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Maize ATR Safeguards Genome Stability During Kernel Development to Prevent Early Endosperm Endocycle Onset and Cell Death^[W]

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ABSTRACT

The Ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases coordinate the DNA damage response (DDR). The roles described for *Arabidopsis thaliana* ATR and ATM are assumed to be conserved over other plant species, but molecular evidence is scarce. Here, we demonstrate that the functions of ATR and ATM are only partially conserved between *Arabidopsis* and maize. In both species, ATR and ATM play a key role in DNA repair and cell cycle checkpoint activation, but whereas *Arabidopsis* plants do not suffer from the absence of ATR under control growth conditions, maize mutant plants accumulate replication defects, likely due to their large genome size. Moreover, contrarily to *Arabidopsis*, maize ATM deficiency does not trigger meiotic defects, whereas the ATR kinase appears to be crucial for the maternal fertility. Strikingly, ATR is required to repress premature endocycle onset and cell death in the maize endosperm. Its absence results in a reduction of kernel size, protein and starch content, and a stochastic lethality of kernels, a process being counteracted by ATM. Additionally, whereas *Arabidopsis atr atm* double mutants are viable, no such mutants could be obtained for maize. Therefore, our data highlight that the mechanisms maintaining genome integrity may be more important for vegetative and reproductive development than previously anticipated.

INTRODUCTION

Due to their sessile lifestyle, plants can be exposed to severe adverse environmental stresses, resulting in aberrant DNA replication and loss of genome integrity, both potentially affecting growth and plant survival (Hu et al., 2016; Nisa et al., 2019). To cope with such aberrations, eukaryotic cells activate highly coordinated cellular networks, collectively termed the DNA damage response (DDR), which are crucial for maintaining genome stability (Nisa et al., 2019). DDR activation relies on two phosphatidylinositol 3 kinase (PI3K)-like protein kinases, called Ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) (Hu et al., 2016). In general, DNA double-strand breaks (DSBs) activate the ATM kinase, whereas ATR is predominantly activated by single-stranded DNA (ssDNA) and defects in replication fork progression (Maréchal and Zou, 2013). ATM is activated by the MRN complex (MRE11, RAD50, and NBS1) that recognizes DSBs (Nisa et al., 2019); ATR responds to the accumulation of the replication protein A (RPA) at ssDNA sites that is generated in response to any lesion that perturbs replication (Iyer and Rhind, 2017).

Remarkably, whereas the upstream kinases ATM and ATR and the proteins that participate directly in the DNA repair mechanisms appear to be largely conserved across eukaryotes, the factors that transduce the DNA damage signal appear to have diverged (Hu et al., 2016; Nikitaki et al., 2018). For instance, plants lack orthologous genes for the canonical signal transducer kinases CHK1/2 and the transcription factor p53 that act downstream of ATM and ATR in mammals (Nikitaki et al., 2018). Instead, Arabidopsis possesses a set of plant-specific proteins, including the SOG1 transcription factor (Yoshiyama et al., 2009; Yoshiyama et al., 2013a) and the SIAMESE/SIAMESE-RELATED (SIM/SMR) family of cyclin-dependent kinase inhibitors (Yi et al., 2014). SOG1 is phosphorylated by ATM in response to DSBs (Yoshiyama et al., 2013b) and can be phosphorylated *in vitro* by ATR (Sjogren et al., 2015). This phosphorylation is crucial for SOG1 activation in response to DSBs (Yoshiyama et al., 2017), representing a direct and immediate connection between DNA damage and the transcriptional activation of DDR genes (Yoshiyama et al., 2009; Bourbousse et al., 2018; Ogita et al., 2018).

In contrast to mammals, where disruption of ATR induces embryonic lethality (Brown and Baltimore, 2000; Maréchal and Zou, 2013; Menolfi and Zha, 2020) and ATM mutations lead to developmental defects (Menolfi and Zha, 2020), Arabidopsis *atr* and *atm* knockout mutants do not show any vegetative developmental defect under non-stressed conditions (Garcia et al., 2003; Culligan et al., 2004), although *atm* mutant plants display partial sterility (Garcia et al., 2003). Even the *atr atm* double mutant is viable, but fully infertile due to severe defects during meiosis (Culligan and Britt, 2008). In spite of these discrepancies at the organismal scale, the respective roles of ATM

and ATR in DNA damage signalling appear to be conserved between plants and other eukaryotes. Indeed, in *Arabidopsis*, loss of ATM results in hypersensitivity to genotoxic agents that induce DSBs either directly (e.g. exposure to γ -irradiation) or indirectly during the replication process (e.g. crosslinking agents), as observed in all eukaryotes (Garcia et al., 2003; Culligan et al., 2006; Menolfi and Zha, 2020). Likewise, the *Arabidopsis atr* mutants are hypersensitive to replication-blocking agents (Culligan et al., 2004), as other eukaryotes (Friedel et al., 2009; Segurado and Tercero, 2009; Jossen and Bermejo, 2013). However, ATR deficiency leads to high sensitivity to DSBs in yeast and mammals (Segurado and Tercero, 2009; Jossen and Bermejo, 2013), whereas *Arabidopsis atr* mutant plants show only a slight sensitivity to ionizing irradiation, suggesting only a minor role of this kinase in the response to DSBs in plants (Culligan et al., 2004; Culligan et al., 2006).

The described roles for ATR and ATM in *Arabidopsis* have been assumed to be conserved in other plants such as crops, but molecular evidence is scarce (Manova and Gruszka, 2015; Szurman-Zubrzycka et al., 2019; Zhang et al., 2020), and some differences may exist between plant species. Indeed, recently, the characterization of both kinases in *Physcomitrella patens* revealed differences with their *Arabidopsis* orthologs, with PpATR playing a predominant role in the transcriptional response to DSBs, rather than PpATM (Martens et al., 2020) and triggering the formation of stem cells from differentiated tissue in response to DSBs (Gu et al., 2020).

So far, the roles of ATR and ATM have not been studied during the development of crop seeds. The maize seed consists of three main compartments: the seed coat or pericarp, of maternal origin, and the embryo and endosperm, the two products of double fertilization (Doll et al., 2017). Endosperm development begins with a triploid (3C) cell formed from the two polar nuclei of the ovule and one sperm cell (Domínguez and Cejudo, 2014). After fertilization, mitotic cell proliferation begins, and at approximately ten to twelve days after pollination (DAP), the endosperm cells transition from a mitotic cell cycle to endoreduplication, resulting in a dramatic increase in nuclear volume and DNA content that peaks at 16 to 20 DAP. Grain filling begins simultaneously through the accumulation of storage compounds, such as starch and proteins, necessary to support seedling growth following germination. During the final stages of seed development, the starchy endosperm undergoes PCD, but these cells and their contents remain intact in the mature grain. The cell death of the starchy endosperm is critical to facilitate the rapid mobilization of the storage reserves for the growth of the germinated embryo. Premature induction of PCD would limit reserve deposition and thus jeopardize germination (Young et al., 1997; Young and Gallie, 2000; Domínguez and Cejudo, 2014).

Here, we demonstrate that the functional roles of the ATR and ATM kinases are only partially conserved between Arabidopsis and maize, as exemplified by the accumulation of replication defects in maize ATR-deficient plants and differences in fertility defects between the two species. Moreover, our data reveal a crucial role for the ATR kinase in proper kernel development, playing a pivotal role in repressing premature endocycle onset and cell death of the endosperm.

RESULTS

Generation of CRISPR/Cas9 Mutants for the Maize *ATR* and *ATM* Genes

Maize orthologs of the Arabidopsis *ATR* and *ATM* genes were initially identified using the PLAZA platform (Van Bel et al., 2018). For the *AtATR* gene (AT5G40820), this resulted in a one-on-one relationship with the Zm00001d014813 gene, encoding a protein with 67.2% sequence similarity. The *ATM* gene (AT3G48190) showed a one-on-one orthology with the maize Zm00001d040166 gene, although the encoded protein shares only a 47.7% sequence similarity. While the *AtATM* gene contains 63 exons and is spread over 23 kb, the *ZmATM* gene has 78 exons and is spread over more than 123 kb.

With both orthologous genes identified, we designed two single guide RNAs (sgRNAs) to induce mutations in the coding sequence of the maize *ATR* and *ATM* genes using CRISPR/Cas9. We introduced these constructs into immature maize embryos via Agrobacterium-mediated transformation to generate knockouts of the maize *ATR* and *ATM* genes. For each construct, T₀ mutant regenerated plants were crossed with B104 wild-type plants. Within the offspring, plants were selected for heterozygosity for the mutation and absence of the Cas9 transgenes. These plants were subsequently self-pollinated to obtain isogenic wild-type and homozygous mutant plants. We selected two independent CRISPR/Cas-induced mutant lines for both genes, named ATR-A and ATR-B, representing *Zmatr* mutants, and ATM-A and ATM-B, representing *Zmatm* mutants. While the ATR-B and ATM-A mutant alleles were caused by an indel that generates a frameshift, ATR-A and ATM-B were deletion mutants of hundreds of base pairs (**Supplemental Figure 1**).

Conserved Role of ZmATR in the Response to Replicative Stress

In all eukaryotes studied so far, ATR is required for responses to replicative stress such as fork stalling, which is associated with hypersensitivity to replication-blocking agents (Friedel et al., 2009; Segurado and Tercero, 2009; Jossen and Bermejo, 2013). The main characteristic of Arabidopsis *atr* mutants is a rapid loss of meristem activity due to inability to slow down the replication process in the presence of stalled replication forks (Culligan et al., 2004). To confirm that maize ATR shares this conserved role, we challenged the two *Zmatr* independent lines and their corresponding wild-type plants with hydroxyurea (HU), which is an inhibitor of ribonucleotide reductase. HU treatment depletes cellular deoxyribonucleotide (dNTP) pools, and thereby induces stalling of replication forks. The sensitivity to DNA stress was determined by measuring root and shoot length after three days of growth within medium supplemented with HU. Although both the wild-type and *Zmatr* mutant roots grew at very similar rates in medium without genotoxin (**Figures**

1A and 1B), the shoot growth in *Zmatr* mutant lines was clearly 40 to 50% smaller in comparison with their wild-type plants (**Figures 1A and 1C**). When treated with HU, the wild-type root growth was not affected, whereas the *Zmatr* mutant roots were reduced by ~60% in comparison with untreated plants (**Figures 1A and 1B**). A similar effect was observed for *Zmatr* mutant shoot length with a decrease of ~40% compared with ~15-20% in wild-type plants (**Figures 1A and 1C**). As expected, ZmATM is not involved in the stabilization of stalled forks, as the *Zmatm* mutant lines did not display inhibition of growth under treatment nor under control conditions (**Figures 1A, 1D, and 1E**).

To determine whether the ZmATR and ZmATM kinases are involved in controlling the transcriptional activation induced by replicative stress, we monitored the expression level changes of several DDR-related genes, including the putative maize orthologous genes for *RAD51A*, *RAD51B*, *BRCA1*, *XRI*, *RAD54* involved in homologous recombination (HR), *RAD7A* that participates in nucleotide excision repair, and the *RNR1* and *TSO2* ribonucleotide reductase subunits that function in the synthesis of nucleotides (Roa et al., 2009; Sánchez-Pons et al., 2011; Lahari et al., 2018). To map the rapid transcriptional changes, *Zmatr* and *Zmatm* mutant plants were treated with 5 mM HU for 90 min. For all genes tested, the early replication stress response was ZmATR-dependent (**Supplemental Figure 2**). These results show that ZmATR has a conserved role in the replicative stress response.

ZmATR Has a Predominant Role in the Response to DSBs, Whereas ZmATM Plays a Minor Role

To explore the roles of ZmATR and ZmATM in response to DSBs, we evaluated the sensitivity of the corresponding mutants to zeocin, which is a radiomimetic agent that generates mainly DSBs. Unexpectedly, *Zmatr* mutants displayed a pronounced root growth sensitivity to 75 μ M zeocin, showing a reduction of root length of ~60% compared with the untreated plants, whereas wild-type plants did not show a significant reduction of root growth (**Figures 2A and 2B**). Intriguingly, with 75 μ M of zeocin, no increased sensitivity was observed for the *Zmatm* mutants compared with wild-type plants, which did show a ~10% reduction in root length (**Figure 2D**). Nevertheless, at zeocin treatment with 150 μ M, a clear difference of reduction in root growth was observed in *Zmatm* mutants compared with wild-type roots. Moreover, under all conditions, we observed a slight inhibition of shoot growth, which was similar between all genotypes examined (**Figures 2C and 2E**).

To confirm these results on DSB sensitivity, we also exposed the seedlings to γ -irradiation, which is known to also induce DSBs. Two-day-old seedlings were treated with a dose of 75 Gy.

Root and shoot lengths were measured after four days of recovery. Under these treatment conditions, the *Zmatr* mutant lines clearly were more sensitive to the treatment compared with wild-type plants (**Figures 3A, 3C and 3D**), but no differences were observed for the *Zmatm* mutant (**Figures 3B, 3E and 3F**). To examine whether ZmATM is required to respond to higher levels of DNA damage as observed with zeocin treatment, we irradiated with doses of 250 and 500 Gy. Only at 500 Gy, shoot growth in *Zmatm* was severely affected (**Figures 3B, 3E and 3F**) and most of the shoots showed accumulation of anthocyanins, revealed by purple coloring (**Figure 3B, lowest panel**). In summary, our results show that ZmATR plays a crucial role in the DDR triggered by DSBs in maize, as described for yeast and mammals (Cimprich and Cortez, 2008; Blackford and Jackson, 2017), whereas ZmATM contributes to the response under severe DNA-damaging conditions only.

