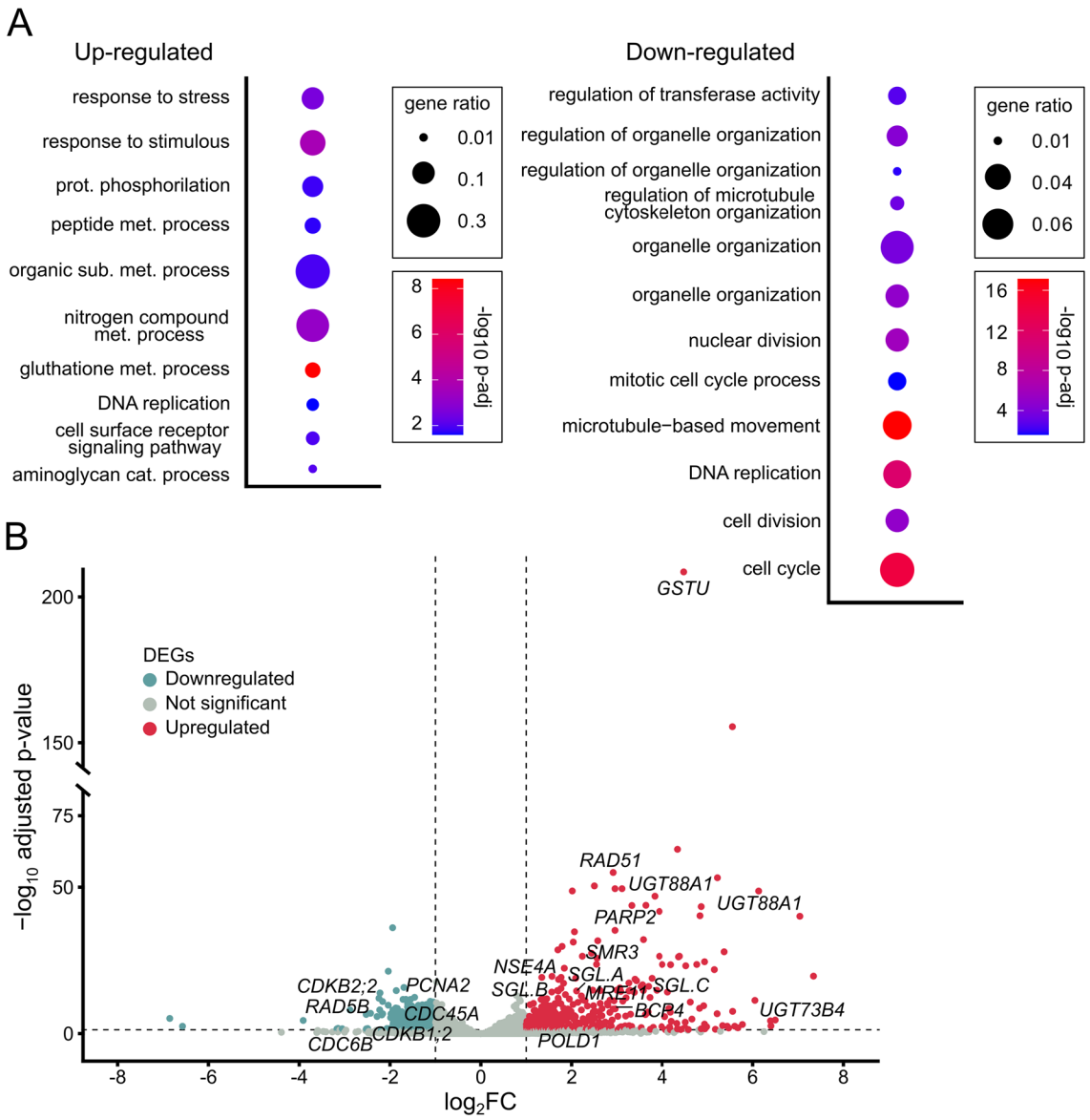

Figure 5. Zeocin effect on gene expression in wild-type barley. (**A**) Biological processes enriched among significantly up-regulated and down-regulated genes in zeocin versus mock treated wild-type (Sebastian) plants. Redundant GO terms were removed manually, based on *P*-value. The full list of GO terms can be found in Suppl. Dataset 3. Statistical significance was determined by Fisher's one-tailed test with g:SCS algorithm correction. Gene ratio represents the number of genes found in the category compared to the total number of genes in the query. (**B**) Volcano plot representing all genes detected as differentially expressed in wild-type (Sebastian) plants treated with zeocin relative to mock treatment. Horizontal dashed line indicates genes passing $P < 0.05$. Vertical dashed lines separate genes with $\log_2$ Fold Change ≤ -1 (blue) or $\log_2$ Fold Change ≥ 1 (red). Specific marker genes were highlighted by names.

H2A variants included one copy of *H2A.Z* and two copies of DDR-associated variant *H2A.X*. This is in line with down-regulation of CAF-1 histone chaperone subunit coding gene *FAS1*. Zeocin-induced down-regulation of replication coupled chromatin modifiers included *VARIANT IN METHYLATION 1* (*VIM1*), and two histone methyltransferases (one uncharacterized and the other homologous to *ARABIDOPSIS TRITHORAX-RELATED PROTEIN 6*). There were also several DDR-associated down-regulated genes, most notably barley homolog of *BARD1*.

Zeocin up-regulated genes pointed to specific directions. The up-regulation of two *PARP* genes and *MRE11* homologs indicated presence of both SSBs and DSBs, respectively. We also saw up-regulation of three RPAs: *RPA70C.C*, *RPA2.K* and *RPA1A* that suggest an increased amount of single stranded DNA. At the level of DDR signaling, we found up-regulation of three *SGL* factors *SGL.A*, *SGL.D*, and *SGL.C*, but not *SOG1*. The up-regulation of barley homolog of Arabidopsis CDK inhibitor *SMR3* is in agreement with the alterations in cell cycle and enhanced levels of endoreduplication. The other responses, presumably downstream of SOG1/SGLs

involved several genes known to code factors involved in the HR: *RAD51*, *BRCA1*, *RecQL3* helicase, the SMC5/6 complex subunit *NSE4.A*, SMC hinge domain protein *GAMMA-IRRADIATION AND MITOMYCIN C INDUCED 1* (*GMI1*), or *BRCT5 DOMAIN CONTAINING PROTEIN 1* (*BCP1*).

The comparison of gene expression in zeocin-treated and untreated WT plants showed activation of oxidative stress response, DDR, signatures of halted replication and shift towards endoreduplication cycle.

Effects of *hvatr.g* on gene expression under mock conditions

We examined the effects of *ATR* mutation on transcriptome by comparing expression in *hvatr.g* (Fig. 6). The *hvatr.g* plants showed a significant (adjusted-$p \leq 0.05$ and a $\log_2$ fold change ≤ -1 or $\log_2 FC \geq 1$) up-regulation of 1150 and down-regulation of 1457 genes relative to WT plants (Supplemental Dataset 2). Gene ontology analyses of the up-regulated genes suggested a connection to only a few categories of biological processes, most related to protein production and/or modifications, as in GO:0006749 (glutathione metabolic process), GO:0006468 (protein phosphorylation), or GO:0006575 (sulfur compound metabolic process). Additional gene categories include xenobiotic export and transport from cell (GO:0046618, GO:0042908). Categories of down-regulated processes show a miss-regulation of transmembrane transport, especially concerning nitrate (GO:0015706, GO:1902025) (Fig. 6A, Supplemental Dataset 3).

Among the most significantly upregulated transcripts was 64-fold upregulated *TFIIIA* (Fig. 6B), which is known to regulate *5S rDNA* transcription. Furthermore, *hvatr.g* plants had over 182-fold higher expression of

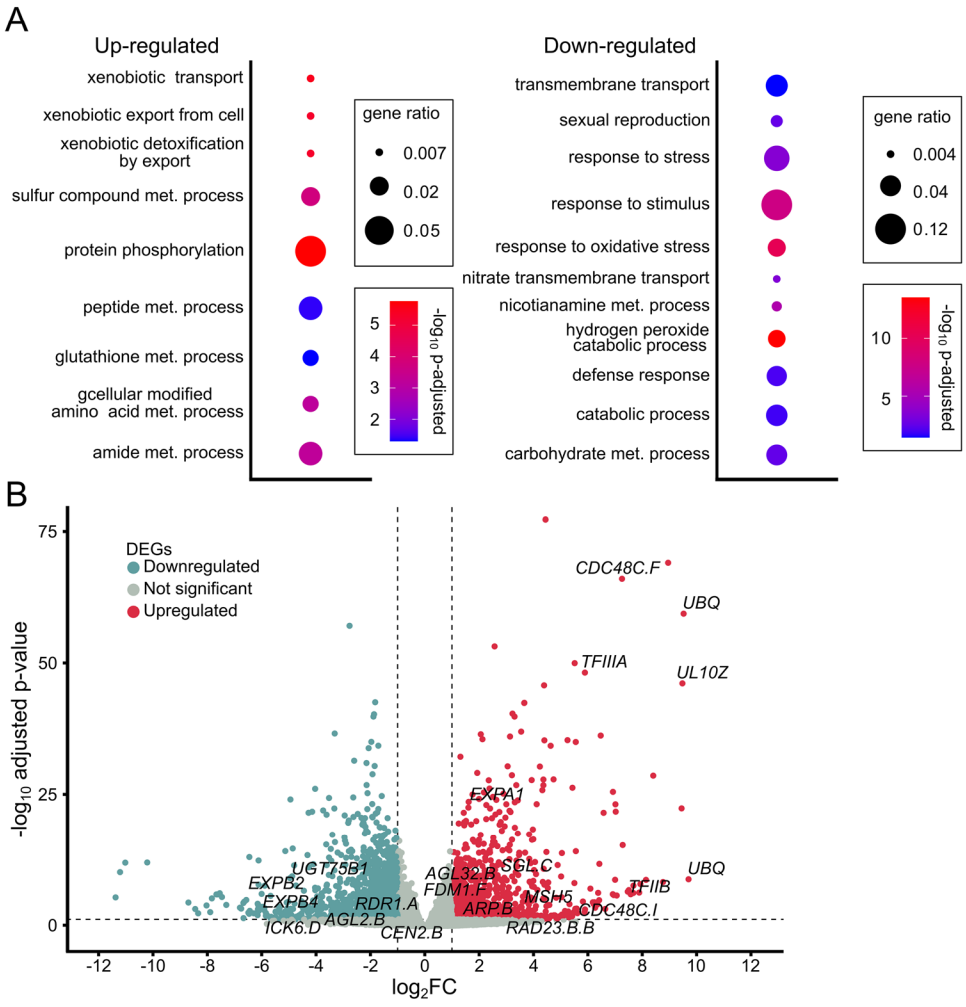

Figure 6. Effects of *ATR* mutation on gene expression in barley. (**A**) Gene enrichment analysis for biological processes in *atr* mutant compared to wild-type (Sebastian). Redundant GO terms were removed manually, based on *P*-value. The full list of GO terms can be found in Suppl. Dataset 3. Statistical significance was determined by Fisher's one-tailed test with g:SCS algorithm correction. Gene ratio represents the number of genes found in the category compared to the total number of genes in the query. (**B**) Volcano plot representing all genes detected as differentially expressed in *atr* plants relative wild-type (Sebastian) plants. Horizontal dashed line indicates genes passing $P < 0.05$. Vertical dashed lines separate genes with $\log_2$ Fold Change ≤ -1 (blue) or $\log_2$ Fold Change ≥ 1 (red). Specific marker genes were highlighted by names.

TFIIB that is involved in the formation of the RNA polymerase II (POL II) transcription preinitiation complex. A very prominent group of the top up-regulated genes were related to ubiquitinylation: *UBIQUITINs*, *UBIQUITIN EXTENSION PROTEINS* and *F-BOX PROTEINS*. The *hvatr.g* plants showed also up-regulation in genes of the DDR pathway like *SGL.C*, suggesting increased genome instability even at mock conditions. Furthermore, there was an up-regulation of the *MutS* homolog 5 (*MSH5*) that was implicated in mismatch repair in Arabidopsis[28] and nucleotide excision repair and proteolysis associated factors *RADIATION SENSITIVE 23* (*RAD23*) and *CELL DIVISION CYCLE* (*CDC48*).