Previous studies in Arabidopsis suggested that in comparison with ATM, ATR plays only a minor role in the response to DSBs, because Arabidopsis *atm* mutant plants display an enhanced sensitivity to γ -irradiation, whereas *atr* mutants only show slight sensitivity in comparison with the wild type (Culligan et al., 2004; Culligan et al., 2006). To confirm these observations, we challenged Arabidopsis *atr* and *atm* mutant plants to zeocin and the related drug bleomycin that both generate mainly DSBs. Interestingly, we found that both mutants displayed an increased sensitivity to both DSB-inducing agents in comparison with the wild type (**Supplemental Figures 3A and 3B**). These results suggest that next to ATM, ATR is also crucial for resistance to DSBs in Arabidopsis.

In Arabidopsis, it is known that the ATR and ATM kinases transduce the DNA stress signal to the SOG1 transcription factor, which controls the transcriptional induction of DNA repair genes (Yoshiyama et al., 2013a; Hu et al., 2016; Yoshiyama et al., 2017). We examined in maize whether the transcriptional response to DSBs is affected in *atr* and *atm* mutants. Because γ -irradiation can generate ROS and other types of stresses that lead to the generation of ssDNA (Kim et al., 2019), we only used zeocin to avoid such secondary effects. The same group of genes mentioned above for the response to HU was employed to evaluate the early transcriptional response to zeocin, by harvesting root tips for RT-qPCR analysis after 3-h and 6-h treatment. Wild-type maize plants presented a rapid and strong transcriptional induction to this DNA-damaging agent (**Supplemental Figure 2**), as previously reported for Arabidopsis cells (Adachi et al., 2011). The loss of either ATR or ATM impeded the early transcriptional response to DSBs for all the genes evaluated (**Supplemental Figure 2**). These observations appear contrasting with previous observations published for Arabidopsis, where ATR was reported to play only a minor role in the transcriptional response to DSBs (Culligan et al., 2006; Ricaud et al., 2007). A possible explanation is the source

of material, being whole *Arabidopsis* seedlings compared with maize root tips. Therefore, to check whether the role of ATR in response to DSBs differs between maize and *Arabidopsis*, we decided to evaluate the transcriptional response in the *Arabidopsis* root tips. Three cyclin-dependent kinase inhibitors (*SMR4*, *SMR5*, and *SMR7*) that are transcriptionally activated by DNA damage (Yi et al., 2014) and DNA repair genes (*RAD51A*, *RAD51B*, *BRCAL*, *XRI-1*, *RAD7A*, *CYCB1;1* and *PARP2*) were included in the analysis. Our results displayed that in *Atatr* root tips the early transcriptional response was attenuated dramatically by zeocin treatment for most of the genes tested, whereas *Atatm* displayed a full loss of transcriptional induction (**Supplemental Figure 4**). These results show that ATR helps to achieve a proper transcriptional induction in response to DSBs in proliferating tissues in both plant species.

Lack of ATR and ATM in Maize Leads to Impaired DNA Repair and Activation of Checkpoints in Response to DNA Stress

One essential mechanism to maintain genome integrity is the accumulation of γ H2AX at DNA lesions in an ATM- and ATR-dependent manner (Friesner et al., 2005; Amiard et al., 2010; Maréchal and Zou, 2013; Waterworth et al., 2019). Upon DNA damage, both kinases phosphorylate the histone variant H2AX to generate γ -H2AX foci that recruit repair factors to the break sites (Turinetti and Giachino, 2015; Waterworth et al., 2019). To determine whether the observed sensitivity to DNA-damaging agents of the *Zmatr* and *Zmatm* mutants was associated with the loss of repair capacity and accumulation of DNA damage, we performed immunodetection of γ H2AX foci on root tip nuclei of untreated and treated plants. The number of foci per nucleus varied widely. To visualize the results, we grouped the nuclei population in five categories, depending on the number of foci present per nucleus. Strikingly, all nuclei observed of untreated wild-type plants showed γ H2AX foci (**Figure 4**), indicating the natural occurrence of replication fork collapse during maize genome replication. The *Zmatr-b* and *Zmatm-a* root tips showed a higher number of foci per nucleus compared with its respective wild-type line (**Figures 4A and 4B**).

In response to replication stress induced by 24 h exposure to HU, the γ H2AX foci number per nucleus increased in the wild-type ATR and ATM lines, with around 80% of the nuclei showing ≥ 51 γ H2AX foci. *Zmatr-b* mutant nuclei showed a hyperaccumulation of DNA damage foci, with around 49% nuclei presenting ≥ 200 foci, where in the isogenic wild-type line this category represented only 29% (**Figure 4B**). *Zmatm-a* displayed a less dramatic accumulation of foci number per nucleus, with only 42% nuclei showed ≥ 101 γ H2AX foci, while in its wild-type line around 57% nuclei were in this category (**Figure 4B**).

We also treated the roots for 24 h with zeocin. Strikingly, we observed a hyperaccumulation of γ H2AX foci in *Zmatr-b* mutant nuclei, while the *Zmatm-a* mutant displayed only a slight increase of γ H2AX foci (**Figure 4A**). Indeed, while the wild-type ATR-B and ATM-A nuclei showed around 62% nuclei with ≥ 101 γ H2AX foci, the population of *Zmatm-a* mutant nuclei in this category was 40%. In the case of *Zmatr-b* mutant, almost 80% of nuclei displayed this degree of accumulation of DNA damage foci (**Figure 4B**). These results are consistent with above-mentioned hypersensitivity of *Zmatr* mutants to γ -irradiation and zeocin, which generate mainly DSBs.

To know whether *Zmatr* and *Zmatm* mutants are impaired in the activation of cell cycle checkpoints, we examined the cell cycle progression by performing flow cytometry on root tips after 24 h of treatment with HU or zeocin. In untreated root tips, no differences in the proportion of G1, S, and G2 nuclei were observed between the ATR-A wild-type and *Zmatr-a* mutant (**Supplemental Figure 5A**). After 24 h treatment with HU, in wild-type root tips, the population of nuclei in S-phase was enriched as was expected because HU triggers the activation of the intra S-phase checkpoint (Cools et al., 2011). However, in the *Zmatr-a* mutant, the nuclei population that arrested in the S-phase was less than in the wild type, and there was an increase in the fraction of nuclei in G1/S. In contrast, the *Zmatm-a* mutant did not display differences with the wild type in response to HU (**Supplemental Figure 5A**).

The profiles of flow cytometry in response to zeocin were less clear and the differences non-significant (**Supplemental Figure 5A**). Therefore, because in other eukaryotes ATM is required for adequate activation of a G2 checkpoint in response to DSBs (Blackford and Jackson, 2017), we determined the number of mitotic events per root tip in wild-type ATM-A and mutant *Zmatm-a*. No differences were observed between both lines in untreated root tips (**Supplemental Figure 5B**). However, in response to zeocin after 24 h, the wild-type roots showed a drastic decrease in the mitotic figures of 175 in average per untreated root to 44 mitoses per treated root tip, whereas *Zmatm-a* mutant roots displayed an average of 94 per treated root tip (**Supplemental Figure 5B**). These results indicate that ZmATM is required for proper activation of a G2 checkpoint in response to DSBs generated by zeocin.

The Maize *atr* Mutant Displays Defective Kernel Development

Similar to *Atatr* and *Atatm* mutants, their counterparts in maize are visually indistinguishable from wild-type plants at adult stage when they are grown under optimal greenhouse conditions, despite the fact that *Zmatr* seedlings were smaller in the first days post-germination in vitro. In contrast to *Atatr* plants that are fully fertile (Culligan et al., 2004), both *Zmatr* mutants displayed smaller cobs (**Figure 5A**; **Supplemental Figure 6A**), with a reduced kernel number per cob (**Figure 5E**),

compared with wild-type plants. Intriguingly, although the *Zmatr* cobs appeared to be fully fertilized at early stages post-fertilization (16 and 28 DAP), a small population of kernels displayed an abnormal phenotype, showing a brown and wrinkled appearance (**Figure 5B**). These kernels died before maturity, resulting in gaps in the mature cobs (**Figures 5A; Supplemental Figure 6A**). The *Zmatr* kernels that did reach maturity were smaller in size and weight compared with wild-type kernels (**Figures 5C and 5D**). The *Zmatm* mutant plants did not display defects on fertility (**Figure 5F**), contrary to what has been reported for *Atatm* mutants, which are semi-sterile (Garcia et al., 2003). The *Zmatm* mutant lines are indistinguishable from wild-type plants in the cob and kernel phenotypes (**Figures 5F and 5G; Supplemental Figure 6B**), number of kernels per cob (**Figure 5I**), and mature kernel weight (**Figure 5H**).

To gain a better insight into the reasons for the apparent lethality shown for some *Zmatr* mutant kernels, we counted the percentage of abnormal kernels per cob. The abnormal kernel phenotype was observed between 5 to 16% (**Supplemental Table 1**), which is not consistent with the expected 25% Mendelian ratio for an embryo lethal mutation, thus rather suggesting a stochastic frequency. Since Arabidopsis ATM is crucial for pollen viability (Garcia et al., 2003), we examined whether the abnormal kernel phenotype may be associated with a defective pollen phenotype through viability staining on pollen from wild-type and *Zmatr* mutant plants. Nearly all the pollen from *Zmatr* mutant plants were viable, similar to pollen of wild-type plants (**Supplemental Figure 7A**). To investigate the maternal effect, reciprocal backcrosses with B104 wild-type plants were performed. Pollen from wild-type plants were not able to rescue the *Zmatr* mutant cob phenotype. In contrast, *Zmatr* mutant plants used as pollen donor for the wild type resulted in normal cob development (**Supplemental Figure 7B**). These reciprocal crosses hint to a maternal fertility defect in the *Zmatr* plants.

Lack of ZmATR and ZmATM Results in Endogenous DNA Damage in the Embryo

To determine whether lack of ZmATR or ZmATM may lead to the accumulation of DNA damage in the embryo, which is a developmental stage with a high rate of DNA replication and thus potentially sensitive to endogenous replicative stress, we analyzed γ H2AX foci accumulation in embryos at 16 DAP. As observed in root tips, all nuclei of *Zmatr-a* and *Zmatr-b* embryos showed a higher number of foci per nucleus compared with their respective wild-type lines (**Figures 6A and 6B**). In the *Zmatr* embryo, the majority of nuclei had ≥ 51 foci, being 68.9% for *Zmatr-a* and 80.2% in *Zmatr-b*, while for wild-type lines the percentage of the nuclei with this foci number were 27.6% and 23.0%, respectively (**Figure 6B**). The *Zmatm* mutant embryo nuclei also displayed an increase of foci number, although this was less outspoken compared with the *Zmatr* mutant embryos (**Figure**

6A). For instance, *Zmatm-a* and *Zmatm-b* nuclei with ≥ 51 γ H2AX foci were 49.5% and 65.7% respectively, whereas their corresponding wild-type lines showed a percentage of 24.5% and 38.7%. The higher accumulation of DNA damage sites within the *Zmatr* embryonic nuclei was reflected by at least 40% of the nuclei analyzed showing ≥ 100 γ H2AX foci compared with their wild-type lines, in which only 2.1 to 2.6% of their nuclei displayed this amount of DNA damage foci. In the *Zmatm* mutant nuclei only between 11.0 to 18.1% of nuclei contained ≥ 100 γ H2AX foci (**Figure 6B**). Thus, these results indicate that lack of ATR leads to a significant accumulation of DNA damage in the embryo. Moreover, although *Zmatm* mutant plants do not show macroscopic differences, they also show increased endogenous DNA damage at least during the embryo development.

Loss of ZmATR Activity Triggers Premature Endoreduplication in the Endosperm

ATR and ATM are relatively more strongly expressed in embryo and endosperm cells of maize, both in mitotic phase and throughout the endoreplication phase (**Supplemental Figure 8**), suggesting that both kinases might have a relevant role during endosperm development. To test whether the expression levels of cell cycle-related genes are affected during endosperm development in the *Zmatr* and *Zmatm* mutants, we analyzed through RT-qPCR the expression at 10, 12, 14, 16, and 19 DAP. The expression levels for almost all evaluated genes with exception of *CDK2* were reduced in *Zmatr* endosperm at 19 DAP in comparison with corresponding wild-type endosperm, whereas at earlier time points, the significant differences were variable (**Supplemental Figure 9A**). These results suggest that at 19 DAP, most cells of *Zmatr* endosperm exited the mitotic cell cycle. In *Zmatm* endosperm, a slight decrease in the expression of cell cycle-related genes at 14 DAP was observed, but at later time points, expression levels recovered to those of wild-type endosperm.

Following the exit of the mitotic cell cycle, the endosperm cells undergo endoreplication (Sabelli and Larkins, 2009). To determine the effects of lack of ATR or ATM on the endoreplication process, developing endosperms of at least three independent cobs were analyzed by flow cytometry. Lack of ZmATM did not lead to changes in endoreplication level (**Supplemental Figure 10**). By contrast, the mean ploidy of *Zmatr-a* and *Zmatr-b* endosperm nuclei was significantly increased at 14 DAP relative to wild-type endosperm, and this phenomenon was sustained up to 19 DAP (**Figure 7A**). This increase was attributed to increased frequencies of 12C, 24C, and 48C endosperm nuclei in comparison with wild-type endosperm (**Figure 7B**): around 32% of the nuclei in the wild-type endosperm were between 12C and 48C, whereas these classes represented around 40% of nuclei in the *Zmatr* mutants (**Figure 7B**).

We next investigated whether lack of ZmATR affected the endoreduplication as consequence of changes in the expression levels of genes associated with DNA replication such as *MINICHROMOSOME (MCM) 2-7 GENE FAMILY* and *PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)*. The transcript levels were measured by RT-qPCR on RNA extracted from wild-type and *Zmatr-b* endosperms at 10, 12, 14, 16, and 19 DAP. Strikingly, reduced expression levels were observed for all evaluated genes at 19 DAP compared with wild-type endosperm (**Supplemental Figure 9B**), contrasting with the increased endoreduplication observed at this developmental time point. This result suggests that the increased occurrence of endoreduplication might be caused by the regulation at post-translational level such as changes of phosphorylation on replisome proteins.

Cell Death Is Enhanced in *ZmATR* Endosperm

Upon completion of endoreduplication, starchy endosperm cells undergo PCD, resulting in extensive DNA degradation (Young and Gallie, 2000). We evaluated whether the pattern and timing of cell death in *Zmatr* and *Zmatm* mutant endosperms were affected. First, the viability of endosperm cells during kernel development was examined by staining fresh sections with Evans blue, a dye that is excluded from living cells with intact plasma membranes, thereby staining only the cytoplasm of non-viable cells (Young et al., 1997). Between 16 and 22 DAP, staining within the central endosperm was more prominent and clearly more extended in *Zmatr* endosperm compared with wild-type endosperm (**Figure 8A**). To quantify these observations, we determined the cell death area (%) per endosperm of at least seven kernels from three different cobs. At the three time points evaluated (16, 19, and 22 DAP), the cell death area (%) was significantly higher in both *Zmatr* mutants in comparison with their respective wild-type lines (**Figure 8B**). In contrast, *Zmatm* mutants did not show differences with their wild-type plants, except for time point 22 DAP in the *Zmatm-b* mutant, for which a significant increase in the cell death area (%) was observed (**Supplemental Figure 11**). However, because this result was not reproducible for the other independent line, it might rather be caused by variability than as a consequence of the lack of ATM.

To study further the cell death within *Zmatr* endosperm, we determined the changes of expression of two lytic proteins that accompany the progression of PCD in the root cap, the S1-P1 nuclease *BFN1* and the aspartic protease *PASPA3-2*, both known to be expressed in the endosperm of Arabidopsis (Farage-Barhom et al., 2008; Fendrych et al., 2014; Moussu et al., 2017). At 19 DAP, the expression of *PASPA3-2* and *BFN1-2* was 1.6- and 2-fold, respectively, higher in *Zmatr* than in wild-type endosperm (**Figure 8C**). Together, these results indicate that lack of ATR leads to more prominent and extended cell death in the endosperm likely by an earlier transition to cell

death. Although this phenomenon was observed in viable *Zmatr* kernels, abnormal *Zmatr* kernels presented massive cell death in the whole endosperm at 16 DAP (**Supplemental Figure 12**), which was consistent with the loss of viability of these kernels.

To determine whether the increased endoreduplication and early cell death affected starch and storage protein synthesis in the *Zmatr* kernels, we first evaluated the expression of genes involved in the protein (*19KDa zein*) and starch synthesis (*Opaque1*, *Opaque10*, *SS4*, *SSIIa*, and *Shrunken 2*) (He et al., 2019). The expression levels of genes linked to starch synthesis displayed a significant increase of more than 2.5-fold at 19 DAP in *Zmatr-b* endosperm compared with wild-type endosperm (**Supplemental Figure 13A**). Next, we compared starch and protein (non-zein and zein) contents in *Zmatr-b* and wild-type endosperms. While no differences were observed at 19 DAP, mature endosperm of *Zmatr-b* kernels showed a significant reduction in the starch and zein protein content (**Supplemental Figures 13C and 13E**), consistent with the smaller size of the *Zmatr* kernels (**Figures 5C**). In *Zmatm* endosperm at 19 DAP, a lower accumulation of starch was found (**Supplemental Figure 13B**), but no difference was observed in mature endosperm (**Supplemental Figure 13C**). Likewise, although *Zmatm* endosperm displayed a significant reduction of starch and protein synthesis-related genes (**Supplemental Figure 13A**), these changes did not affect the final accumulation of these nutrients in mature kernels. Together, our results indicate that lack of ZmATR, but not of ZmATM, leads to early induction of endoreduplication and cell death in starchy endosperm, affecting starch and protein storage, resulting in the reduction of mature kernel size.

The Presence of Either ZmATR or ZmATM Is Essential for Survival

To determine whether the *Zmatr Zmatm* double mutant is viable, we crossed the *Zmatr-b* and *Zmatm-b* single mutants. No progeny with homozygous mutations in both genes were obtained after genotyping 200 seedlings from the F₂ generation and 100 plants from the F₃ generation. To gain more insight into the basis for the apparent lethality associated with the double mutant, the *atr*^{-/-} *atm*^{+/-} and *atr*^{+/-} *atm*^{-/-} sesquimutant lines, single mutants and wild-type plants were recovered for further analysis during the development. The sesquimutant plants displayed a slight but significant reduction in the plant height in comparison with the single mutants and wild-type lines of adult plants that grew under optimal conditions in the greenhouse (**Figure 9A**).

To better understand the basis for the apparent kernel lethality associated with the double mutation, we performed viability staining on pollen from the sesquimutant and single mutant plants. Contrary to single mutants, where no differences in the pollen viability were observed with wild-type plants, part of the pollen of sesquimutant plants was non-viable (**Figure 9B**). Strikingly,

despite the sesquimutants displaying a population of non-viable pollen, full cobs were obtained after self-pollination, although the percentage of abnormal kernels was dramatically increased in comparison with the *Zmatr* single mutant (**Figure 9C**). To evaluate whether the phenotype of abnormal kernels is consistent with a Mendelian ratio, we estimated the percentage of abnormal kernels per cob (**Figure 9C**). Interestingly, the kernels with lethal phenotype were between 35 to 45%, rather than the 25% that would be expected for a lethality associated uniquely to double mutant kernels; the early death of some *Zmatr* single mutant plus sesquimutant kernels likely contributed to this higher percentage.

DISCUSSION

ATR and ATM Signalling Functions Are Largely Conserved Among Arabidopsis and Maize Upon Exposure to DNA-Damaging Agents

Maize *atr* mutants show hypersensitivity towards HU, which initially induces stalling of replication forks by depleting cellular deoxyribonucleotide pools, but also results in DSB formation because of fork collapse (Singh and Xu, 2016). We found that the early transcriptional DDR induced by HU was ATR-dependent and that lack of *ZmATR* fails to arrest cells in the S-phase, accompanied by a strong accumulation of γ -H2AX foci that are indicative for DNA break accumulation in response to HU. It suggests that *Zmatr* mutant plants are not able to stabilize the stalled forks, similarly to what is described for other species. Together, these results show that *ZmATR* has a conserved role in the response to replicative stress. Likewise, as observed in Arabidopsis, we found that *ZmATM* plays an important role in the induction of cell cycle arrest after DSB induction, as illustrated by the high frequency of mitotic events observed in *Zmatm* mutants exposed to zeocin.

On the other hand, we found that the DSB-induced DDR depends on both ATR and ATM. Maize *atr* mutants show an excessive accumulation of DSBs after treatment with zeocin, to a level similar to what was observed upon exposure to HU, indicating that the loss of ATR-dependent DDR leads to an impairment in DNA damage repair. In contrast, maize *atm* mutants were far less sensitive to DSB-inducing agents than *Zmatr* mutants and did not show a strong level of γ H2AX accumulation when challenged with zeocin. The number of foci was even significantly reduced compared with wild-type nuclei, suggesting that in maize, like in Arabidopsis, ATM plays a more prominent role than ATR in the phosphorylation of γ H2AX (Friesner et al., 2005; Waterworth et al., 2019). Because γ H2Ax has been shown to facilitate HR in both mammals and Arabidopsis (Maréchal and Zou, 2013; Biedermann et al., 2017), we may hypothesize that the absence of ATR leads to replication stress that activates an ATM-dependent increase of γ H2AX foci. Under normal growth conditions, the relatively low levels of DSBs can be repaired by HR, explaining the viability of the *Zmatr* mutant plants. However, when such plants are challenged by exogenous inducers of DSBs, the break repair machinery fails, leading to massive accumulation of DNA damage. Consistent with this idea, it has been observed that mammalian cells deficient for ATR display elevated chromosomal fragmentation after DSB induction due to defects in repair by HR (Wang et al., 2004).

ATR and ATM transduce the DNA stress signals to SOG1, whose phosphorylation is crucial for the transcriptional response to DNA damage, including the transcriptional induction of genes needed for the activation of a transient cell cycle arrest and PCD (Yoshiyama et al., 2009;

Yoshiyama et al., 2017; Bourbousse et al., 2018; Ogita et al., 2018). We found that the early transcriptional response to DSBs in maize was dependent on ZmATM, as occurs in Arabidopsis (Culligan et al., 2006; Ricaud et al., 2007). Furthermore, in both Arabidopsis and maize, ATR participates in the early transcriptional response to DSBs in root tip meristems. This important role of ATR in the DSB response had probably been overlooked in Arabidopsis due to the use of whole plantlets for the analysis of changes in gene expression, rather than focusing on meristems as we did here. This would suggest that the respective roles of ATM and ATR vary according to the cell type, ATR playing a more crucial role in proliferating cells, in which DNA damage can interfere with the replication process, whereas ATM may be more prominent in differentiated cells. In addition, the relative contribution of ATR appears to be species specific. Indeed, in Arabidopsis *atr* mutants, the transcriptional activation of DDR genes is only dramatically attenuated, whereas in *Zmatr* it is completely absent. Consistently, the relative importance of ATR and ATM for the regulation of the transcriptional response to DSBs has diverged between eukaryotes. In yeast, Tel1/ATM contributes only marginally to direct DSB repair and is of little importance in checkpoint control (Mantiero et al., 2007), playing only a secondary role in signalling pathways (Craven et al., 2002; Jaehnig et al., 2013), whereas these processes are dominated by Mec1/ATR (Gasch et al., 2001; Watson et al., 2004). Likewise, it has been shown for the moss *Physcomitrella patens* that the early transcriptional response induced by bleomycin (DSBs inducer) was largely dependent on ATR and only marginally on ATM (Martens et al., 2020).

Although many functions can be compensated between ATR and ATM in diverse eukaryotes (Tomimatsu et al., 2009; Maréchal and Zou, 2013; Menolfi and Zha, 2020), including Arabidopsis (Friesner et al., 2005; Amiard et al., 2010; Roitinger et al., 2015; Waterworth et al., 2019), other roles are non-redundant or performed predominantly by one of the two kinases, and thus lack of any of them leads to developmental defects as a consequence of genetic instability (Murga et al., 2009; Maréchal and Zou, 2013; Menolfi and Zha, 2020). In maize, this partial functional redundancy between the two kinases appears to be conserved: while at the adult vegetative stage, the *Zmatr* and *Zmatm* single mutants are visually indistinguishable from wild-type plants, like their counterparts in Arabidopsis (Garcia et al., 2003; Culligan et al., 2004), the sesquimutant plants display a slight but significant reduction in the stature and the double mutant is lethal, demonstrating that at least one of the kinases is required for an adequate development. This observation contrasts with Arabidopsis, where the *atr atm* double mutant is viable (Culligan and Britt, 2008).

Essential Role of Maize ATR in Proliferating Cells

Contrary to Arabidopsis, where *atr* and *atm* mutant root nuclei do not show spontaneous appearance of γ H2AX (Friesner et al., 2005; Amiard et al., 2010), we found that maize tissues with a high division rate, such as the developing embryo and root tips of *atr* mutants, display an increased presence of the DNA damage marker γ H2AX in comparison with wild-type plants, suggesting that mutants suffer from replicative stress and/or DNA breakages in proliferative tissues. The maize genome is at least 17-fold bigger than the Arabidopsis genome, and contains much more complex repetitive regions (e.g., microsatellites and quasi-palindromic AT-rich repeats) (Haberer et al., 2005; Schnable et al., 2009). These characteristics probably make its genome more prone to replicative stress, implying that ATR plays a more essential role in species with large versus small genomes. Consistent with this hypothesis, *atr* mutant plants have been isolated in barley, whose genome size is of a same order of magnitude as maize, and around 60% of root meristem cells displayed endogenous DNA damage, resulting in plants with reduced size (Szurman-Zubrzycka et al., 2019).