The strongly down-regulated genes in *hvatr.g* were eight copies of *NICOTIANAMINE SYNTHASE* (Supplemental Dataset 2). These genes should be strongly expressed in roots, where it regulates intake of iron[29]. In rice it was shown to be under direct control of NAC-family proteins during drought stress[30]. The same pathway in rice up-regulates the expression of genes involved in membrane modification genes and transport[30]. Even more remarkable was the decrease in the expression of transmembrane transporters. Among the 71 down-regulated transmembrane transport-related genes, 25 are associated with the transport of nitrogen-based compounds (Supplemental Fig. 3B). Comparison of up-regulated and down-regulated genes with products involved in nitrogen transport or metabolism, confirmed this further (example genes Supplemental Fig. 3A, full gene list Supplemental Dataset 4). The most prevalent down-regulated genes in this group were *HIGH AFFINITY NITRATE TRANSPORTERS* and *NRT1/PTR FAMILY TRANSPORTERS* (6 genes each).

In summary, *hvatr.g* plants show a well notable pattern of transcriptional changes that includes a mix of DDR, transcriptional and translational responses. The groups of up- and down-regulated genes also indicate enhanced turnover of genic products from the transcription, through protein synthesis, modification and degradation.

Differences in reaction to zeocin caused by *hvatr.g* mutation

To assess the DNA damage response in plants lacking the functioning ATR kinase, we looked on differentially expressed genes in zeocin and mock-treated *hvatr.g* plants. There were in total 424 genes up-regulated and 622 genes down-regulated in zeocin-treated compared to mock-treated *hvatr.g* plants (Fig. 7A, Supplemental

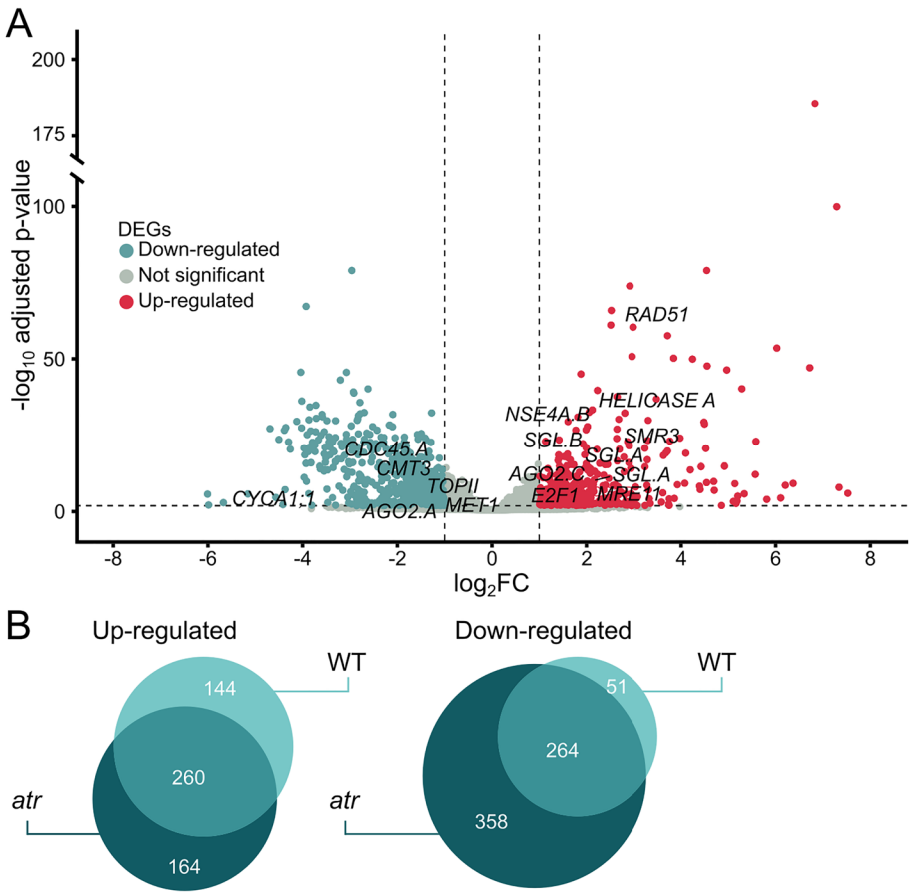

Figure 7. Response of *atr* mutant plants to zeocin treatment. (**A**) Volcano plot representing all genes differentially expressed in zeocin-treated versus mock-treated *atr* mutant plants. Horizontal dashed line indicates genes passing $P < 0.05$. Vertical dashed lines separate genes with $\log_2$ Fold Change ≤ -1 (blue) or $\log_2$ Fold Change ≥ 1 (red). Specific marker genes were highlighted by names. (**B**) Venn diagrams showing differentially expressed genes after zeocin treatment in wild-type and *atr* mutant plants relative to the mock treatment of the same genotype.

Dataset 2, Supplemental Fig. 2). Gene enrichment analysis showed categories similar to those described in zeocin-treated WT plants (Supplemental Fig. 3). To filter for the ATR-specific responses, we visualized the data as Venn diagrams of genes with significantly changed expression by zeocin-treatment in WT and *hvatr.g* plants (Fig. 7B, Supplemental Dataset 5).

The majority of zeocin-induced transcriptional changes in WT were ATR-independent with 260 genes up-regulated (64.4% out of 404) and 264 genes down-regulated (83.8% out of 315) (Fig. 7B, WT and overlap). This included all major up-regulated DDR genes related to SSB repair (*PARP*), translesion synthesis (*REV3*), direct hydrolysis (*TDP*) and HR (*MRE11, SGL.D, SGL.C, SMR3, RAD54, BRCA1, BCP1, BCP4, GMI1, NSE4.A*). Similarly, genes down-regulated by zeocin treatment in ATR-independent manner included many positive regulators of cell cycle, replication factors and histones (Supplementary Dataset 4). When looking on dependence of transcriptional change on ATR upon zeocin treatment, we found 144 up-regulated genes (35.6% out of 404) and 51 down-regulated genes (16.2% out of 315). The DDR associated genes up-regulated in ATR dependent manner included *RECQ HELICASE 3 (RECQL3)* and *DNA POLYMERASE DELTA catalytic SUBUNIT 1* (*POLD1*). Both proteins are involved in processing lagging strand during replication where RECQL3 unwinds it and shows a substrate preference for nicked Holliday junctions[31] and POLD performs its synthesis[32].

Finally, the last category represented zeocin-induced transcriptional changes unique to *hvatr.g* plants. There were 358 such down-regulated genes among which dominated 67 histone genes of all types (2× *H1*, 17× *H2A* in different variants including two copies of *H2A.X* variant associated with marking the DNA damage sites, 17× x *H2B*, 5× *H3.1* and 26× *H4*). The down-regulated replication factors included large subunit of DNA primase, *MCM3* and GINS complex subunit *PSF1*, and cell cycle genes *MAD2*, *AURORA KINASE 1.A* (*AUR1.A*) as well as microtubule-associated protein *TORTIFOLIA1*. From the DDR genes, we found significant down-regulation of *RECQL2* helicase, RecQ-mediated genome instability protein 1 (*RMI1*), two uncharacterized DNA ligases and *F BOX-LIKE17* (*FBL17*). The FBL17 is interesting candidate as its loss of function mutant was identified in an *atr* phenotype suppressor screen in Arabidopsis and was described as *SUPPRESSORS OF ATR 1* (*SOAT*)[33]. When inspecting 164 genes up-regulated by zeocin treatment in *hvatr.g* and not in WT, we found *APURINIC ENDONUCLEASE-REDOX PROTEIN* (*ARP*) that is a major endonuclease involved in base excision repair (BER), RAD23 that has a role in NER and interacts with the 26S proteasome components, and also ESSENTIAL MEIOTIC ENDONUCLEASE 1B (EME1B) that is known to interact with endonuclease MUS81. Up-regulation of these components indicates specific shift in the use of DDR pathways towards nucleolysis, BER and NER and in *hvatr.g* plants.

Discussion

We established in vitro DNA damage treatment conditions for barley, generated a list of barley DDR genes including the *SOG1* and *SGL* family and performed transcriptomic analysis of WT and *hvatr.g* plants in response to zeocin treatments.

A protocol for efficient induction of DNA damage under controlled in vitro conditions is not well established for barley. Our initial attempts to treat whole sterilized barley seeds according to established protocols in Arabidopsis failed. The reasons were high frequency of fungal contamination, highly variable responses to chemical treatments and complexity of root phenotype (multiple roots emerging). Based on extensive testing and modifications, we propose a protocol using dissected mature embryos and their cultivation on solid media containing genotoxins. Dissecting embryos represents an additional and demanding experimental step, but it helped in several ways. First, it greatly reduced fungal contaminations that occurred on whole seeds, possibly due to the complex surface of barley seeds. Second, it excluded endosperm which provided energy for the germinating embryo, allowing for minimal contact of the roots with the zeocin-containing media. Third, the germination showed less variation between the individuals and the experiments.

To induce DNA damage, we applied zeocin, a phleomycin D1/bleomycin-type antibiotic with radiomimetic effects. While DSBs are commonly caused by radiomimetic drugs, and bleomycin has been shown to induce DNA fragmentation[34], research also revealed that only about 10% of DNA alterations caused by bleomycin involve both strands, and within this percentage, only a fraction are actual DSBs[35]. The ratio of SSBs to DSBs caused by bleomycin varies depending on concentration and chromatin compaction, ranging from 3:1 to 20:1[18,36,37]. Presence of both DSBs and SSBs was confirmed on DNA from bleomycin-treated barley root tissues via Comet assays[38]. Therefore, zeocin is a relatively broad-spectral DNA damage inducer that can potentially serve as a genotoxin for testing sensitivity of a wide range of DDR mutants. Induction of SSBs and to a lower extent also other non-DSB types of DNA damage by phleomycin type antibiotics[27] also justifies use of these chemicals for treatments of *atr* mutants. Although we did not assess the amount of DNA-SBs directly in our experiments, inhibitory effects of zeocin treatments on plant growth and root tissue differentiation indicated that it was effective already at the lowest applied dose (100 µg/ml). This was further supported by up-regulation of the DDR marker genes *BRCA1* and *RAD51*. The effects on the shoot were less prominent, most likely because they were not in a direct contact with the drug-containing media.

Analysis of ATR mutant plants confirmed shorter but more seminal roots in *hvatr.g* compared to WT[21]. This is a principal difference compared to phenotypic analysis of dicots, where the primary root offers a simple proxy for the plant growth under both mock and genotoxic stress conditions. The exact reason for this phenotype is unclear but we propose several speculative models to it. Presumed replication-coupled defects in *hvatr.g* mutant plants might shift the ration between the root elongation by active cell division versus initiation of the new roots. In wild-type plants, formation of too many new roots might be suppressed via interaction of ATR with the factors that integrate DDR with cell cycle such as SOG1 and WEE1[7,39,40]. Yet another mechanism might include metabolic problems because *hvatr.g* plants exhibited a down-regulation of transmembrane transport genes, most notably the nitrate and nitrogen-compound transporters. We also observed down-regulation of 13 *EXPANSIN* genes,

which could potentially impact cell growth and root number in *hvatr.g* plants. *EXPANSINS* were part of a root number quantitative trait locus in barley, although no specific gene was conclusively confirmed as the causative factor[41]. We also searched for misregulated genes known to be involved in barley root development. The only such candidate was down-regulated *PME5* (*PECTINESTERASE 11*), which has previously been linked to root length regulation[42]. However, to answer this in an unbiased way, a complex forward-directed suppressor screen would have to be carried out in the *hvatr.g* background, seeking double mutants that restore wild-type-like phenotype.

Genome-wide transcriptomics upon DNA damage proved useful in exploring plant DDR[7,22,43]. Therefore, it was interesting to observe transcriptional changes upon zeocin treatment in barley. A prominent effect was a response to oxidative stress. This is consistent with the notion that bleomycin type antibiotics, including zeocin, mediate production of superoxides and free oxygen radicals[44,45]. The upregulated enzymes included GSTUs and UGTs that use glutathione to detoxify reactive oxygen species. Glutathione is a buffer protein used against redox active molecules. In plants, some GSTUs have also strong antioxidative roles, with some classes of GSTU having peroxidase activity[46]. Furthermore, UGTs catalyze activation reactions of most secondary metabolites, including antioxidative molecules by addition of sugar moieties[47,48].