Contrasting Requirements for ATM and ATR During Meiosis and Early Stages of Development in Plants

Intriguingly, contrasting requirements in different species have been observed for plant ATM proteins during meiosis: ATM deficiency results in semi-sterility in Arabidopsis and full infertility in rice (Garcia et al., 2003; Zhang et al., 2020), a phenomenon that we did not observe in maize. In Arabidopsis, although ATM deficiency alone impacts meiotic DSB repair leading to partial sterility, the *atr atm* double mutant displays full sterility and accumulation of chromosomal fragmentation, indicating partial functional redundancy between the two proteins during meiosis (Culligan and Britt, 2008). This suggests that, in maize, ATR may function redundantly with ATM in meiotic cells. Consistent with this idea, the maize sesquimutants showed the appearance of non-viable pollen, whereas single mutants did not display differences compared with wild-type plants. However, how ZmATR and ZmATM participate in meiosis and/or female gametophyte development remains to be revealed. Notably, the production of non-viable pollen in the sesquimutants could either be due to meiotic defects or to defects occurring later, during the development of gametophytes.

Conversely, whereas lack of ATR does not affect the fertility in Arabidopsis and barley (Culligan et al., 2004; Szurman-Zubrzycka et al., 2019), in maize it resulted in a slight reduction of fertility, as reflected by a smaller cob size, decrease in the number of seeds per cob, and early abortion of some kernels. However, this effect likely relates to severe replicative stress during the

early stages of embryo and endosperm development rather than to meiotic defects, since pollen viability was unaffected in *Zmatr* mutants.

ATR Prevents Early Endosperm Endoreplication and Programmed Cell Death

As mentioned above, mutant maize *atr* cobs show the stochastic appearance of abnormal kernels, which is characterized by an early death before maturity. As we observed that lack of ATR activity results in an increase of replicative stress in embryos of viable kernels, we may hypothesize that the viable *Zmatr* kernels achieve to cope properly with the endogenous DNA damage, while the abnormal kernels accumulate higher levels of breakages of DNA and genetic instability, eventually triggering early death of the kernel.

In the Arabidopsis root meristem, DSBs trigger cell differentiation, which includes the transition into the endocycle (Adachi et al., 2011). Furthermore, the excessive accumulation of DNA damage activates PCD in meristematic cells when the DNA damage cannot be repaired (Fulcher and Sablowski, 2009; Furukawa et al., 2010). In Arabidopsis, both endoreduplication and PCD are triggered by the perception of DNA damage through ATR and/or ATM (Fulcher and Sablowski, 2009; Furukawa et al., 2010; Adachi et al., 2011). The single mutants display a delay in the induction of PCD, but they do not lose the capacity to activate this process (Furukawa et al., 2010), while the endoduplication activation is not affected (Furukawa et al., 2010; Adachi et al., 2011). By contrast, induction of endoreduplication and PCD are fully compromised in double mutants (Furukawa et al., 2010; Adachi et al., 2011).

Considering that endoreduplication and PCD are an intrinsic part of maize endosperm development, and those processes are induced by DSBs in Arabidopsis through an ATM/ATR-dependent pathway, we may hypothesize that during the mitotic division of endosperm cells, lack of ZmATR leads to the accumulation of DSBs that act as a signal driving an early transition into the endocycle. During the subsequent events of DNA replication, even more DNA damage may accumulate, which would lead to a premature onset of PCD.

The early onset of endocycle and PCD in *Zmatr* kernels is reminiscent of the early senescence observed in *Atatm* mutants, in which the level of DSBs increases but the DNA repair efficiency decreases, because AtATM represses DSB-induced expression of genes associated with senescence (Li et al., 2020). Consistent with this idea, the endosperm of non-viable *Zmatr* kernels displays an early cell death phenotype. Likewise, the *Zmatr Zmatm* double mutant kernels abort early, indicating that in maize, the two kinases also have partially redundant functions in these mechanisms: ZmATM could be involved but has a minor role, which in its absence can be fully compensated by ZmATR. Furthermore, the elevated expression of both kinases during endosperm

development supports the idea that ATM and ATR have a crucial role during the endosperm development in wild-type kernels. Likely both kinases are involved directly in the signaling of DSBs generated by DNA degradation.

The viable *Zmatr* kernels display a reduction in the weight and size of mature seeds, accompanied with a decrease in the starch and proteins content as a consequence of an accelerated kernel maturity process in the endosperm triggered by early endoreduplication and PCD. This reduced accumulation of the storage nutrients may jeopardize the germination, which explains the early shoot growth phenotype of the *Zmatr* seedlings, when they still rely on nutrients supplied by the endosperm. However, this delay of growth may also be related with the importance of DDR during the germination of seeds (Waterworth et al., 2015; Waterworth et al., 2016).

In summary, we found that the necessity of the ATM and ATR kinases under non-stress conditions appears to be much higher in maize compared with Arabidopsis, with absence of ATR resulting in both vegetative and reproductive phenotypes that are partially offset by ATM. Our data highlight that the mechanisms involved in the maintenance of genome integrity may be more important for plant development than previously anticipated and indirectly suggest that the presence of a non-optimal DDR might severely affect the yield of field-grown crop plants.

METHODS

Plant Medium and Growth Conditions

Maize seedlings were grown to maturity in soil in a 24°C growth chamber with a 16-h light/8-h dark regime, 55% relative humidity and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation at plant level. For seedling analysis, the maize seeds were germinated using a paper roll system. Seeds were sterilized for 3 min in 100% ethanol, then submerged in 5% NaClO for 30 min, and finally the seeds were rinsed three times with sterilized water. Twelve seeds were spread out over the paper roll, subsequently the roll paper with seeds was transferred to a recipient with liquid 0.5 × Murashige and Skoog (MS) medium (Basalt Salt Mixture M0221; Duchefa, The Netherlands).

Arabidopsis plants were grown *in vitro* under long-day conditions (16-h light/8-h dark, Lumilux Cool White lm, 50 to 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 21°C on solidified medium (half-strength MS (2.151 g/L), 10 g/L sucrose, and 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES), adjusted to pH 5.7 with 1 M KOH and 8 or 10 g/L agar). The T-DNA insertion mutant lines *atr-2* and *atm-2* were previously described (Garcia et al., 2003; Culligan et al., 2004). *Arabidopsis thaliana* seeds were sterilized with 5% NaClO for 20 min and subsequent washing with sterile water. To obtain homogeneous germination, the seeds were vernalized for 2 days at 4°C.

Vector Construction

The CRISPR construct was adapted from Xing et al. (2014). The pBUN411 plasmid was digested by HindIII, leading to two fragments of 2 kb (hereafter called fragment A) and 11.5 kb. The 11.5-kb fragment was further digested with SpeI, resulting in two fragments of 8.5 kb (called fragment B) and 3 kb. The pP+ plasmid was digested with XbaI and HindIII and was ligated to fragments A and B. This resulted in the whole backbone of pBUN411 being replaced by the backbone of pP+, containing a spectinomycin resistance cassette and a more efficient origin of replication. This new vector was called pBUN411-Sp. Using the CRISPR-P *in silico* gRNA design tool (Lei et al., 2014), we selected both for ATR and ATM two possible target sites. Designed primers with the target sites were used to perform PCR on the pCBC-MT1T2 plasmid (Xing et al., 2014), resulting in a fragment containing the desired target sites and the correct sites for ligation into pBUN411-Sp. This was done for both targeted genes. The vector contained the *bar* (bialaphos resistance) marker gene for selection of transformed callus in plants.

Maize Transformation and Selection of CRISPR/Cas Lines

Immature embryos of the maize B104 inbred line were transformed using *Agrobacterium tumefaciens* EHA101 containing the pBUN411-Sp expression constructs. Transformed calli were selected on increasing concentrations of phosphinothricin after which transformed T₀ shoots were regenerated as described before (Coussens et al., 2012). For genotyping, DNA was extracted from transformed T₀ shoot or leaf material in the next generations (approximately 2 cm²) using the Wizard® Genomic DNA purification kit (Promega, WI, USA). We screened T₀ transformants through PCR (primers are listed in Supplemental Table 2), followed by Sanger sequencing to identify CRISPR/Cas9-induced mutations in the targeted genes. The plants were screened for absence of the *bar* marker by ammonium well assay (Coussens et al., 2012), followed by genotyping for the absence of the Cas9 gene construct. Two independent *atm* and *atr* mutants were selected and backcrossed with B104 wild-type plants. These lines were upscaled and screened in F2 to obtain wild-type and homozygous mutant plants (*Zmatm* or *Zmatr*) in the same genetic background.

Genotoxic Treatments

Maize seeds were germinated in a paper roll system for 3 days to ensure a straight root orientation. At this time point, seedlings of similar size (2.5.-3.5 cm) were transferred to a falcon tube (50 mL) with liquid 0.5 × MS medium (mock or supplemented with genotoxin) that was used like growth hydroponic system. Treatments were performed with 2.5 mM HU (Sigma-Aldrich, MO, USA) or zeocin (75 µM or 150 µM). After 3 days of treatment, the plantlets were analyzed for root and shoot lengths.

For treatment with γ-irradiation, the maize seeds were grown hydroponically using the paper roll system. After 2 days, the seedlings were treated with γ-irradiation using a ¹³⁷Cs source (C.I.S. Bio, Gif sur Yvette, France) at doses of 75, 250 or 500 Gy, and then returned for growth under hydroponic conditions. After 5 days, the root and shoot lengths were measured.

To evaluate the sensitivity to genotoxic agents in Arabidopsis, seedlings were grown for 4 days on 0.5 × MS and transferred to genotoxin-supplemented medium (5.0 µM zeocin or 0.2 µg/mL bleomycin). After 10 days, the plantlets were analyzed for root length. All the measurements were performed using the ImageJ software package (https://imagej.net/ImageJ_1.x).

RNA Extraction and RT-qPCR

The RNA extractions for maize were performed in pools of ten root tips (1-2 mm) or endosperms of nine independent kernels (three half endosperms per cob of three independent cobs were mixed to perform the RNA extraction). Total RNA was extracted using a Direct-zol RNA MiniPrep Plus kit

(Zymo Research, CA, USA). For Arabidopsis experiments, seeds were germinated on control medium on a nylon mesh and transferred 5 d after germination to control medium or medium supplemented with 20 μ M zeocin for the indicated time, after which at least 200 root tips (1-2 mm) were harvested. RNA was isolated with the RNeasy Mini kit (Qiagen, Germany) and was treated on-column with the RQ1 RNase-Free DNase (Promega, WI, USA).

RNA quality and concentration were determined by Nanodrop (Thermo Fisher Scientific, MA, USA). The iScript cDNA synthesis kit (Bio-Rad, CA, USA) was used to prepare cDNA from 1 μ g of RNA according to the manufacturer's instructions. Quantitative RT-PCR was performed in a 384-well plate on a LightCycler 480 (Roche, Belgium) with SYBR Green I Master mix (Roche, Belgium) in a final volume of 5 μ L and 0.25 μ M primer concentration. Each reaction was done with three technical and three biological repeats. *EF- α* and *18S rRNA* primers were used for normalization of the maize data, while for Arabidopsis three reference genes were used: *EMB2386*, *PAC1* and *RPS26E*. Relative expression values were manually calculated using the $2^{-\Delta\Delta CT}$ method. Primers were designed using Primer3Plus (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>). Primers used for qRT-PCR are listed in Supplemental Table 2.

Immunostaining of γ -H2AX

Root tips of 3-day-old seedlings or embryos of 16 DAP were fixed overnight in 4% paraformaldehyde in a solution of 1x PME (50 mM Pipes pH 6.9, 5 mM MgSO₄, 1 mM EGTA) and then washed three times for 5 min in 1x PME. For one experimental replication, ten root meristems or six embryos were pooled respectively for nucleus isolation. The dissected tissues were chopped in Galbraith's buffer (45 mM MgCl₂, 20 mM MOPS, 30 mM sodium citrate, 0.1% [v/v] Triton X-100, adjusted to pH 7.0 using 1 M NaOH). After filtering through a 50- μ m CellTrics filter and centrifugation under 200 g for 10 min at 4°C, the supernatant was removed. The nuclei were resuspended in 30 μ L nuclei extraction buffer and spread on slides. The samples were left to dry for 1 h at room temperature.

Immunostaining was performed as described (Amiard et al., 2010), with the following modifications. Each slide was incubated overnight at 4°C with 80 μ L of a rabbit anti-plant γ -H2AX antibody (kindly provided by Charles White, CNRS, Clermont- Ferrand, France) in a 1:600 dilution in fresh blocking buffer (3% BSA in 1x PBS). Slides were washed three times for 5 min in 1x PBS solution and then incubated for 3 h at room temperature in 100 μ L blocking buffer containing Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Invitrogen, CA, USA), diluted 1:1000 in fresh blocking buffer. Finally, DNA was counterstained with 2 μ g/mL DAPI for

30 min, after which slides were washed in 1x PBS and mounted in mounting medium. Imaging of the nuclei was done using an Olympus FV1000 confocal microscope.

Mitosis Phase Counting

The number of mitotic events per root tip was examined in untreated and treated root tips with 150 μ M zeocin. Root tips were fixed overnight in 4% paraformaldehyde in a solution of 1x PME (50 mM Pipes pH 6.9, 5 mM MgSO₄, 1 mM EGTA) and then washed three times for 5 min in 1x PME. Root apices were digested in enzyme mix (1% w/v cellulase, 0.5% w/v cytohelicase, 1% w/v pectolyase in 1x PME) for 2 h at 37°C. After three washes with 1x PME, root apices were squashed gently between the slide and a coverslip, and frozen in liquid nitrogen. Afterwards, the coverslip was removed, and the slides were left to dry for 1 h at room temperature. The slides were stained with 2 μ g/mL DAPI for 30 min, after which slides were washed in 1x PBS and mounted in mounting medium. Imaging of the nuclei was done with an epifluorescence microscope (Axio Imager.Z2.). The number of mitoses were counted by eye.

Pollen Staining

Fresh pollen from tassels was collected in the morning in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid). Staining was done with a modified Alexander stain as reported by Peterson et al. (2010). The pollen was spread on slides, and then the staining solution was applied. After 10-15 min of incubation, samples were visualized under a microscope (Olympus BX51). Pollen from at least six independent plants was analyzed.

Flow Cytometry

For maize ploidy analysis of roots, seeds were germinated using the paper roll system. Control and treated root tips (1-2 mm) of 3-day-old seedlings were analyzed. For one experimental replication, ten root meristems were analyzed and three replications per treatment were used. The root tips were chopped with a razor blade in 1 mL of Gif nuclei-isolation buffer (45 mM MgCl₂, 30 mM sodium citrate, 60 mM MOPS, 1% (w/v) polyvinylpyrrolidone 10 000, pH 7.2) containing 0.1% (w/v) Triton X-100, supplemented with 5 mM sodium metabisulphite and RNase (5 U/mL). Propidium iodide was added to the filtered supernatants to a final concentration of 50 μ g/mL. The samples were analyzed using a Cyflow SL3 flow cytometer (Partec-Sysmex) with a 532-nm solid state laser (30 mW) excitation and an emission collected after a 590-nm long-pass filter. For cell cycle analysis, we used the algorithm available in the Flomax software.

For endosperm analysis, tissue was obtained by removing the seed coat and embryos from each kernel. At least three kernels were analyzed for each plant. Three individual ears were used for flow cytometry analysis. The endosperm was rapidly chopped on a glass Petri dish using a razor blade in 1 mL ice-cold Galbraith's buffer (45 mM MgCl₂, 20 mM MOPS, 30 mM sodium citrate, 0.1% [v/v] Triton X-100, adjusted to pH 7.0 using 1 M NaOH). The homogenate was passed through two layers of a 100-μm nylon mesh and filtered through a 50-μm CellTrics filter. Then the nuclei were precipitated (200 g, 10 min, 4°C), and the supernatant was removed. The nuclei were suspended in 200 μL nuclei extraction buffer, after which 1 mL staining buffer was added (Cystain UV Precise P, Sysmex Partec, Germany). The mix was filtered through a 50-μm CellTrics filter (Sysmex – Partec) and analyzed by the Cyflow MB flow cytometer (Sysmex Partec, Germany). For each sample, at least 10,000 nuclei were collected and analyzed using a logarithmic scale display. The Cyflogic software was used for ploidy measurements. Mean C-values were calculated according to the following equation: $[(n_3 \times 3) + (n_6 \times 6) + (n_{12} \times 12) + \dots] / \text{total number of nuclei}$, where n_3 = total number of nuclei in the 3C peak, etc.

Evans Blue Staining

Fresh kernels were collected at 19, 22 and 25 DAP for each wild-type and mutant line. Near-median longitudinal hand sections (approximately 3 mm thick) were stained in 0.1% (w/v) Evans blue (C.I 23860) for 2 min as reported by Young et al. (1997). Stained sections were washed with water for 30 min and photographed. The analyses were carried out from at least seven kernels of three independent cobs for each genotype.

Analysis of Total Protein and Starch Contents

The total protein and starch contents of the kernels were analyzed as described (Zhang et al., 2019), with some modifications. Twenty endosperms of immature (19 DAP) or mature kernels from the same ear were pooled as a single replicate. Three biological replicates were used for the analysis. Flour was obtained by grinding dry endosperm under liquid nitrogen, and then lyophilized overnight.

Starch content determination was performed following the method described by instructions of the Total Starch Assay Kit (Catalog: K-TSTA-50A, Megazyme, Australia) based on the use of thermostable α-amylase and amyloglucosidase. Analyses were performed on 100 mg of flour.

For the measurement of protein content, approximately 50 mg of flour was used for the content analysis of zein and non-zein proteins. The non-zein proteins were extracted by adding 1 mL of the buffer (12.5 mM sodium borate, 5% SDS, 2% 2-mercaptoethanol, proteases inhibitors) to

50 mg flour and incubated overnight at 37°C with constant shaking. Subsequently, the samples were centrifugated at 12,000 g at room temperature for 15 min to precipitate debris. Supernatant was collected and absolute ethanol was added to a final concentration of 70%. The samples were incubated at 37°C for 2 h, then centrifugated at 12,000 g for 10 min at room temperature. The supernatant was transferred to a new tube (zein fraction), and then dried in a Speedvac and resuspended in water. The pellet was used to extract non-zein proteins with 1 mL buffer (12.5 mM sodium borate, 5% SDS, 2% 2-mercaptoethano, proteases inhibitors). The mix was incubated at 37°C for 2 h, and then centrifugated for 15 min at 12,000 g and room temperature. The pellet was washed twice with 70% ethanol, air-dried, and resuspended in 8 M Urea. Protein quantification was performed with the Compat-Able Protein Assay Preparation Reagent Kit (Catalog: 23215, Thermo Fisher Scientific, MA, USA) and the Pierce BCA Protein Assay Kit (Catalog: 23225, Thermo Fisher Scientific, MA, USA) following manufacturer's manual.

Statistical Analysis

All statistical analyses were performed using the R software (<https://www.r-project.org/>).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Map of CRISPR/Cas9 Mutations

Supplemental Figure 2. Early Transcriptional Response in *Zmatr-b* and *Zmatm-a* Root Tips Treated with HU or Zeocin

Supplemental Figure 3. *Atatr* and *Atatm* Mutants Display Sensitivity to Zeocin and Bleomycin, Both DSB Inducers

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AUTHOR CONTRIBUTIONS

J.A.P.G., T.E. and L.D.V. conceived and designed the research. J.A.P.G., T.E., I.A., M-U.N., H.V.d.D. and C.R. performed the experiments. G.C., M.V.L., and L.P. did the maize transformations. J.A.P.G., T.E., I.A., C.R. and L.D.V analyzed data. J.A.P.G. and L.D.V. wrote the article. All authors read, revised, and approved the article.

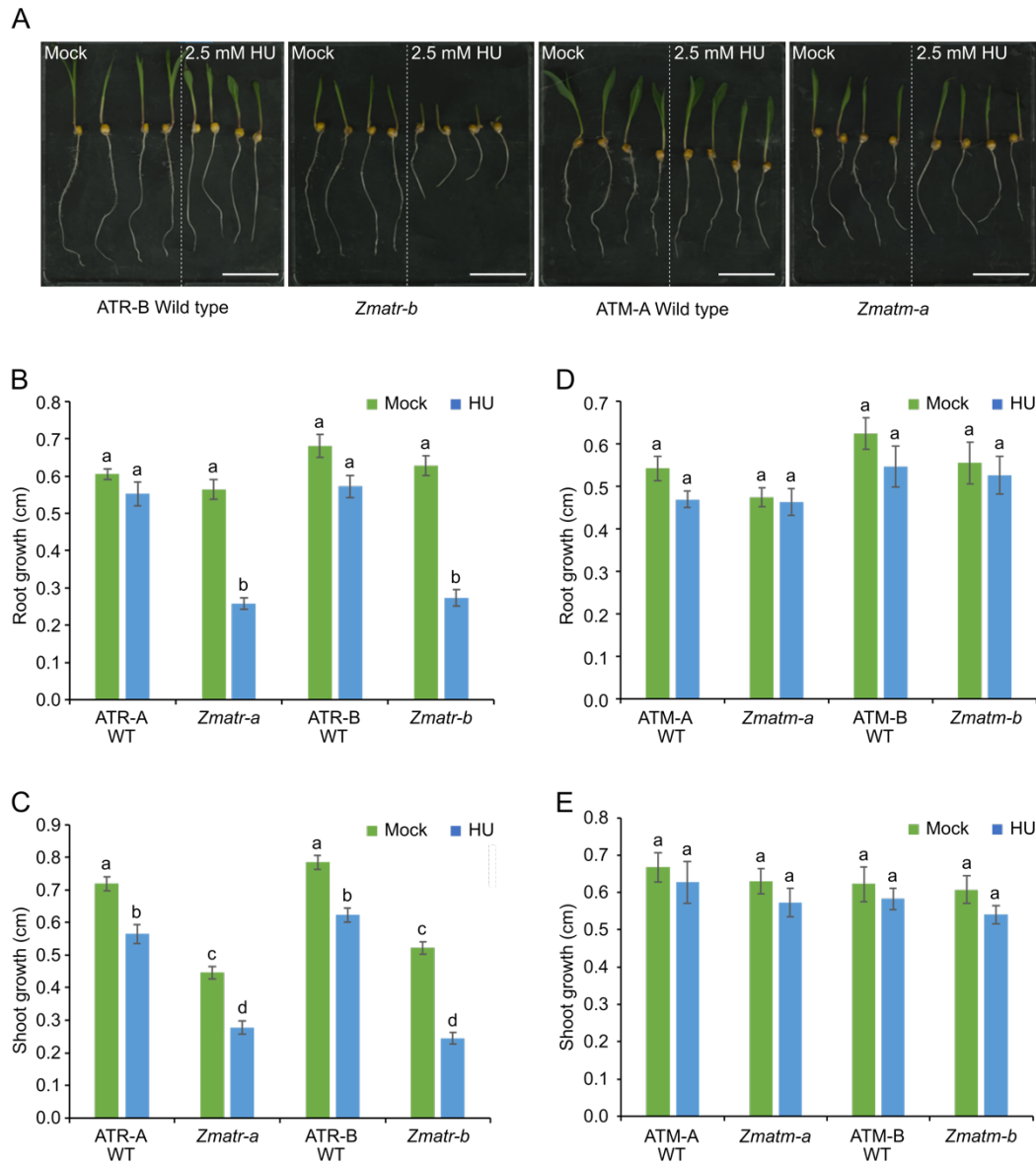


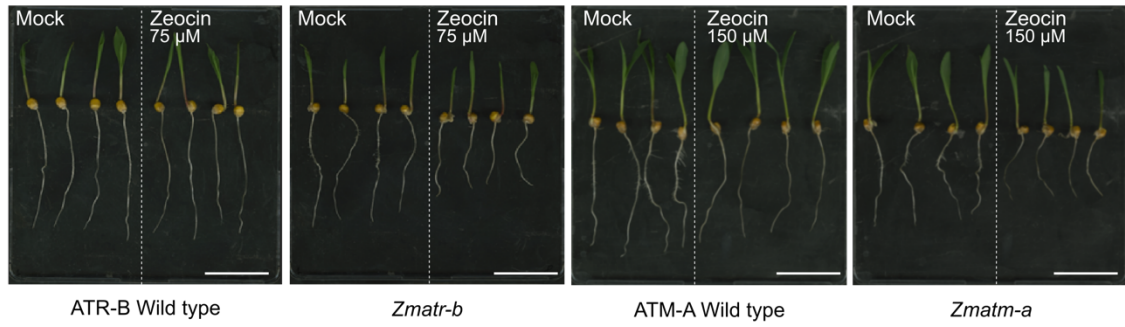
Figure 1. *Zmatr* Plants Are Hypersensitive to Replicative Stress Induced by HU.

(A) Representative wild-type and mutant seedlings following growth for 3 days under control conditions (Mock) or in the presence of 2.5 mM HU. Scale bars = 5.5 cm.

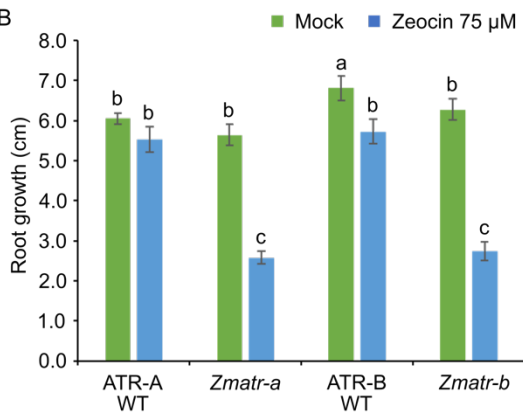
(B and D) Quantification of root growth of ATR **(B)** and ATM **(D)** wild-type and mutant seedlings treated as in **(A)**.

(C and E) Quantification of shoot growth of ATR **(C)** and ATM **(E)** wild-type and mutant seedlings treated as in **(A)**. Letters indicate statistically different growth for each genotype ($P < 0.0001$, mixed model analysis, Tukey correction for multiple testing). Values are average \pm SE; $n = 8-10$.