Many zeocin-induced transcriptional changes included genes directly or indirectly associated with the cell cycle and cell division. The down-regulation of *CYCs* and *CDKs* indicated an inhibition of regular cell cycle progression. Presence of DNA damage generally leads to a halted cell cycle to gain time necessary for the repair[49]. A strong down-regulation of core histone genes and replication factors indicated reduced DNA synthesis. Many histone genes reach the peak of expression during S phase, when massive amounts of histones are needed for packaging of newly synthetized DNA. Interestingly, the down-regulation included also two DDR-associated *H2A.X* histone variant genes. Phosphorylated form of H2A.X (γH2A.X) marks the sites of active DNA damage repair[50]. Because H2A.X is present at specific genomic positions under non-DNA damaging conditions[51], it likely that *H2A.X* is transcriptionally-regulated in the cell cycle stage-dependent manner. Our data suggest (indirectly) that the H2A.X response to DNA damage occurs at the level of post-translational modifications. An obvious consequence of the response at the protein rather than transcriptional level would be a faster reaction time. This could be important in order to pause cell cycle and start the repair before more damage occurs either directly by the mutagen or indirectly by continuation of regular cellular processes. The second prominent trend indicated a shift from mitotic cycling towards endoreduplication. Endoreduplication is a modified cell cycle where G2 phase is followed by another S-phase instead of mitosis[52]. Endoreduplication is part of a standard plant developmental program but can be alleviated by stress[39,53]. In Arabidopsis, the process is controlled by the KIP-RELATED PROTEINS (KRPs) and SIAMESE-RELATED proteins (SMRs), where the first promote mitosis while the latter endoreduplication[54]. We found zeocin-induced up-regulation of barley *SMR3*. In Arabidopsis, SMRs suppress mitosis by repressing A and B type CYCs and B type CDKs and number of these factors was downregulated by zeocin treatment in barley. The other SMR activity is to promote endoreduplication cycle by suppressing expression in the signaling cascade consisting of CYCDD, CDKA, RBR1, E2Fs and FBL17 and leading to KRPs[54]. In barley, we found transcriptionally down-regulated ERF factor *DEL1* and *FBL17*. Barley plants show generally low levels of endoreduplication in somatic tissues under normal conditions but the frequency is higher in specialized tissues such as some endosperm developmental stages[20]. Interestingly, there was a significant increase in the frequency of endoreduplicated nuclei in the roots of zeocin-treated plants. This is in agreement with the molecular signatures in our RNA-seq analysis and suggests that barley performs adjustments to its cell cycle in responses to genotoxic stress via evolutionarily conserved SMR-dependent pathway.

To focus RNA-seq on DDR components, we created list of barley DDR genes using protein homologies with Arabidopsis candidates[22]. Through selection, filtering and manual curation steps, we identified 421 barley candidates. Some, like the barley *BRCA1* homolog had lower similarity to Arabidopsis and were added manually. The list also included homologs of Arabidopsis DDR response master regulator *SOG1*, and its closely related genes *ANAC044* and *ANAC085*[26,55]. We found five members of this family in barley. Comparisons with rice and maize suggested that SOG1 is a single copy gene in barley. In contrast, the other four family members clustered with one clade with the Arabidopsis ANAC044, ANAC085 and rice SGL. This suggested that these copies represent barley *SGLs*. Interestingly, *SGLs* were more transcriptionally responsive to zeocin-induced DNA damage than SOG1 in barley. This aligns with Arabidopsis, where SOG1 shows minimal transcriptional changes to DNA damage, but ANACs are up-regulated[7], and indicates an evolutionarily conserved regulation of SOG1 at the protein level and *SGLs* at the transcriptional level during DDR. Other up-regulated genes included several DNA damage repair factors and positive regulators of HR including *RAD51*, *BRCA1*, *RecQL3*, *NSE4.A*, *GMI1*, or *BCP1*.

Transcriptomic data of *hvatr.g* showed activated DDR even under the mock conditions, consistent with previous findings of increased DNA damage in non-treated *hvatr.g* plants[21]. Up-regulated factors indicated greater utilization of mismatch and NER pathways in ATR mutants. Notably, moderately up-regulated genes in *hvatr.g* included *RAD23* and *CDC48*. RAD23 is connected to proteolysis helping with the cell-cycle progression and stress response[56]. In yeast, Rad23 recognizes a complex of Cdc48 and Ubiquitin ligase E4, facilitating proteasomal degradation. Plant CDC48 homologs have similar roles in protein degradation[57]. The enrichment of genes involved in ubiquitinilation, along with down-regulation of E2 Ub-conjugating enzymes and RGLG2 Ub-ligase E3 in *hvatr.g*, suggests increased protein turnover. Alternatively, elevated CDC48 expression may contribute to methionine metabolism for cell defense in the nicotineamine pathway or facilitate de-condensation of centromeric heterochromatin and/or activation of rDNA genes as observed in Arabidopsis. These pathways potentially have support from other upregulated genes. Notably, *FBL17* was down-regulated in *hvatr.g*, and Arabidopsis *FBL17* mutants were identified as *SUPPRESSORS OF ATR 1* sensitivity phenotype[33], indicating a transcriptional regulatory feedback loop between ATR and FBL17.

Besides of the obvious transcriptional differences between *hvatr.g* and wild-type, it has to be noted that vast majority of the transcriptional response between both genotypes remained unchanged. This is most likely due to a partial functional redundancy of plant ATM and ATR kinases as described in Arabidopsis[58]. Therefore, a

barley *atm atr* double mutant would be needed to uncover the whole spectrum of genes controlled by these key DDR kinases. Alternatively, analysis of barley *sog1* mutant might be methodologically easier and possibly even more informative option[7,8]

In conclusion, our study indicates that barley exhibits a conserved response to chemically-induced DNA-SBs. We observed molecular signatures of oxidative stress response, that is consistent with the zeocin expected mode of action and responses to both DNA single and double strand breaks. We also identifies some genes that could be possible targets of modifying mitotic division and endoreduplication in barley. This study offers valuable resources for further detailed investigations into barley's DDR, its associations with other stresses, and plant development.

Materials and methods

Plant materials and growth conditions

We used barley cultivars Golden Promise and Sebastian (WT), and *hvatr.g* TILLING mutant allele of *ATR* (HORVU.MOREX.r3.7HG0748510) gene[21]. In vitro plant cultivation was done in an air-conditioned phytochamber with a long day regime (16 h light, 150 µmol m^{-2} s^{-1}, 21 °C, 8 h dark, 19 °C). Plants used for seed production were grown in the climate-controlled phytotron under long day conditions (16 h light, 150 µmol m^{-2} s^{-1}, 15 °C, 8 h dark, 13 °C, 65% humidity). Plant materials used in this work are a part of cultivated cereal varieties and were not resourced in field. Golden Promise (Acc. No. HOR 16645) is available at the Leibniz Institute of Plant Genetics and Crop Plant Research, IPK, Genebank, Gatersleben, Germany and Sebastian (Acc. No. 03C0602773) at the Germplasm Resource Information Network (GRIN), Prague, Czech Republic. Mutant *hvatr.g* is available at *Hor*TILLUS (*Hordeum*—TILLING—University of Silesia) database upon request from M.S. Experimental research on plant material in this study, including its collection, complied with the relevant institutional, national, and international guidelines and legislation.

DNA damage assays

Barley seeds were surface sterilized with 70% ethanol (v/v) for 5 min, followed by 10 min treatment with 8% sodium hypochlorite (v/v) and final triple wash with ddH_2O. Sterilized seeds were imbibed overnight in sterile water at 4 °C in dark. The following day, embryos were carefully excised under binocular in a sterile laminal flow-hood from the seeds and placed scutellum side down on ½ MS medium with 0.6% agarose (w/v) or medium supplemented with zeocin (ThermoFisher Scientific, Cat. no. R25001) in 107 × 94 × 96 mm boxes (Duchefa, Cat. no. S1686). In place of a lid, another container was sealed to the one containing medium with embryos with a parafilm (total height 192 mm). Containers were placed in a phytochamber (Percival Scientific) and plants were grown for 14 days after which they were carefully pulled out of the medium for measurements. All the measurements were completed using the ImageJ plugin SmartRoot[59]. Experiments were performed in three biological replicates. For testing the different concentrations of zeocin required for wild-type barley (*cv.* Golden Promise) phenotypic response each replicate contained 18–20 individual plants. In the DNA damage assays comparing the phenotypic response to zeocein of *hvatr.g* and wild-type *cv.* Sebastian, each replicate had 9–10 plants per genotype. Statistical significance was tested with One-way ANOVA with post hoc Tukey HSD in Minitab (www.minitab.com).

Propidium iodide staining and root microscopy

Barley plants *cv.* Golden Promise grown for seven days on a control or 100 µg/ml zeocin media were used to assess root morphology at microscopic level. Three representative plant samples were chosen for imaging. The whole root was stained by pseudo-Schiff propidium iodide staining as described (Coiro and Truernit[60]). Incubation with propidium iodide and Schiff reagent lasted for 48 h. Following the overnight incubation with chloral hydrate solution roots were mounted on glass in water. Imaging was performed using Leica TCS SP8 STED3X confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with an HC PL APO CS2 10×/0,4 DRY objective, hybrid detectors (HyD), and the Leica Application Suite X (LAS-X) software version 3.5.5 with the Leica Lightning module (Leica, Buffalo Grove, IL, USA). For propidium iodide acquisition, laser excitation at 534 nm and emission at 550–730 nm were used. The maximal projection pictures were constructed from aligned Z-stack images of approximately 250–300 µm steps, containing 45 individual optical sections. The images were post-processed by Leica Lightening software module.

Flow-cytometry

The nuclear ploidy measurements were done on 20 Golden Promise plants grown from dissected embryos for seven days on solid ½ MS medium with or without 300 µg/ml zeocin. Two to three root apical meristems from each individual plant were chopped using a razor blade directly into 500 ml Otto I buffer (0.1 M citric acid, 0.5% Tween 20 v/v). Nuclei suspension was filtered through a 50 µm nylon mesh into a fresh tube, mixed with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \times 12H_2O$) containing 2 µg DAPI (4′,6-diamidino-2-phenylindole) fluorescent stain. Ploidy was measured on a Partec PAS I flow cytometer with WT barley leaf tissue as a standard. Statistical significance was assessed by a two-sample T-Test in Minitab.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Golden Promise seeds were surface sterilized, embryos dissected, and grown on ½ MS medium with 0.6% agarose. After 7 days, young seedlings were moved to liquid ½ MS medium without or with zeocin (300 µg/ml). Sampling was performed at strict time points 0.5, 1 and 6 h after the beginning of treatment. All root apical meristems (RAMs) from a single plant were dissected and flash-frozen in liquid nitrogen. Three plants were taken for each treatment and stored until use at − 80 °C. RNA extraction was performed by RNeasy Mini Kit

(Qiagen, Cat. no. 74104) according to manufacturer's instructions with on column DNAse I treatment. cDNA was constructed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific™, Cat. no. K1631). The qPCR was performed with the HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Cat. no. 08-24-205 0000S) in CFX96 Touch Real-Time PCR Detection System (BioRad). Mann–Whitney U-test was performed in Minitab to assess statistical significance of the data.

RNA-sequencing

Plant material for RNA-sequencing was prepared similarly to RT-qPCR experiments with following modifications: 500 µg/ml zeocin treatment was applied for 6 h. Genotypes used were wild-type *cv.* Sebastian and mutant *hvatr.g*. Quality of the total RNA was checked on BioAnalyzer 2100 with RNA 6000 Pico Chips (Agilent) and samples with RNA integrity number > 8.1 were processed further. RNA sequencing was performed in three biological replicates for every experimental point at Novogene (UK) Company Limited using 150 bp paired-end protocol. At least 60 million paired-end reads were produced for each sample. The sequencing reads were deposited at Gene Expression Omnibus under ID GSE235051.The raw reads were trimmed using Trim Galore v.0.4.1 (www.bioinformatics.babraham.ac.uk/projects/trim_galore) and aligned to the 3rd version of reference genome of the *H. vulgare* cv. Morex[61] using HiSat2 v.2.1.0 genomic mapper[62]. Aligned reads were assigned to the genes according to the genome annotation using Subread v.1.5.2 software[63] and raw read counts were normalized to TPM expression levels. Differential expression analysis was performed using DESeq2 v.1.38.3 package[64] in R v.4.2.2 software (www.r-project.org). DEGs were identified according to the Benjamini–Hochberg FDR-adjusted *p*-value (< 0.05).

GO Term enrichment analysis

For the assessment of Gene Ontology an online tool gProfiler was used (www.biit.cs.ut.ee/gprofiler/gost). The tool uses g:SCS algorithm for correction of *p*-values. Output was manually curated to filter out redundant GO terms.

Identification of DNA damage response and repair genes in barley

The amino acid sequences of 321 Arabidopsis DNA damage repair genes[22] were BLASTed[65] to the set of 83,661 barley genes[66]. Subsequently, all barley candidates were BLASTed back to Arabidopsis to confirm best similarity. The candidates confirmed in both directions of reciprocal BLAST were taken for further analysis. They were filtered by the BLAST E-value (≤ 0.01), comparison of protein lengths, and alignment lengths (40% and more was accepted for both parameters).

Phylogeny

The amino acid sequences of NAC domains found in *A. thaliana* SOG1, ANAC044, ANAC85, ANAC005, ANAC006; *Oryza sativa* SOG1, SGL, *Zea mays* NACTF99, NACTF08, and *Hordeum vulgare* HORVU.MOREX.r3.2HG0175540 (*SGL.B*), HORVU.MOREX.r3.2HG0175550 (*SGL.C*), HORVU.MOREX.r3.2HG0175560 (*SGL.D*), HORVU.MOREX.r3.6HG0590960 (*SGL.A*), HORVU.MOREX.r3.7HG0670800 (*SOG1*) were retrieved from The Arabidopsis Information Resource (TAIR, www.arabidopsis.org), The Rice Annotation Project Database (RAP-DP, www.rapdb.dna.affrc.go.jp), Maize Genetics and Genomics Database (www.maizegdb.org) and BARLEX (www.barlex.barleysequence.org) databases respectively, all accessed on May 2nd, 2023. The alignments of proteins were performed in MEGA software (www.megasoftware.net) using MUSCLE. Prepared alignments were graphically shown using SnapGene (www.snapgene.com), amino acids were highlighted based on consensus sequence and their physicochemical properties. The maximum likelihood phylogeny reconstruction was computed with MEGA using bootstrap method with 1000 iterations. The substitution model used was JTT with gamma distributed rates with five categories (+G).

Data availability

The datasets presented in this manuscript can be found in the text and figures, supplementary materials and RNA-seq reads were deposited in the NCBI Gene Expression Omnibus online repository under the number: GSE235051. The following barley genes and/or their products were mentioned in this study: *ASF1B* (HORVU.MOREX.r3.1HG0084850), *ATR* (HORVU.MOREX.r3.7HG0748510), *BARD1* (HORVU.MOREX.r3.2HG0181390), *BCP1* (HORVU.MOREX.r3.6HG0554520), BRCA1 (HORVU.MOREX.r3.1HG0078370), CAX5 (HORVU.MOREX.r3.4HG0337640.1), CDC48 (HORVU.MOREX.r3.2HG0105790, HORVU.MOREX.r3. 2HG0105790), CDKB1;2 (HORVU.MOREX.r3.5HG0463930), CDKB2;2 (HORVU.MOREX.r3.4HG0384440), CHR1.B (HORVU.MOREX.r3.4HG0338270), CMT3 (HORVU.MOREX.r3.6HG0628050), CYC6B (HORVU.MOREX.r3.3HG0301080), CYCA1.A (HORVU.MOREX.r3.3HG0249410), CYCA3;1.A (HORVU.MOREX.r3.4HG0342640), CYCB1;2.A (HORVU.MOREX.r3.3HG0259030), CYCB1;2.B (HORVU.MOREX.r3.1HG0069480), CYCB1;2.C (HORVU.MOREX.r3.3HG0295540), CYCB1;3.A (HORVU.MOREX.r3.1HG0069490), CYCB1;3.A (HORVU.MOREX.r3.1HG0069550), CYCB2;3.B (HORVU.MOREX.r3.7HG0751620), CYCD3;3 (HORVU.MOREX.r3.5HG0467900), FAS1 (HORVU.MOREX.r3.5HG0501270), FBL17.A/SOAT1 (HORVU.MOREX.r3.5HG0433490), GMI1 (HORVU.MOREX.r3.1HG0058570), MET1 (HORVU.MOREX.r3.2HG0151710), MRE11 (HORVU.MOREX.r3.7HG0715110), MSH5 (HORVU.MOREX.r3.1HG0068200), NICOTIANAMINE SYNTHASE (HORVU.MOREX.r3.4HG0415050), NSE4.A (HORVU.MOREX.r3.7HG0724600), PME5 (HORVU.MOREX.r3.2HG0189320.1), RAD23 (HORVU.MOREX.r3.6HG0569510), RAD51 (HORVU.MOREX.r3.7HG0721560), RecQL 3 (HORVU.MOREX.r3.6HG0620770), RGLG2 (HORVU.MOREX.r3.7HG0677600), RPA1A (HORVU.MOREX.r3.6HG0620000), RPA2.K (HORVU.MOREX.r3.6HG0597130), RPA70C.C (HORVU.MOREX.r3.1HG0021720), SGL.A (HORVU.MOREX.r3.6HG0590960), SGL.D (HORVU.MOREX.r3.2HG0175560), SGL.C (HORVU.MOREX.

r3.2HG0175550), SGL.B (HORVU.MOREX.r3.2HG0175540), SOG1 (HORVU.MOREX.r3.7HG0670800), TFIIB (HORVU.MOREX.r3.1HG0079200), TFIIIA (HORVU.MOREX.r3.6HG0548800), UBC2 (HORVU.MOREX.r3.5HG0517500), Ub-LIGASE E3 (HORVU.MOREX.r3.3HG0229520), VIM1 (HORVU.MOREX.r3.1HG0000630). Additionally, the following non-barley genes and/or their products were mentioned in this work. The Arabidopsis ANAC044 (AT3G01600), ANAC085 (At5g14490), SOG1 (AT1G25580). The rice (*Oryza sativa*) SOG1 (Os06g0267500) and SGL (Os02g0594800) and the maize (*Zea mays*) NACTF99 (Zm00001eb280480) and NACTF8 (Zm00001eb280490).

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References

1. Ciccia, A. & Elledge, S. J. The DNA damage response: Making it safe to play with knives. *Mol. Cell* **40**, 179–204 (2010).
2. Tibbetts, R. S. & Abraham, R. T. PI3K-related kinases. In *Signaling Networks and Cell Cycle Control: The Molecular Basis of Cancer and Other Diseases* (ed. Gutkind, J. S.) 267–301 (Humana Press, 2000). https://doi.org/10.1007/978-1-59259-218-0_15.
3. Garcia, V. *et al.* AtATM is essential for meiosis and the somatic response to DNA damage in plants. *Plant Cell* **15**, 119–132 (2003).
4. Culligan, K., Tissier, A. & Britt, A. ATR regulates a G2-phase cell-cycle checkpoint in *Arabidopsis thaliana*. *Plant Cell* **16**, 1091–1104 (2004).
5. Helton, E. S. & Chen, X. p53 modulation of the DNA damage response. *J. Cell. Biochem.* **100**, 883–896 (2007).
6. Yoshiyama, K. O. SOG1: A master regulator of the DNA damage response in plants. *Genes Genet. Syst.* **90**, 209–216 (2016).
7. Bourbousse, C., Vegesna, N. & Law, J. A. SOG1 activator and MYB3R repressors regulate a complex DNA damage network in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E12453–E12462 (2018).
8. Ogita, N. *et al.* Identifying the target genes of SUPPRESSOR OF GAMMA RESPONSE 1, a master transcription factor controlling DNA damage response in Arabidopsis. *Plant J.* **94**, 439–453 (2018).
9. Velemínský, J., Zadražil, S., Pokorný, V., Gichner, T. & Švachulová, J. Repair of single-strand breaks and fate of N-7-methylguanine in DNA during the recovery from genetical damage induced by N-methyl-N-nitrosourea in barley seeds. *Mutat. Res. Mol. Mech. Mutagen.* **17**, 49–58 (1973).
10. Velemínský, J., Zadražil, S., Pokorný, V. & Gichner, T. DNA repair synthesis stimulated by mutagenic *N*-methyl-*N*-nitrosourea in barley seeds and free embryos. *Mutat. Res. Mol. Mech. Mutagen.* **44**, 43–51 (1977).
11. Velemínský, J., Pokorný, V., Šatava, J. & Gichner, T. Post-replication DNA repair in barley embryos treated with *N*-methyl-*N*-nitrosourea. *Mutat. Res. Mol. Mech. Mutagen.* **71**, 91–99 (1980).
12. Lupu, A., Nevo, E., Zamorzaeva, I. & Korol, A. Ecological–genetic feedback in DNA repair in wild barley, *Hordeum spontaneum*. *Genetica* **127**, 121–132 (2006).
13. Vu, G. T. H. *et al.* Repair of site-specific DNA double-strand breaks in barley occurs via diverse pathways primarily involving the sister chromatid. *Plant Cell* **26**, 2156–2167 (2014).
14. Gruszka, D., Marzec, M. & Szarejko, I. The barley EST DNA replication and repair database (bEST-DRRD) as a tool for the identification of the genes involved in DNA replication and repair. *BMC Plant Biol.* **12**, 88 (2012).
15. Volkova, P. Y. *et al.* Early response of barley embryos to low- and high-dose gamma irradiation of seeds triggers changes in the transcriptional profile and an increase in hydrogen peroxide content in seedlings. *J. Agron. Crop Sci.* **206**, 277–295 (2020).
16. Szurman-Zubrzycka, M. E. *et al.* HorTILLUS—A rich and renewable source of induced mutations for forward/reverse genetics and pre-breeding programs in barley (*Hordeum vulgare* L.). *Front. Plant Sci.* **9**, 216 (2018).
17. Jaskowiak, J. *et al.* Al-tolerant barley mutant hvatr.g shows the ATR-regulated DNA damage response to maleic acid hydrazide. *Int. J. Mol. Sci.* **21**, 8500 (2020).
18. Povirk, L. F., Wübter, W., Köhnlein, W. & Hutchinson, F. DNA double-strand breaks and alkali-labile bonds produced by bleomycin. *Nucleic Acids Res.* **4**, 3573 (1977).
19. Hu, Z., Cools, T. & De Veylder, L. Mechanisms used by plants to cope with DNA damage. *Annu. Rev. Plant Biol.* **67**, 439–462 (2016).
20. Nowicka, A. *et al.* Dynamics of endoreduplication in developing barley seeds. *J. Exp. Bot.* **72**, 268–282 (2021).
21. Szurman-Zubrzycka, M. *et al.* ATR, a DNA damage signaling kinase, is involved in aluminum response in barley. *Front. Plant Sci.* **10**, 1299 (2019).
22. Bouyer, D. *et al.* Genome-wide identification of RETINOBLASTOMA RELATED 1 binding sites in Arabidopsis reveals novel DNA damage regulators. *PLOS Genet.* **14**, e1007797 (2018).
23. Anderson, H. J. *et al.* Arabidopsis thaliana Y-family DNA polymerase η catalyses translesion synthesis and interacts functionally with PCNA2. *Plant J.* **55**, 895–908 (2008).
24. Murozuka, E., Massange-Sánchez, J. A., Nielsen, K., Gregersen, P. L. & Braumann, I. Genome wide characterization of barley NAC transcription factors enables the identification of grain-specific transcription factors exclusive for the Poaceae family of monocotyledonous plants. *PLoS ONE* **13**, e0209769 (2018).
25. Gorbatova, I. V. *et al.* Studying gene expression in irradiated barley cultivars: PM19L-like and CML31-like expression as possible determinants of radiation hormesis effect. *Agronomy* **10**, 1837 (2020).
26. Takahashi, N. *et al.* A regulatory module controlling stress-induced cell cycle arrest in Arabidopsis. *eLife* **8**, e43944 (2019).
27. Povirk, L. F. DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: Bleomycin, neocarzinostatin and other enediynes. *Mutat. Res. Mol. Mech. Mutagen.* **355**, 71–89 (1996).
28. Adé, J., Belzile, F., Philippe, H. & Doutriaux, M.-P. Four mismatch repair paralogues coexist in *Arabidopsis thaliana*: AtMSH2, AtMSH3, AtMSH6-1 and AtMSH6-2. *Mol. Gen. Genet. MGG* **262**, 239–249 (1999).
29. Mizuno, D. *et al.* Three nicotianamine synthase genes isolated from maize are differentially regulated by iron nutritional status. *Plant Physiol.* **132**, 1989–1997 (2003).
30. Lee, D.-K. *et al.* The rice OsNAC6 transcription factor orchestrates multiple molecular mechanisms involving root structural adaptions and nicotianamine biosynthesis for drought tolerance. *Plant Biotechnol. J.* **15**, 754–764 (2017).
31. Kobbe, D., Blanck, S., Focke, M. & Puchta, H. Biochemical characterization of AtRECQ3 reveals significant differences relative to other RecQ helicases. *Plant Physiol.* **151**, 1658–1666 (2009).
32. Garg, P., Stith, C. M., Sabouri, N., Johansson, E. & Burgers, P. M. Idling by DNA polymerase δ maintains a ligatable nick during lagging-strand DNA replication. *Genes Dev.* **18**, 2764–2773 (2004).
33. Pan, T. *et al.* A novel WEE1 pathway for replication stress responses. *Nat. Plants* **7**, 209–218 (2021).
34. Vega-Muñoz, I., Herrera-Estrella, A., Martínez-de la Vega, O. & Heil, M. ATM and ATR, two central players of the DNA damage response, are involved in the induction of systemic acquired resistance by extracellular DNA, but not the plant wound response. *Front. Immunol.* **14**, 2385 (2023).
35. Povirk, L. F. & Houlgrave, C. W. Effect of apurinic/apyrimidinic endonucleases and polyamines on DNA treated with bleomycin and neocarzinostatin: specific formation and cleavage of closely opposed lesions in complementary strands. *Biochemistry* **27**, 3850–3857 (1988).