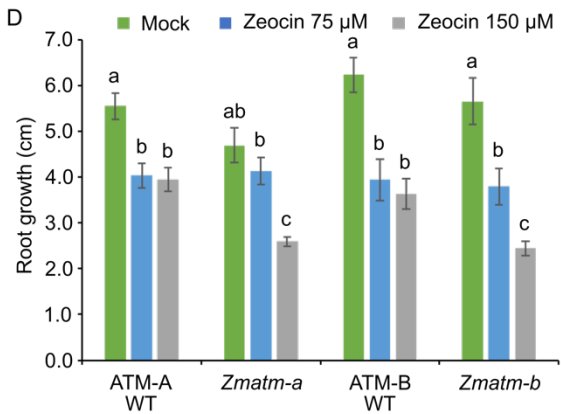
A



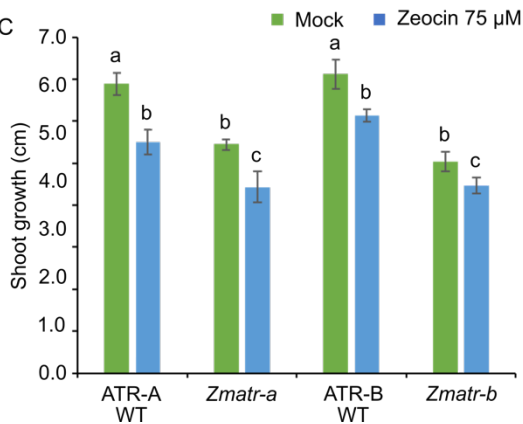
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D



C



E

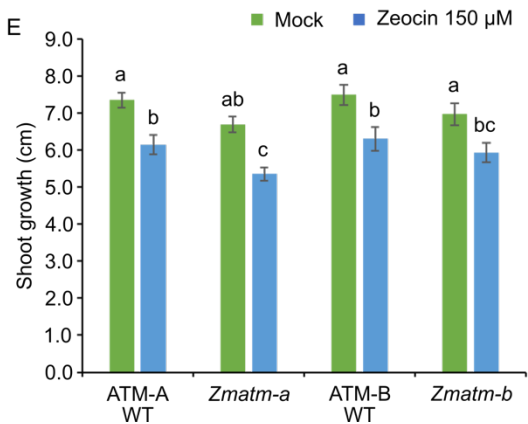


Figure 2. Both Maize ATR and ATM Kinases Are Necessary for Resistance to Zeocin.

(A) Representative wild-type and mutant seedlings following growth for 3 days under control conditions (Mock) or in the presence of 75 or 150 μM zeocin. Scale bars = 5.5 cm.

(B and D) Quantification of root growth of ATR **(B)** and ATM **(D)** wild-type and mutant seedlings treated as in **(A)**.

(C and E) Quantification of shoot growth of ATR **(C)** and ATM **(E)** wild-type and mutant seedlings treated as in **(A)**. Letters indicate statistically different growth for each genotype ($P < 0.0001$, mixed model analysis, Tukey correction for multiple testing). Values are average \pm SE; $n = 8-10$.

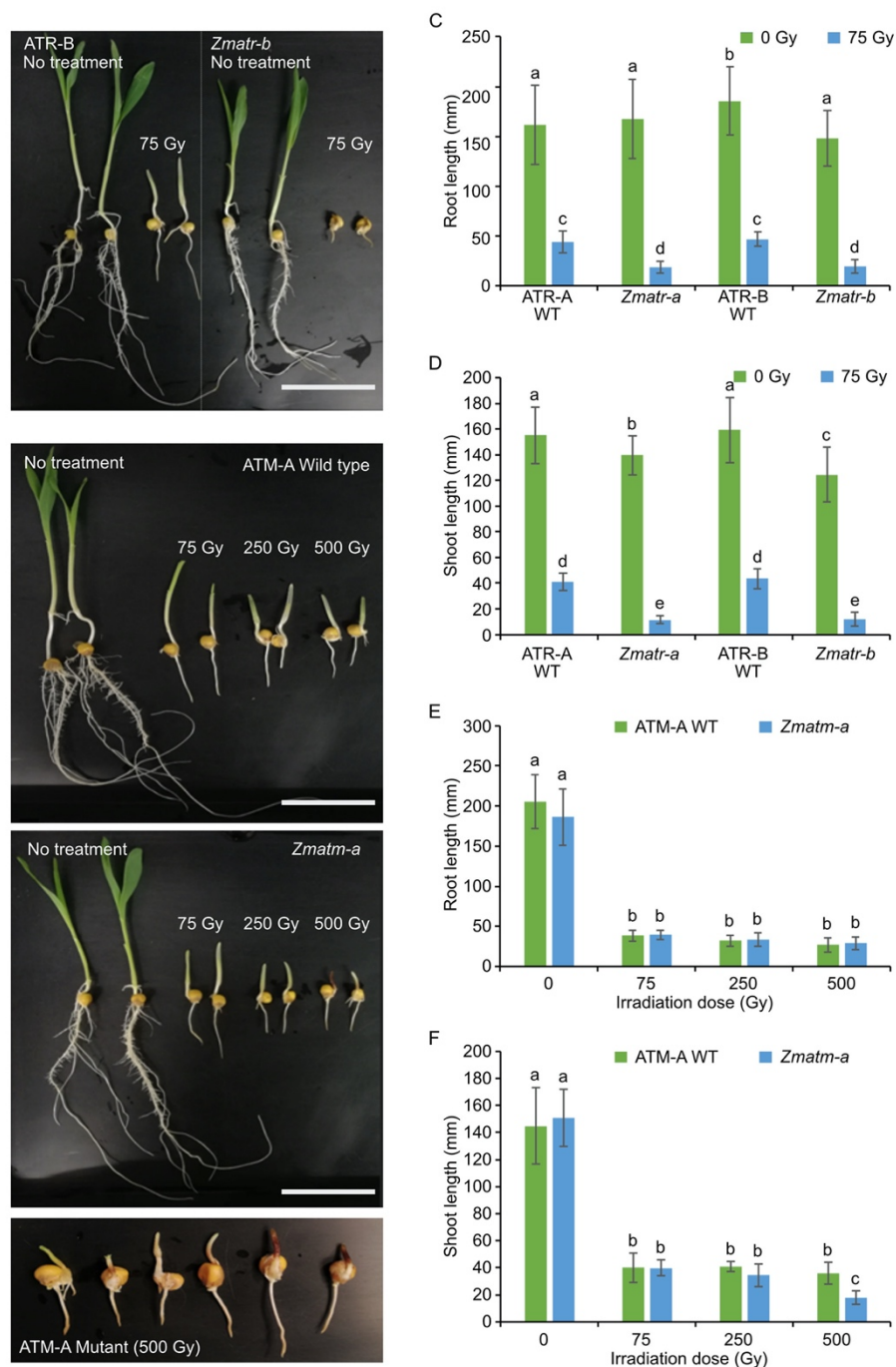


Figure 3. Maize ATR Plays a Predominant Role in the Response to γ -Irradiation-Induced DNA damage.

(A and B) Representative 7-day-old ATR **(A)** and ATM **(B)** wild-type and mutant seedlings grown under control conditions (no treatment) or γ -irradiated at doses of 75, 250 or 500 Gy on day 2. Scale bars = 6.5 cm.

(C and D) Average root **(C)** and shoot **(D)** length of ATR wild-type and mutant seedlings.

(E and F) Average root **(E)** and shoot **(F)** length in ATM wild-type and mutant seedlings. Letters indicate statistically different growth for each genotype ($P < 0.0001$, mixed model analysis, Tukey correction for multiple testing). Values are average \pm SE; $n = 26-32$.

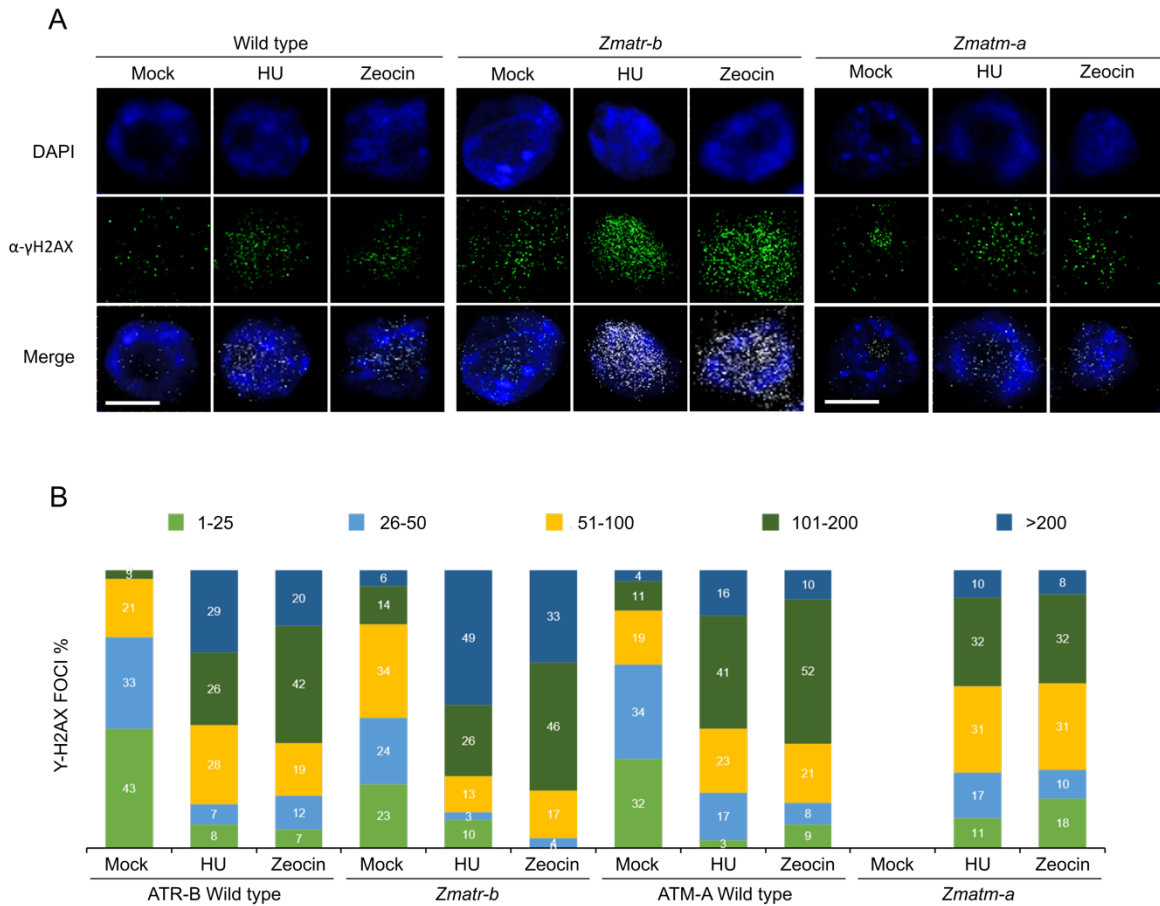


Figure 4. Accumulation of γ H2AX Foci in *Zmatr* and *Zmatm* Root Tips in Response to HU and Zeocin.

(A) Immunostaining of γ H2AX foci accumulation (green) in nuclei stained with DAPI (blue) in maize root tips of wild-type plants and *Zmatr-b* and *Zmatm-a* mutants after 24 h of treatment with 5.0 mM HU or 150 μ M zeocin. Scale bars = 5 μ m.

(B) Quantification of number of γ H2AX foci per nucleus in ATR-B wild-type, ATM-A wild-type, *Zmatr-b* and *Zmatm-a* root tips after 24 h of treatment with HU (5 mM) or zeocin (150 μ M). For each sample, the γ H2AX foci of 100 nuclei were counted and grouped into five categories: 1-25, 26-50, 51-100, 101-200, and more than 200 foci per nucleus.

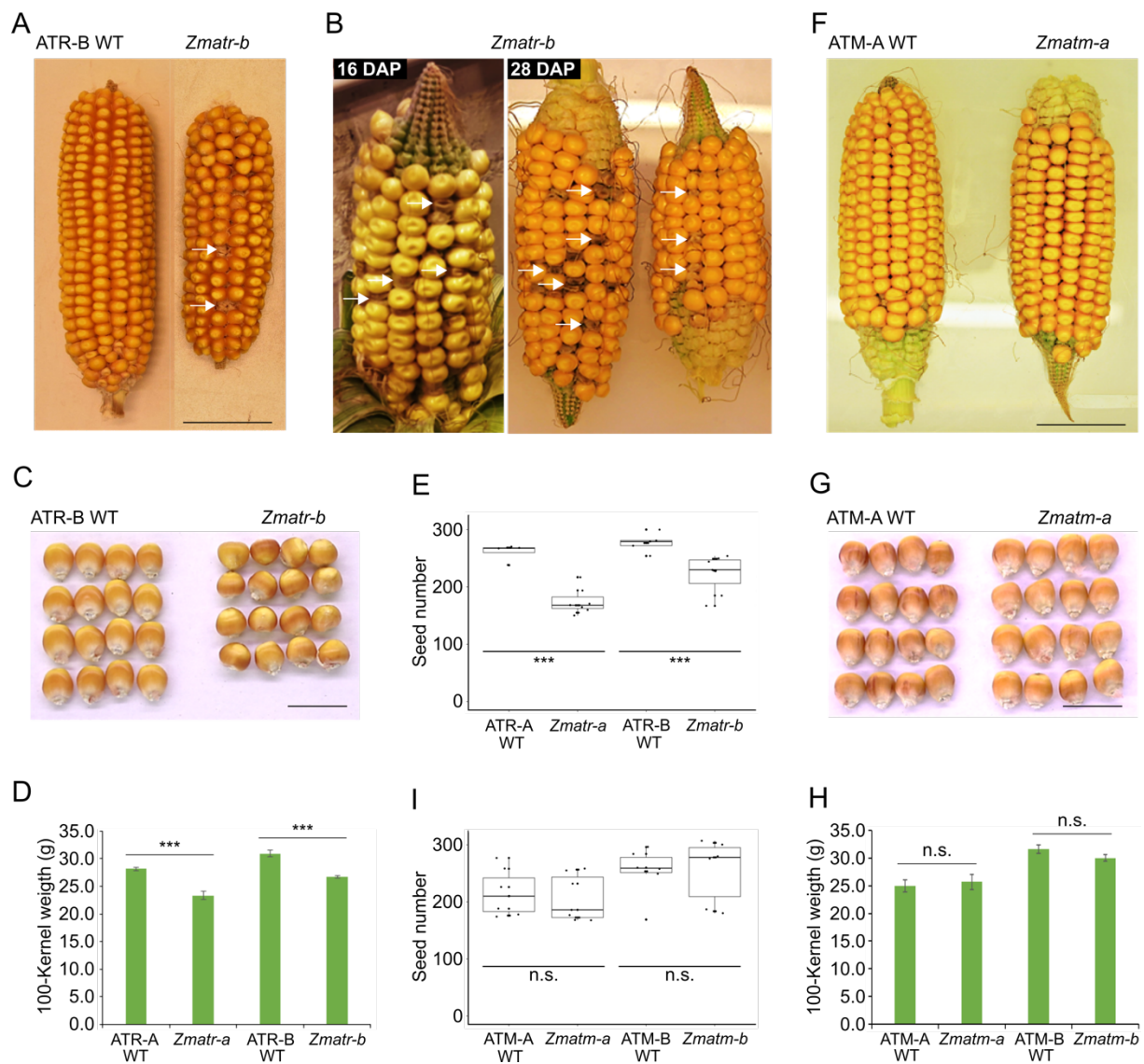


Figure 5. Phenotypic Cobs and Kernels Features of *Zmatr* and *Zmatm* Mutant Plants

(A) Representative mature F₂ ear of wild type (WT) and *Zmatr-b*. Arrows indicate gaps left by early death of kernels. Scale bar = 4 cm.