36. Chen, J., Ghorai, M. K., Kenney, G. & Stubbe, J. Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage. *Nucleic Acids Res.* **36**, 3781–3790 (2008).
37. Shimada, K. *et al.* TORC2 signaling pathway guarantees genome stability in the face of DNA strand breaks. *Mol. Cell* **51**, 829–839 (2013).
38. Georgieva, M. & Stoilov, L. Assessment of DNA strand breaks induced by bleomycin in barley by the comet assay. *Environ. Mol. Mutagen.* **49**, 381–387 (2008).
39. Mahapatra, K. & Roy, S. SOG1 transcription factor promotes the onset of endoreduplication under salinity stress in Arabidopsis. *Sci. Rep.* **11**, 11659 (2021).
40. Chen, H. *et al.* The ATR–WEE1 kinase module promotes SUPPRESSOR OF GAMMA RESPONSE 1 translation to activate replication stress responses. *Plant Cell* https://doi.org/10.1093/plcell/koad126 (2023).
41. Robinson, H. *et al.* Genomic regions influencing seminal root traits in barley. *Plant Genome* https://doi.org/10.3835/plantgenome2015.03.0012 (2016).
42. Abdel-Ghani, A. H. *et al.* Genome-wide association mapping in a diverse spring barley collection reveals the presence of QTL hotspots and candidate genes for root and shoot architecture traits at seedling stage. *BMC Plant Biol.* **19**, 216 (2019).
43. Kilian, J. *et al.* The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.* **50**, 347–363 (2007).
44. Hecht, S. M. Bleomycin: New perspectives on the mechanism of action. *J. Nat. Prod.* **63**, 158–168 (2000).
45. Chankova, S. G., Dimova, E., Dimitrova, M. & Bryant, P. E. Induction of DNA double-strand breaks by zeocin in *Chlamydomonas reinhardtii* and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response. *Radiat. Environ. Biophys.* **46**, 409–416 (2007).
46. Wagner, U., Edwards, R., Dixon, D. P. & Mauch, F. Probing the diversity of the Arabidopsis glutathione S-transferase gene family. *Plant Mol. Biol.* **49**, 515–532 (2002).
47. Chong, J. *et al.* Downregulation of a pathogen-responsive tobacco UDP-Glc:phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress, and weakens virus resistance. *Plant Cell* **14**, 1093–1107 (2002).
48. Simon, C. *et al.* The secondary metabolism glycosyltransferases UGT73B3 and UGT73B5 are components of redox status in resistance of Arabidopsis to *Pseudomonas syringae* pv. tomato. *Plant Cell Environ.* **37**, 1114–1129 (2014).
49. Preuss, S. B. & Britt, A. B. A DNA-damage-induced cell cycle checkpoint in Arabidopsis. *Genetics* **164**, 323–334 (2003).
50. Friesner, J. D., Liu, B., Culligan, K. & Britt, A. B. Ionizing radiation–dependent γ-H2AX focus formation requires ataxia telangiectasia mutated and ataxia telangiectasia mutated and Rad3-related. *Mol. Biol. Cell* **16**, 2566–2576 (2005).
51. Yelagandula, R. *et al.* The histone variant H2A.W defines heterochromatin and promotes chromatin condensation in Arabidopsis. *Cell* **158**, 98–109 (2014).
52. Veylder, L. D., Larkin, J. C. & Schnittger, A. Molecular control and function of endoreplication in development and physiology. *Trends Plant Sci.* **16**, 624–634 (2011).
53. Hase, Y., Trung, K. H., Matsunaga, T. & Tanaka, A. A mutation in the UVI4 gene promotes progression of endo-reduplication and confers increased tolerance towards ultraviolet B light. *Plant J.* **46**, 317–326 (2006).
54. Kumar, N. & Larkin, J. C. Why do plants need so many cyclin-dependent kinase inhibitors?. *Plant Signal. Behav.* **12**, e1282021 (2017).
55. Lang, L. *et al.* The DREAM complex represses growth in response to DNA damage in Arabidopsis. *Life Sci. Alliance* **4**, (2021).
56. Farmer, L. M. *et al.* The RAD23 family provides an essential connection between the 26S proteasome and ubiquitylated proteins in Arabidopsis. *Plant Cell* **22**, 124–142 (2010).
57. Bègue, H., Mounier, A., Rosnoblet, C. & Wendehenne, D. Toward the understanding of the role of CDC48, a major component of the protein quality control, in plant immunity. *Plant Sci.* **279**, 34–44 (2019).
58. Culligan, K. M., Robertson, C. E., Foreman, J., Doerner, P. & Britt, A. B. ATR and ATM play both distinct and additive roles in response to ionizing radiation. *Plant J.* **48**, 947–961 (2006).
59. Lobet, G., Pagès, L. & Draye, X. A novel image-analysis toolbox enabling quantitative analysis of root system architecture. *Plant Physiol.* **157**, 29–39 (2011).
60. Coiro, M. & Truernit, E. Xylem characterization using improved pseudo-Schiff propidium iodide staining of whole mount samples and confocal laser-scanning microscopy. In *Xylem: Methods and Protocols* (eds de Lucas, M. & Etchhells, J. P.) 127–132 (Springer, 2017). https://doi.org/10.1007/978-1-4939-6722-3_11.
61. Mascher, M. Pseudomolecules and annotation of the third version of the reference genome sequence assembly of barley cv. Morex [Morex V3]. *e.* https://doi.org/10.5447/ipk/2021/3. (2021).
62. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **37**, 907–915 (2019).
63. Liao, Y., Smyth, G. K. & Shi, W. The Subread aligner: Fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res.* **41**, e108 (2013).
64. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
65. Camacho, C. *et al.* BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421 (2009).
66. Mascher, M. *et al.* Long-read sequence assembly: A technical evaluation in barley. *Plant Cell* **33**, 1888–1906 (2021).

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Author contributions

A.P., J.D., and J.V. conceived and designed the study. J.V., performed experiments with barley and analyzed RNA-seq data, M.K. processed RNA-seq data, J.Z. developed list of barley DDR genes. M.S.Z. developed *hvatr.g* mutant. All authors analyzed data and interpreted the results. A.P. and J.V. wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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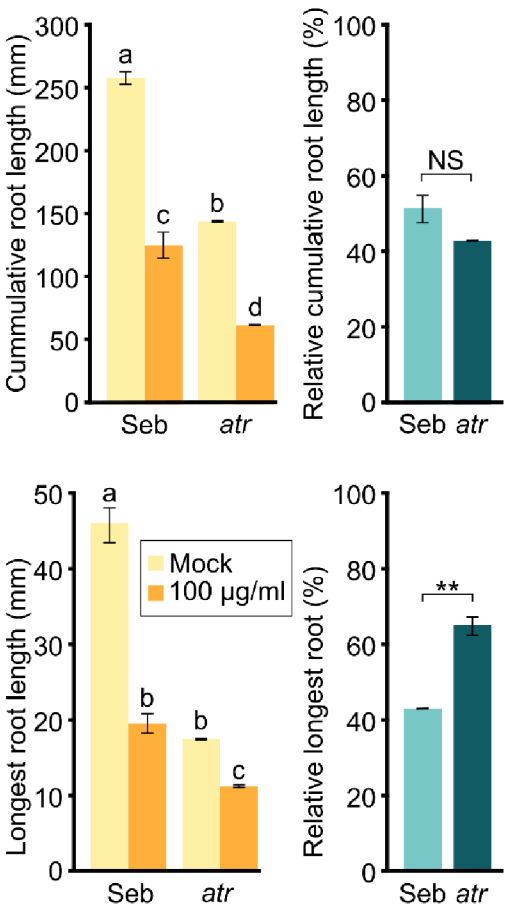
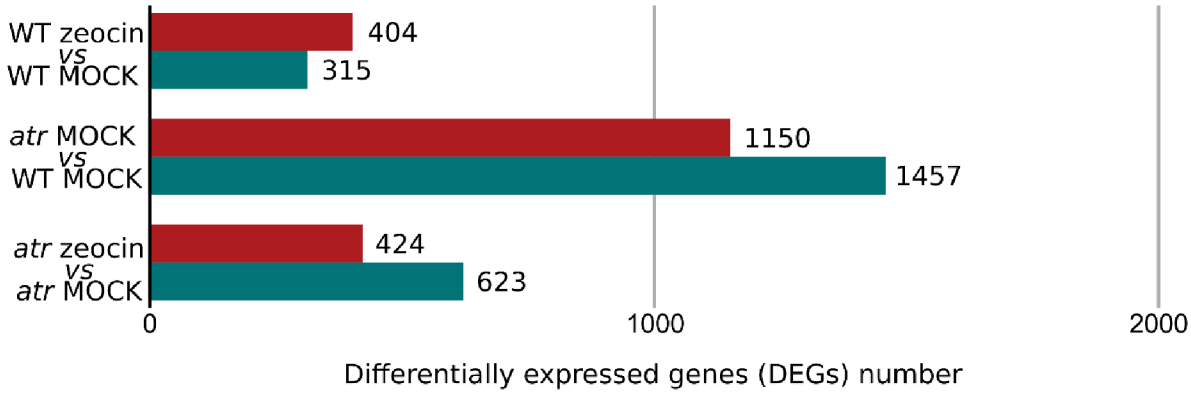
Supplemental Figure 1



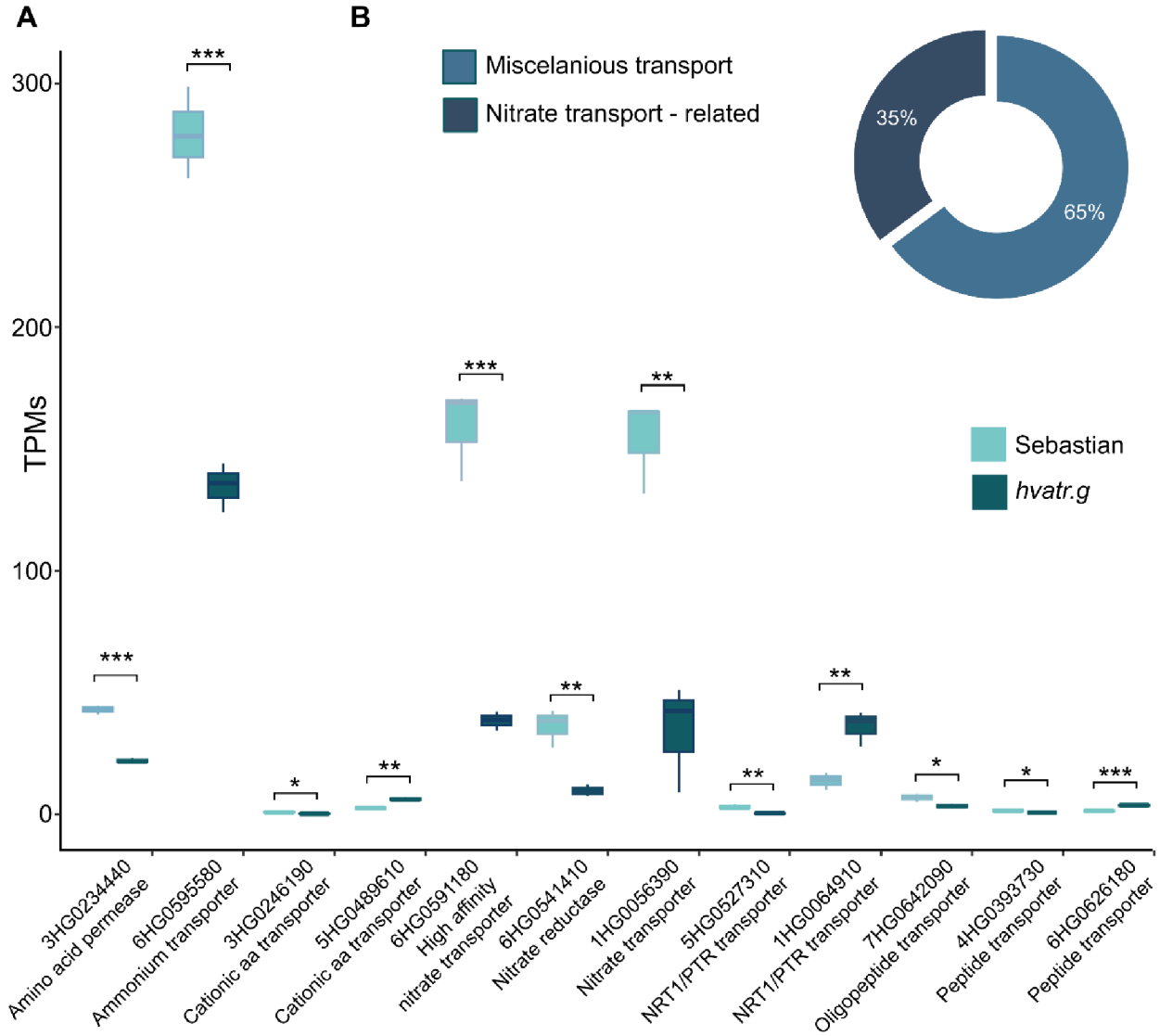
Supplemental Figure 1: Measurements of additional phenotypic traits showing the effect of zeocin treatment on Seb and *atr* plants' growth. Error bars indicate the standard deviation between the means of three biological replicates. For the absolute values represented the letters above columns indicate similarities between samples. The same letters indicate samples that were not significantly different in one-way ANOVA with *post-hoc* Tukey's test ($P < 0.05$). For the assessment of the statistical significance of the relative values the to-sample T-test was used, * $P < 0.05$, ** $P < 0.01$

Supplemental Figure 2



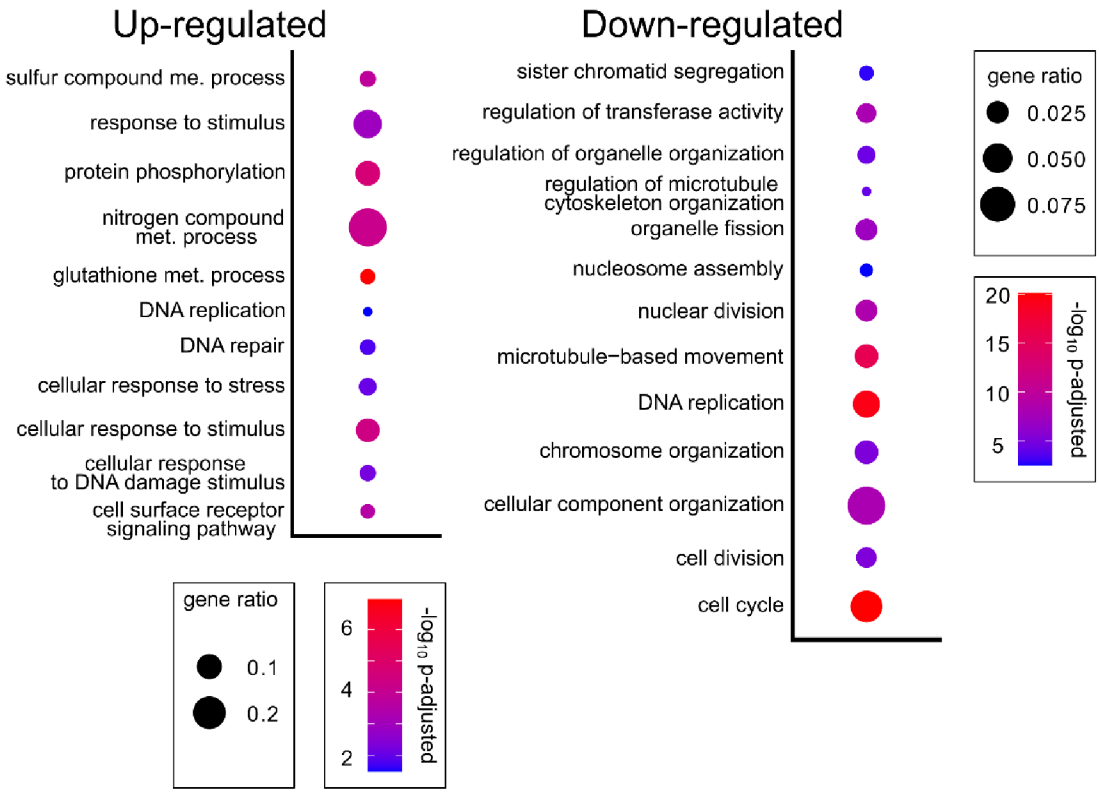
Supplemental Figure 2: Bar plot showing the number of differentially expressed genes (DEGs) found in the transcriptome comparison of wild-type Sebastian treated and untreated plants (WT MOCK vs WT zeocin), *atr* mutant and wild-type plants (*atr* MOCK vs WT MOCK) and *atr* mutant treated and untreated plants (*atr* MOCK vs *atr* zeocin). Up-regulated genes are presented by the red bar, and down regulated by the blue bar.

Supplemental Figure 3



Supplemental Figure 3: (**A**) Bar and whiskers plot showing the expression of selected genes related to nitrate transport in Sebastian (wild type) and *hvatr.g* in transcripts per million (TPMs). The boxplots' hinges are in the 1st and 3rd quartile, with a marked median. Whisker marks show the lowest or highest value within the 1.5 interquartile range below or above hinges. Asterisks represent significant differences in T-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.05$. (**B**) Pie chart showing the percentage of down-regulated genes involved in Transmembrane transport (GO:0055085) involved in nitrate transport.

Supplemental Figure 4



Supplemental figure 4: Biological processes enriched among significantly up-regulated and down-regulated genes in zeocin versus mock treated *hvatr.g* plants. Redundant GO terms were removed manually, based on P – value. The full list of GO terms can be found in Suppl. Dataset 3. Statistical significance was determined by Fisher's one-tailed test with g:SCS algorithm correction. Gene ratio represents the number of genes found in the category compared to the total number of genes in the query.

APPENDIX III:
PUBLISHED ABSTRACTS AND POSTERS

Characterization and functional analysis of candidate protein interactors of SMC complexes

Jovanka Vladejić[1], Beáta Petrovská[1], Jan Vrána[1], Zdeněk Perutka[2], Marek Šebela[2], Nicolas Blavet[1], Tomáš Vlčko[3], Ludmila Ohnoutková[3], Jaroslav Doležel[1] and Aleš Pečinka[1]

In: Abstract of the "Plant Biotechnology: Green for Good V"
Olomouc, Czech Republic 2019
Poster presentation

[1]Institute of Experimental Botany, Centre of the Region Haná for Biotechnological and Agricultural Research, Olomouc, Czech Republic
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Organization of hereditary information within plant nuclei is a highly controlled, yet plastic process that engages a great number of proteins, many of which have not yet been characterized in plants. A group of nuclear proteins, belonging to Structural Maintenance of Chromosomes (SMC) complexes, are crucial for maintenance of nuclear architecture and higher organization of chromatin. Employing the information obtained from UNcleProt database (http://barley.gambrinus.ueb.cas.cz/), which contains detailed outlook of the barley nuclear proteome, we selected several candidate proteins that may be interactors of the SMC complexes. Our research aims to identify and characterize these unknown proteins, as well as distinguish their potential role in genome function.

Functional analysis of candidates was initiated by genotyping commercially available *Arabidopsis thaliana* T-DNA mutants. Should these mutants prove as unsatisfactory, additional CRISPR/Cas9 mutants will be designed and generated. In order to examine the proteins' function in plants with large genomes, CRISPR/Cas9 barley mutants will also be created. Following the transformation analysis, inspection of the protein localization will involve establishment of fusion constructs using fluorescence tags and cloning. Further characterization of the protein will require identifying interacting proteins (e.g. by the pull-down).

Functional analysis of candidate nuclear proteins

Jovanka Vladejić[1], Beáta Petrovská[1], Zdeněk Perutka[2], Jan Vrána[1], Nicolas Blavet[1], Tomáš Vlčko[3], Ludmila Ohnoutková[3], Jaroslav Doležel[1], Marek Šebela[2] & Aleš Pečinka[1]

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Overview

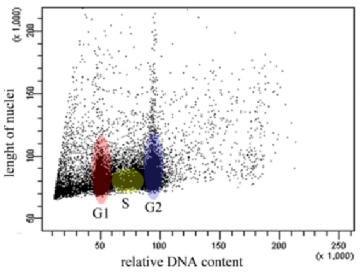
Organization of genetic information within cell nuclei is a complex process leading to the formation of higher chromatin order. Nuclear proteins are responsible for the establishment and maintenance of the stability of these structure. Among these we took particular interest in the unknown proteins, including potential interactors of Structural Maintenance of Chromosomes (SMC) complexes. Relying on the information previously obtained and released in the UNcleProt database, identifying the nuclear proteins of *Hordeum vulgare* (barley) we chose candidate proteins for further functional analysis.

GOAL: Functional characterization of the candidate protein

Milestone 1: *Hordeum vulgare* proteome analysis

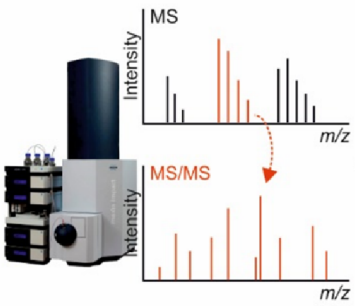
Cell cycle

Flow-assisted nuclei sorting (FANS)



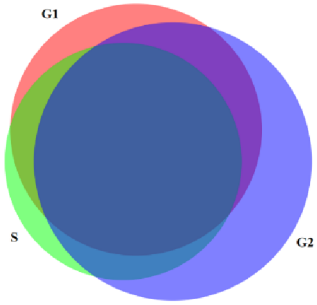
FCM sorting

Protein isolation



MS analysis

Protein identification



1922 identified proteins

UNcleProt

Nuclear proteome database

Large genomes

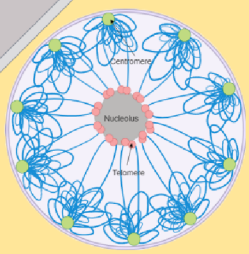
H. vulgare

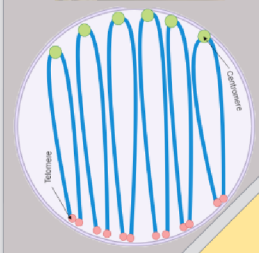
Candidate protein: **NSE2**

CRISPR/Cas9 mutant lines made:

- 30 bp deletion
- 27 bp deletion
- 6 bp deletion
- 1 bp deletion
- 1 bp insertion

NonRabl nuclear organization





Rabl nuclear organization

SMC complex

A.thaliana

Candidate proteins: **Tudor/Agenet domain**

Commercialy available T-DNA mutants

- Mutant selection

Small genomes

CANDIDATE PROTEINS

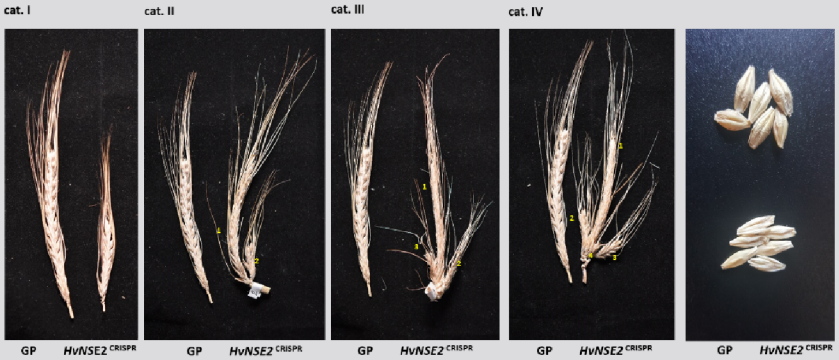
Milestone 2 – Localization

Milestone 3 – Interactions

Milestone 4 –Transcriptomics

Milestone 5 – Phenotypic analysis

HvNSE2 candidate mutant lines show spike and seed phenotype



Spike phenotype

Seed phenotype

This work was supported by the grant Plants as a Tool for Sustainable Global Development(No. CZ.02.1.01/0.0/0.0/16_019/0000827) from ERDF

Characterization of the BRCT5/6 domain-containing protein in *Arabidopsis thaliana*

Jovanka Vladejic[1], Eva Dvořák Tomaštíková[1], Fen Yang[1], Jan Palecek[2] and Ales Pecinka[1]

In: Abstract of the "Mendel Early Career Symposium"
Vienna, Austria 2022
Poster presentation

[1]Institute of Experimental Botany (IEB), Czech Acad Sci, Centre of the Region Hana for Biotechnological and Agricultural Research (CRH), Slechtitelu 31, 77900 Olomouc, Czech Republic.
[2]Functional Genomics and Proteomics, National Centre for Biomolecular Research (NCBR), Faculty of Science, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic.