(B) Representative F₂ ears of *Zmatr-b* at 16 and 28 days after pollination (DAP). Arrows indicate kernels with abnormal phenotype that eventually die.

(C) Representative mature kernels of WT and *Zmatr-b* from a segregated F₂ ear. Scale bar = 1.5 cm.

(D) Comparison of the 100-kernel weight of mature WT and *Zmatr* kernels of two independent lines. Weight was determined in a segregated F₂ population. Values are means \pm SE; n=8-12 (***, P<0.001, Student's *t*-test).

(E) Number of kernels per cob in WT and *Zmatr* plants of two independent lines. Values are means \pm SE; n=7-10 (***, P<0.001, Student's *t*-test).

(F) Representative mature F₂ ear of WT and *Zmatm-a*. Scale bar = 4 cm.

(G) Representative mature kernels of WT and *Zmatm-a* from a segregated F₂ ear. Scale bar = 1.5 cm.

(H) Comparison of the 100-kernel weight of mature WT and *Zmatm* kernels of two independent lines. Weight was determined in a segregated F₂ population. Values are means \pm SE; n=8-9 (n.s., not significant; Student's *t*-test).

(I) Number of kernels per cob in WT and *Zmatm* plants of two independent lines. Values are means \pm SE; n=6-7 (n.s., not significant; Student's *t*-test).

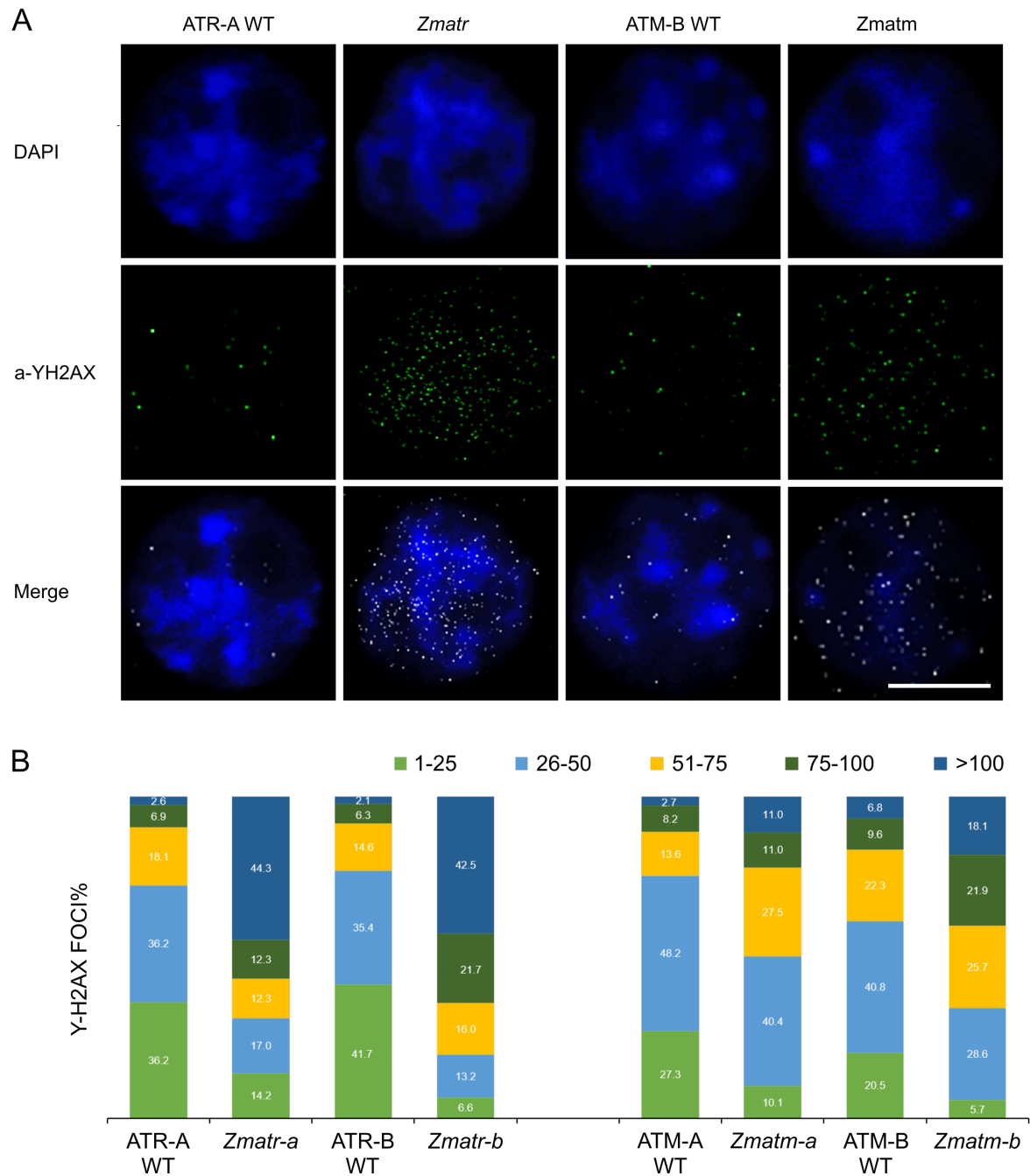


Figure 6. Detection of γ H2AX Foci in *Zmatr* and *Zmatm* Mutant Embryos.

(A) Immunostaining of γ H2AX foci accumulation (green) in nuclei stained with DAPI (blue) of ATR wild-type (WT), *Zmatr*, ATM-WT, and *Zmatm* embryos at 16 days after pollination (DAP). A representative nucleus is shown for each line. Scale bar = 5 μ m.

(B) Quantification of γ H2AX foci in ATR-WT, *Zmatr*, ATM-WT, and *Zmatm* embryos at 16 DAP. For each sample, the γ H2AX foci of 100 nuclei were counted and grouped into five categories: 1-25, 26-50, 51-75, 76-100, and more than 100 foci per nucleus. Two independent lines were analyzed.

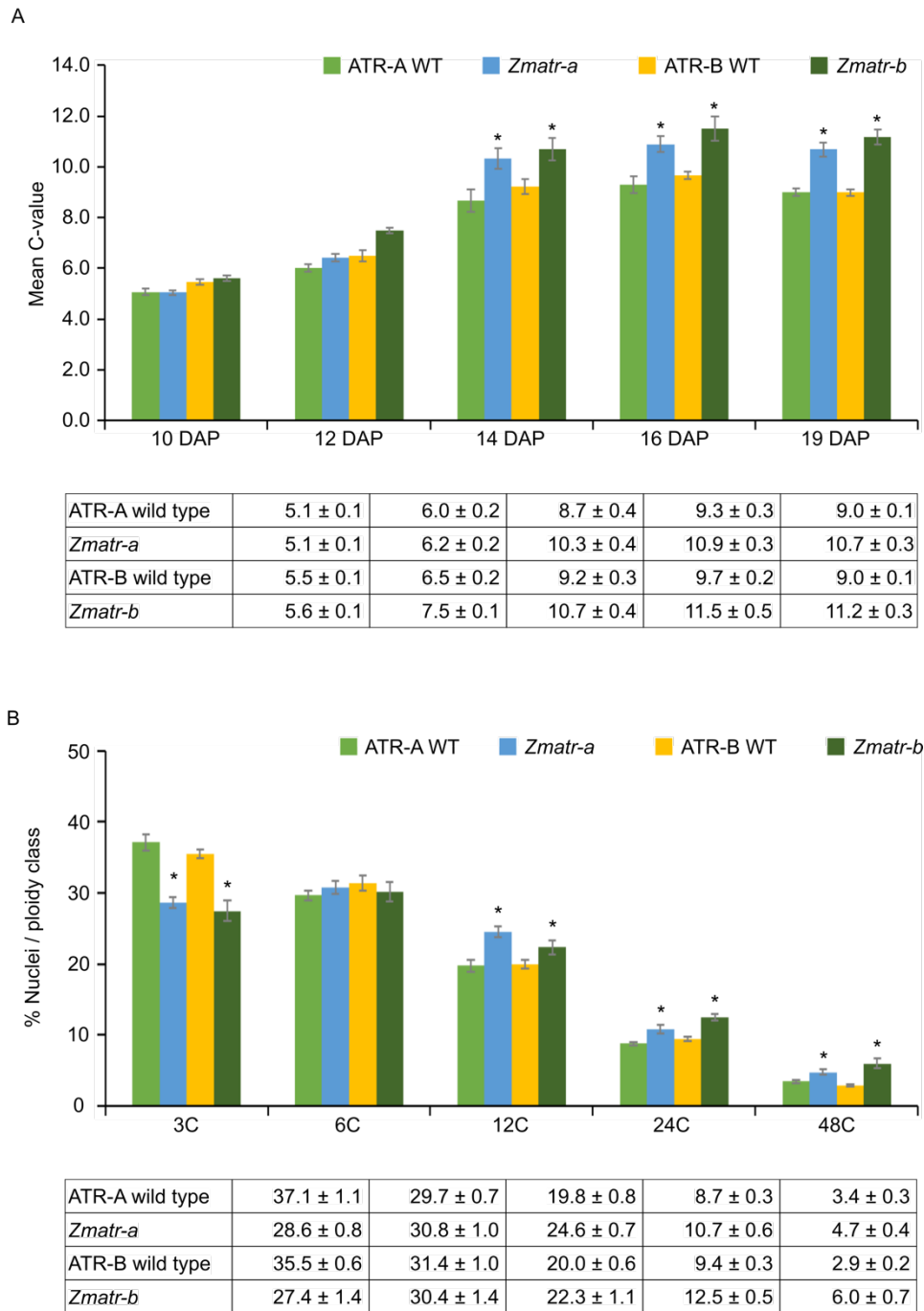


Figure 7. Premature Endocycle Onset in *Zmatr* Endosperm.

(A) The mean C-values in ATR-WT and *Zmatr* endosperm at five developmental stages between 10 and 19 days after pollination (DAP).

(B) Distribution of endosperm nuclei (expressed as a percentage of the total number of nuclei) among different ploidy classes in 19-DAP ATR-WT and *Zmatr* endosperms. Values are means ± SE; n=9-11 (*, $P < 0.001$, Student's *t*-test). All analyses were carried out from at least three kernels of three independent cobs for each genotype.