Structurally and functionally diverse proteins involved in the maintenance of genome stability often contain BRCT-domain(s). BRCT domain was first described in human BRCA1 protein, a famous tumor- suppressant. Since then, many BRCT domain-containing proteins and their function during DNA damage repair, have been described in animals and fungi. However, they remain largely uncharacterized in plants. We aimed to identify proteins containing BRCT5/6 domains in *Arabidopsis thaliana* as well as assess their possible involvement in DNA damage repair. Based on homology and structural similarity to animal BRCT5/6 domains, four Arabidopsis candidate proteins were selected. The initial screening consisted of testing the sensitivity of Arabidopsis mutants in a series of DNA damage assays and revealed one protein required for normal resistance to DNA damage. Hypersensitivity to DNA damage suggested that our candidate is involved in DNA damage repair. Using promotor fusion lines with a β-glucuronidase (GUS) reporter gene it was determined that its expression is highly increased in conditions leading to DNA damage. Double mutants with reporter lines monitoring single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA) types of homologous recombination further confirmed BCP1's role in homology-based repair. Hence, we identified a new DNA repair factor in Arabidopsis.

Characterization of the BRCT5/6 domain-containing protein in *Arabidopsis thaliana*

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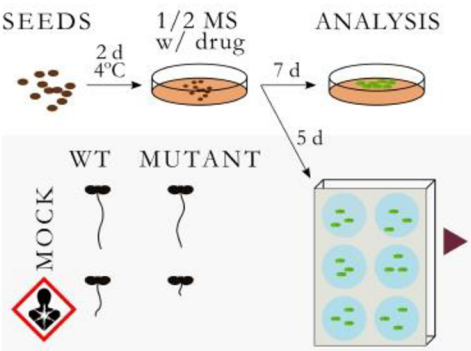
[2] Functional Genomics and Proteomics, National Centre for Biomolecular Research (NCBR), Faculty of Science, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic.

1 INTRODUCTION

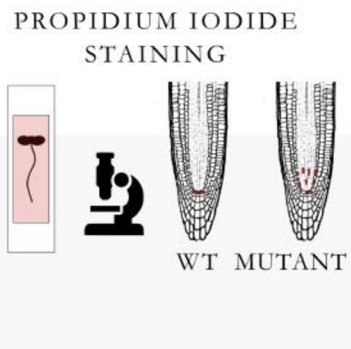
We aimed to identify proteins containing BRCT5/6 domains in *Arabidopsis thaliana* and to assess their possible involvement in DNA damage repair. Based on homology and structural similarity to the animal BRCT5/6 domains, four Arabidopsis candidate proteins were selected. They were subsequently marked as BRCT-containing protein (BCP) 1, 2, 3 and 4. The screening consisted of testing the sensitivity of Arabidopsis mutants in a series of DNA damage assays and revealed one protein required for normal resistance to DNA damage.

2 METHODS

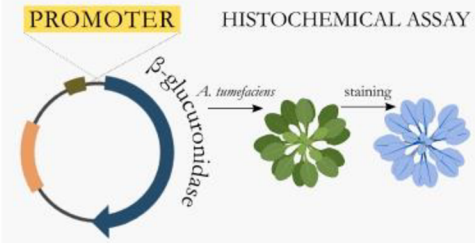
Sensitivity assays



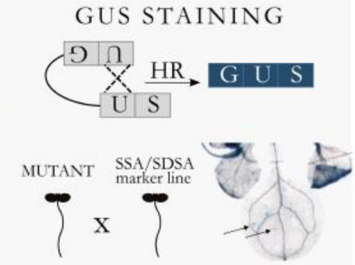
Cell death assays



Cloning and transformation

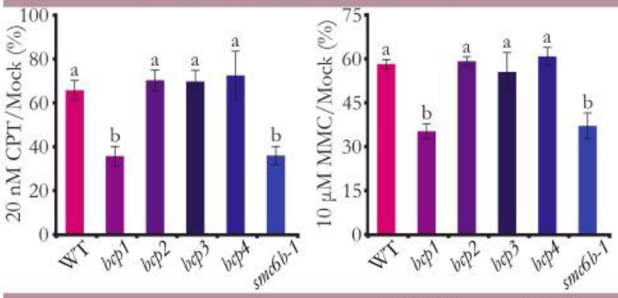


HR assays

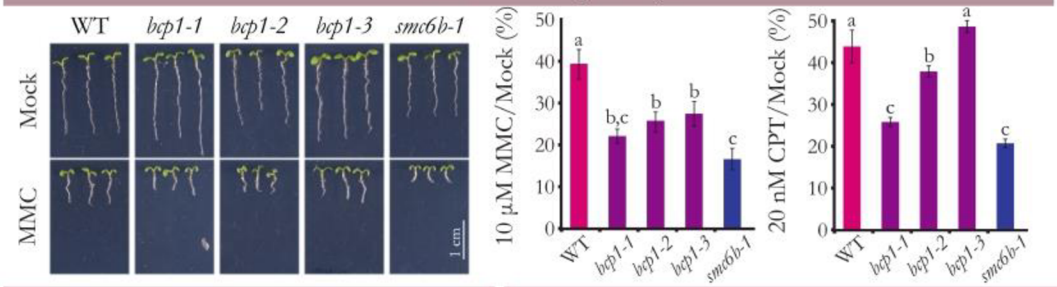


3 RESULTS

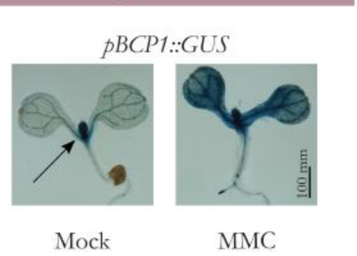
Screen of the candidates



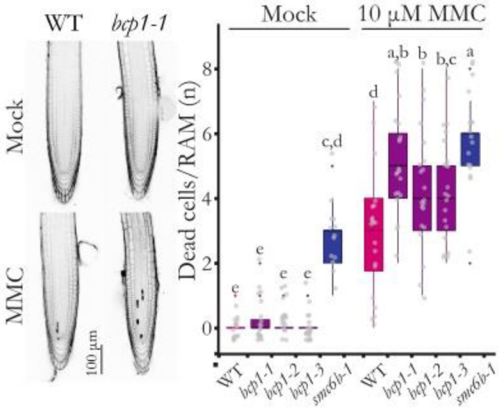
BCP1 sensitivity assays



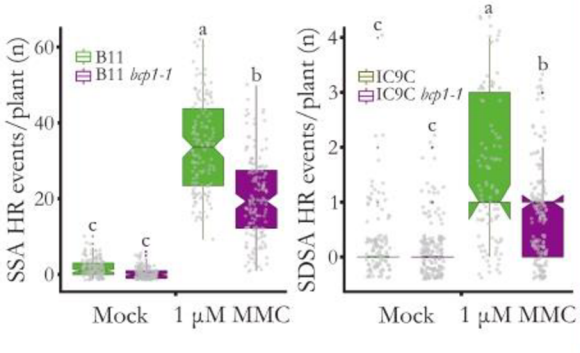
Expression



Cell death assays



HR frequency



4 CONCLUSION

- In the screen testing four candidate proteins, *BCP1* was the most sensitive to genotoxic agents
- *BCP1* is required for repair of various types of DNA damage
- *BCP1* is required for normal frequency of homologous recombination

NOTE: To assess the statistical significance of the results a one-way ANOVA with post -hoc Tukey HSD ($p \leq 0.05$) was applied. Means that do not share a letter are significantly different. .

Institute of Experimental Botany of the AS CR, v. v. i. | Czech Academy of Sciences | C. R. HANA

Characterization of the BRCT5/6 domain-containing protein in *Arabidopsis thaliana*

Jovanka Vladejic[1], Eva Dvořák Tomaštíková[1], Fen Yang[1], , Jaroslav Doležel[1], Jan Paleček[3] and Aleš Pečinka[1,2]

In: Abstract of the "Plant Biotechnology: Green for Good VI"
Olomouc, Czech Republic 2022
Poster presentation

[1] Institute of Experimental Botany (IEB), Czech Acad Sci, Centre of the Region Hana for Biotechnological and Agricultural Research (CRH), Slechtitelu 31, 77900 Olomouc, Czech Republic.
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[3] National Centre for Biomolecular Research (NCBR), Faculty of Science, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic.

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Analysis of BRCT5 domain-containing proteins and a new component of DNA damage repair in Arabidopsis

Jovanka Vladejić[1], Fen Yang[1,2], Eva Dvořák Tomaštíková[1], Jaroslav Doležel[1], Jan J. Paleček[3], Aleš Pečinka[1,2]

[1] Institute of Experimental Botany (IEB), Czech Acad Sci, Centre of the Region Haná for Biotechnological and Agricultural Research (CRH), Šlechtitelů 31, 77900 Olomouc, Czech Republic
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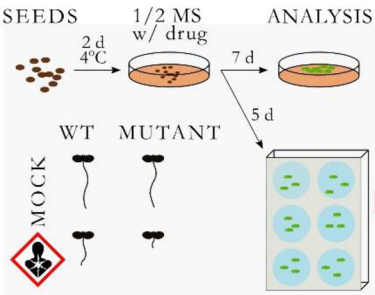
1 INTRODUCTION

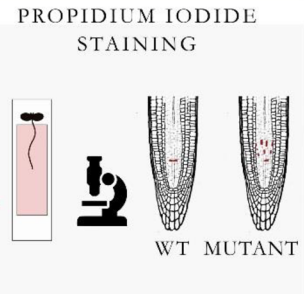
We aimed to identify proteins containing BRCT5 domain in *Arabidopsis thaliana* and to assess their possible involvement in DNA damage repair. Based on homology and structural similarity to the human and fission yeast BRCT5 domains, four Arabidopsis candidate proteins were selected. They were subsequently marked as BRCT-containing protein (BCP) 1, 2, 3, and 4. The screening consisted of testing the sensitivity of Arabidopsis mutants in a series of DNA damage assays, and revealed one protein required for normal resistance to DNA damage.

2 METHODS

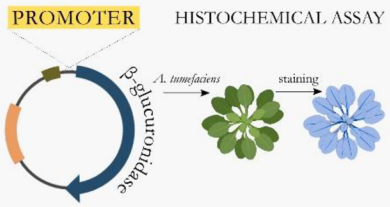
Sensitivity assays



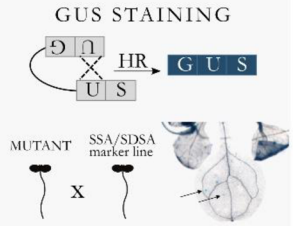
Cell death assays



Cloning and transformation

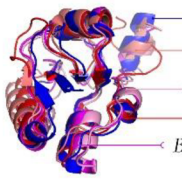


HR assays

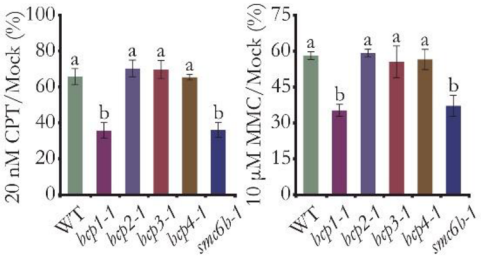


3 RESULTS

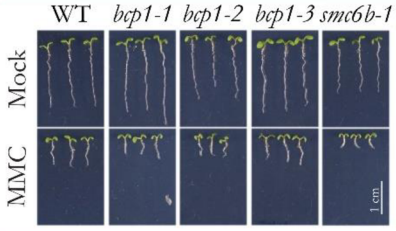
BRCT5 domain model

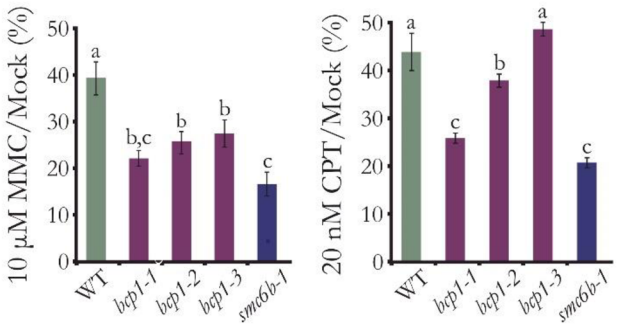


Sensitivity to DNA damage

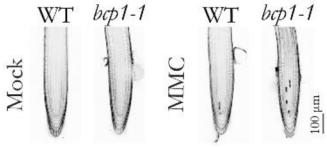


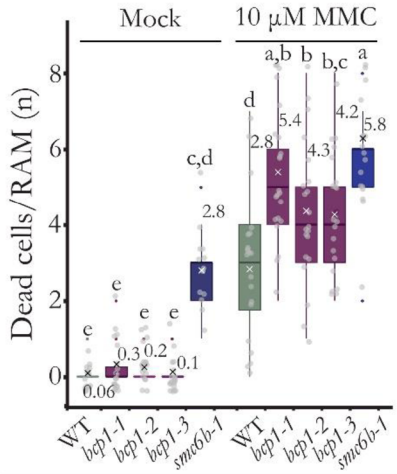
BCP1 sensitivity assays



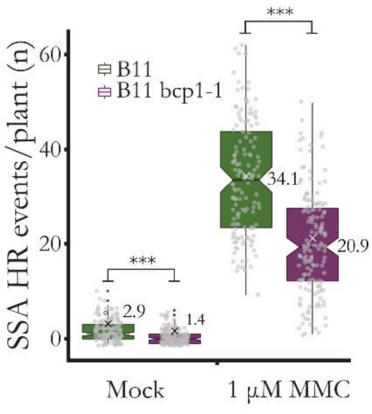


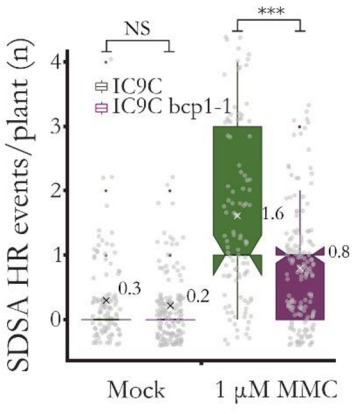
Cell death assays



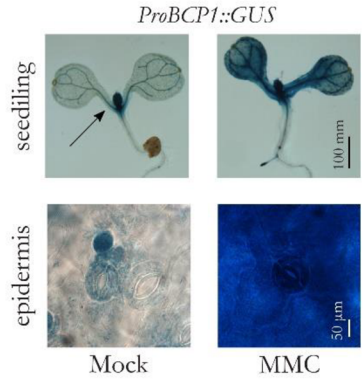


Homologous recombination assays





Expression



4 CONCLUSION

We identified a novel plant-specific DNA repair factor BCP1. BCP1 loss of function mutants are hypersensitive to DNA damage. BCP1 is involved in homology based repair, as shown by the reduced frequency of somatic homologous recombination in the mutant plants.

NOTE: To assess the statistical significance of the results in sensitivity assays a one-way ANOVA with post -hoc Tukey HSD ($p \leq 0.05$) was applied. Means that do not share a letter are significantly different. For assesing the relevance of results in homologous recombination assay a two sample T-test with unequal variance was used. Asterisks represent significant differences * $P < 0.05$, *** $P < 0.001$, NS – not significantly different..

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Czech Academy of Sciences
C. R. HANÁ
EUROPEAN UNION
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Operational Programme Research, Development and Education

Exploring DNA damage response in barley (*Hordeum vulgare*)

Jovanka Vladejic[1,2], Martin Kovacik[1,2], Jana Zwyrtkova[1], Jaroslav Doležel[1], Miriam Szurman-Zubrzycka[3], Ales Pecinka[1]

In: Abstract of the "Mendel Early Career Symposium"
Vienna, Austria 2023
Poster presentation

[1] Institute of Experimental Botany (IEB), Czech Acad Sci, Centre of the Region Hana for Biotechnological and Agricultural Research (CRH), Slechtitelu 31, 77900 Olomouc,
[2] Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Šlechtitelů 27, 77900 Olomouc, Czech Republic
[3] Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland

Maintaining genome integrity presents a particular challenge for plants due to their sedentary lifestyle, which disables direct avoidance of unfavorable external conditions. Additionally, plants' metabolic processes generate reactive molecules as by-products of e.g. photosynthesis, creating internal DNA-damaging conditions. Due to this, plants have developed a unique and strictly regulated web of DNA damage responses (DDR). We initiated analysis of the DDR system in cultivated barley (*Hordeum vulgare*), a temperate cereal model with a large and repeat rich genome. A series of DNA damaging assays was established to describe barley plants' phenotypic response to chemically induced DNA double-strand breaks. The efficacy of assays as a tool to be used for assessing potential new DDR barley mutants was demonstrated. DNA damage response network activation in barley was assessed by transcriptome analysis using RNA sequencing for wild type and mutant in the DDR signaling kinase *ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED* (*ATR*). Considering the barley genome had only recently been sequenced, and it lacks the in-depth gene analysis, a list of potential DNA damage response genes in barley was compiled based on their homology with *Arabidopsis* genes. The comparison of transcripts in wild-type plants and *atr* mutants following genotoxic stress showed the effect a loss of a significant player could have on DNA damage response.

Exploring DNA damage response in cultivated barley

Authors

Jovanka Vladejić[1,2], Martin Kovačik[1,2], Jana Zwyrtková[1], Jaroslav Doležel[1] Miriam Szurman-Zubrzycka[3], Ales Pecinka[1]

Affiliations

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Introduction

Plants have developed a unique and strictly regulated web of DNA damage responses (DDR). We examined the DDR system in cultivated barley, plant with a large (4.8 Gbp/1C) and repeat rich genome.

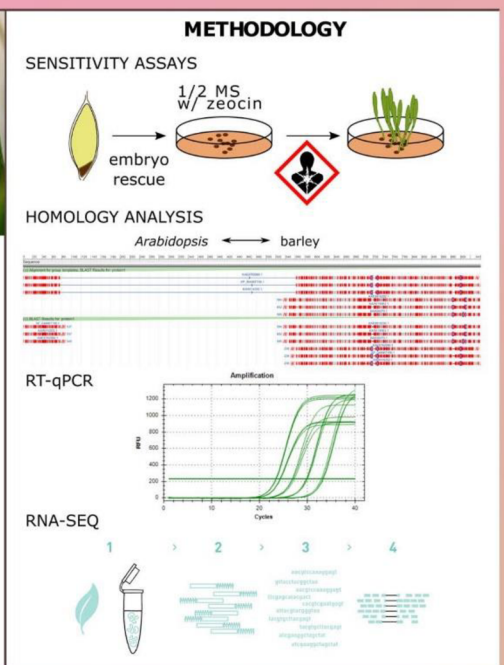
To decribe barley's response to DNA double-strand breaks induced by zeocin, we established and employed a new series of *in vitro* assays.

In the lack of in-depth gene analysis in barley, we compiled a list of candidate DDR genes.

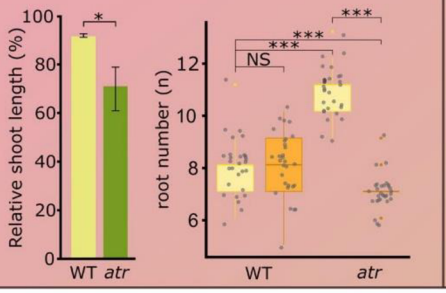
The activation of DDR network in barley was assessed by transcriptome analysis using RNA sequencing for wild type and mutant in the DDR signaling kinase ATAXIA TELANGIECTASIA MUTATED AND RAD3-RELATED (ATR).

The comparison of transcripts in wild-type plants and *atr* mutants following zeocin treatments showed the effect a loss of a significant player could have on DNA damage response.

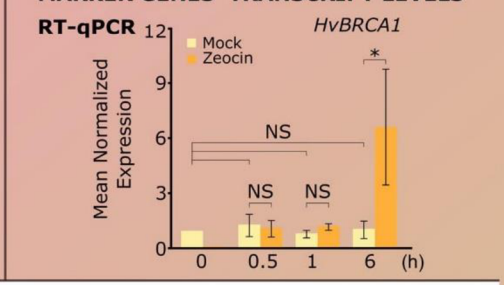
METHODOLOGY



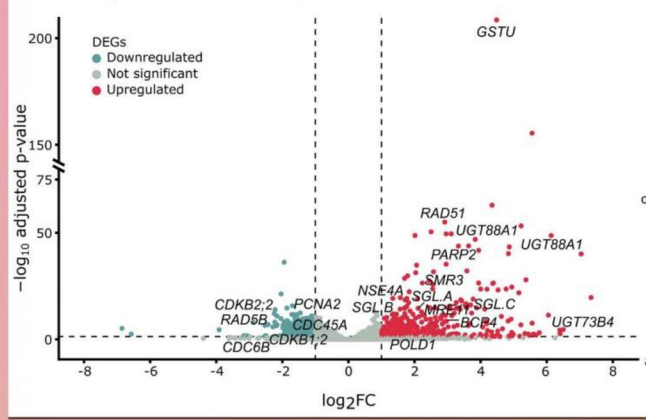
PHENOTYPIC ANALYSIS



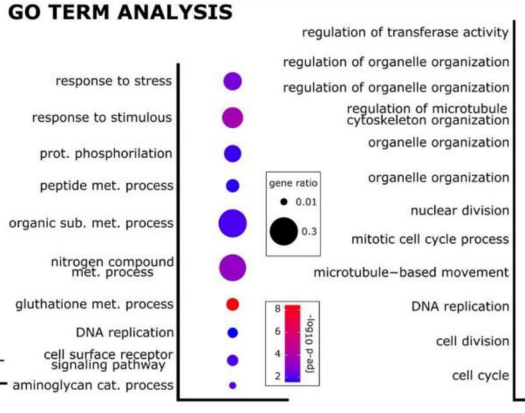
MARKER GENES' TRANSCRIPT LEVELS



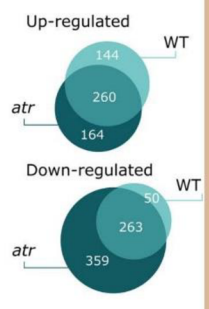
RNA-SEQ ANALYSIS: ZEO/MOCK WT



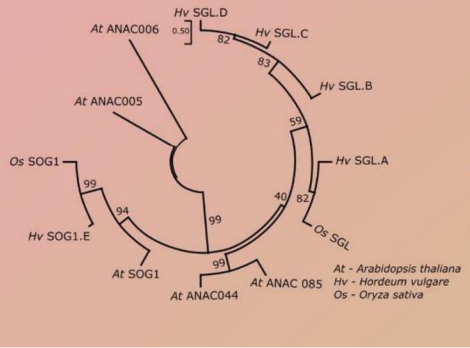
GO TERM ANALYSIS



COMPARISON MOCK VS *atr* TRANSCRIPTOME



PHYLOGENY ANALYSIS OF SOG1 FAMILY



Conclusion

- *In vitro* DNA damage assays were successfully implemented for barley
- Zeocin induced DNA double-strand breaks cause, besides the activation of the DDR and halting of the cell cycle, a strong anti-oxidative response
- Barley has one orthologue of the *Arabidopsis SOG1*, and four homologues of *ANAC044/085*
- There are at least 194 genes whose expression in barley is completely dependant on ATR

Note: To assess the relevancy of results following statistical analysis were used: ANOVA with a *post-hoc* Tukey test, Kruskall-Wallis H-test with *post-con* Conover-Iman, Mann-Whitney. Phylogeny was reconstructed in MEGA software wfollowing the alignment by MUSCLE.

References:

Szurman-Zubrzycka, Miriam, et al. "ATR, a DNA damage signaling kinase, is involved in aluminum response in barley." Frontiers in Plant Science 10 (2019): 1299.

Jaskowiak, Joanna, et al. "Al-tolerant barley mutant hvatr. g shows the ATR-regulated DNA damage response to maleic acid hydrazide." International Journal of Molecular Sciences 21.22 (2020): 8500.

Kurowska, Marzena, et al. "TILLING-a shortcut in functional genomics." Journal of applied genetics 52 (2011): 371-390.

Images

https://www.allgenetics.eu/services/genomics-for-researchers/rna-seq/

Lobo, Ingrid. "Basic local alignment search tool (BLAST)." Nature Education 1.1 (2008).

Exploring DNA damage response in barley (Hordeum vulgare)

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In: Abstract of the "The Czech Plant Nucleus Workshop 2023"
Brno, Czech Republic 2023
Poster and oral presentation

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[2] Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Šlechtitelů 27, 77900 Olomouc, Czech Republic
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