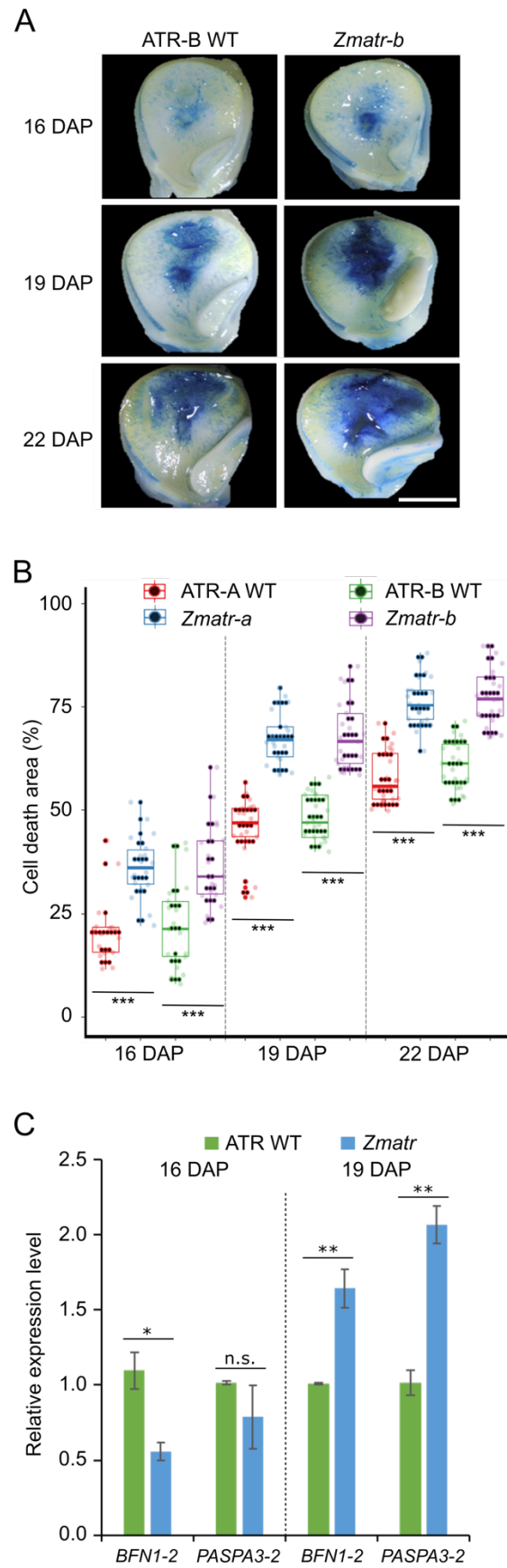


Figure 8. Lack of Maize ATR Results in Enhanced Cell Death in Endosperm.

(A) Progression of endosperm cell death in ATR-B wild-type (WT) and *Zmatr-b* kernels at indicated days after pollination (DAP), as indicated by Evans Blue staining. Dead cells are dark-stained. Scale bar = 3 mm.

(B) Quantification of the percentage of cell death within the endosperm area in ATR-A and ATR-B WT, and *Zmatr-a* and *Zmatr-b* mutants. All analyses carried out from at least seven kernels of three independent cobs for each genotype. Values are means \pm SE; n=21 (***, $P < 0.001$, Student's *t* test).

(C) Expression levels of the *BFN1-2* and *PASPA3-2* transcripts in ATR-B WT and *Zmatr-b* endosperms at 16 and 19 DAP. RNA was extracted of an endosperm pool extracted from at least three kernels of three cobs for each genotype, and the expression levels were measure by quantitative RT-qPCR. Each bar shows the mean \pm SD (** $P < 0.05$; ** $P < 0.01$, Student's *t*-test) of three biological replicates.

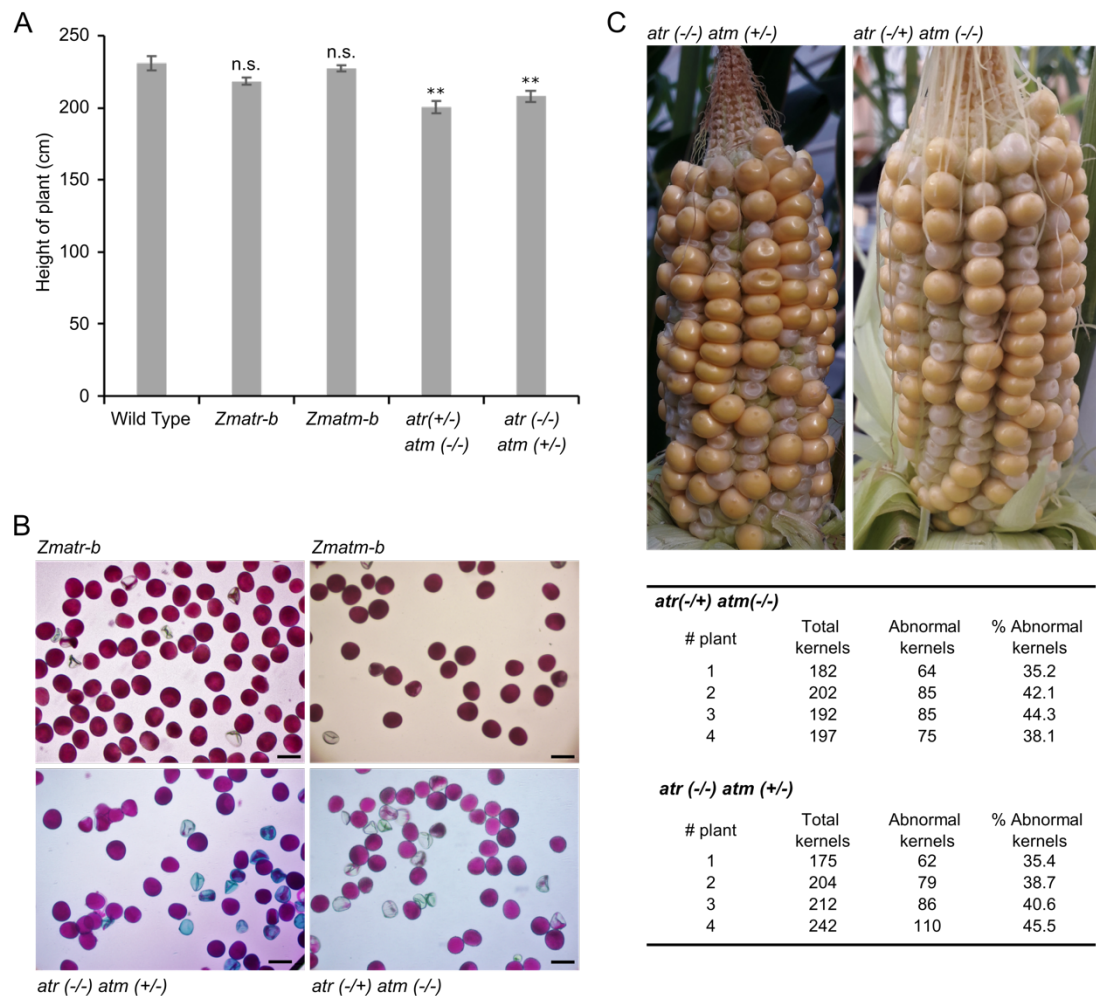
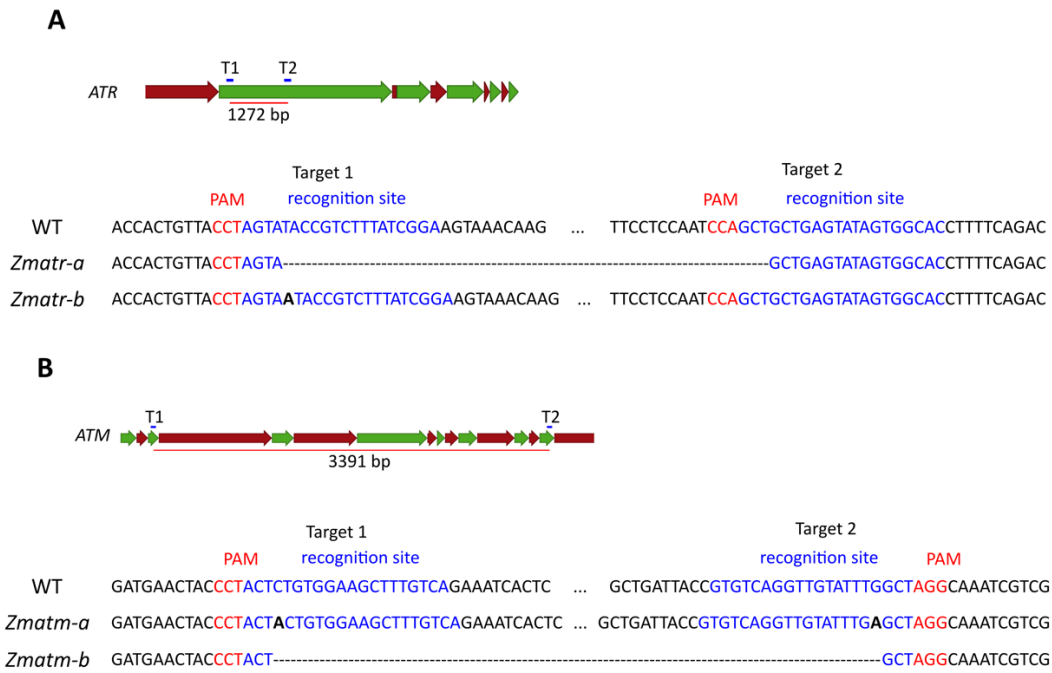


Figure 9. Vegetative and Reproductive Phenotypes of *atm* (+/-) *atr* (-/-) and *atm* (-/-) *atr* (+/-) Sesquimutant Plants.

(A) Height of plants (cm) of wild type, *Zmatr-b* and *Zmatm-b* single mutants, and *atm* (+/-) *atr* (-/-) and *atm* (-/-) *atr* (+/-) sesquimutants. Height was measured from the soil up to the top tassel. Values are means \pm SE; n=21 (**, $P < 0.005$, Student's *t*-test; n.s = no significant).

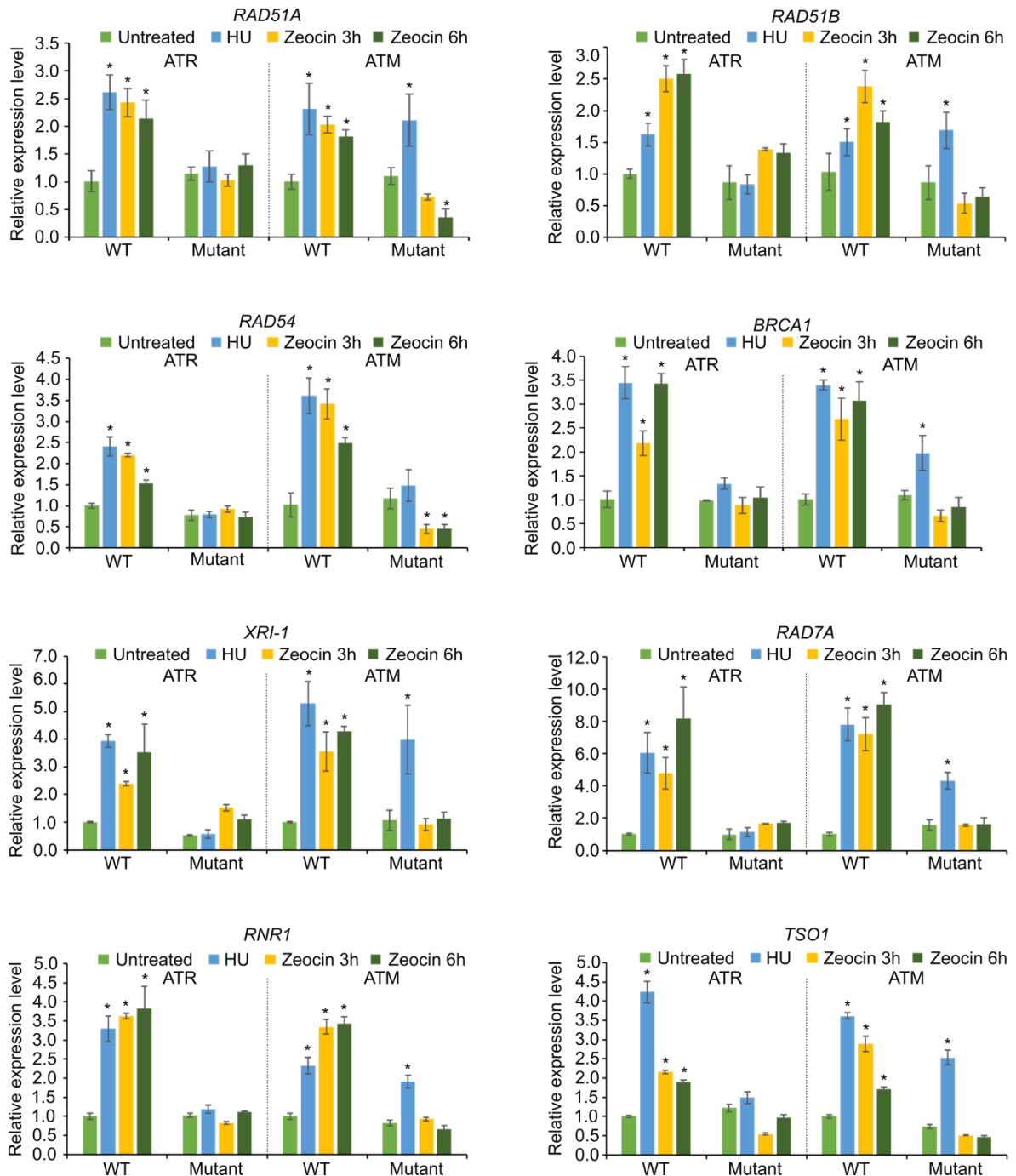
(B) Representative pollen of *Zmatr-b* and *Zmatm-b* single mutants, and *atm* (+/-) *atr* (-/-) and *atm* (-/-) *atr* (+/-) sesquimutants. Viability was visualized by Alexander staining. Scale bars = 50 μ m.

(C) Cob phenotype of the *atm* (+/-) *atr* (-/-) and *atm* (-/-) *atr* (+/-) sesquimutants at 19 DAP (top panel). Percentage of abnormal kernels per cob of *atm* (+/-) *atr* (-/-) and *atm* (-/-) *atr* (+/-) sesquimutants (lower panel).



Supplemental Figure 1. Map of CRISPR/Cas9 Mutations (Supports Figures 1 and 2).

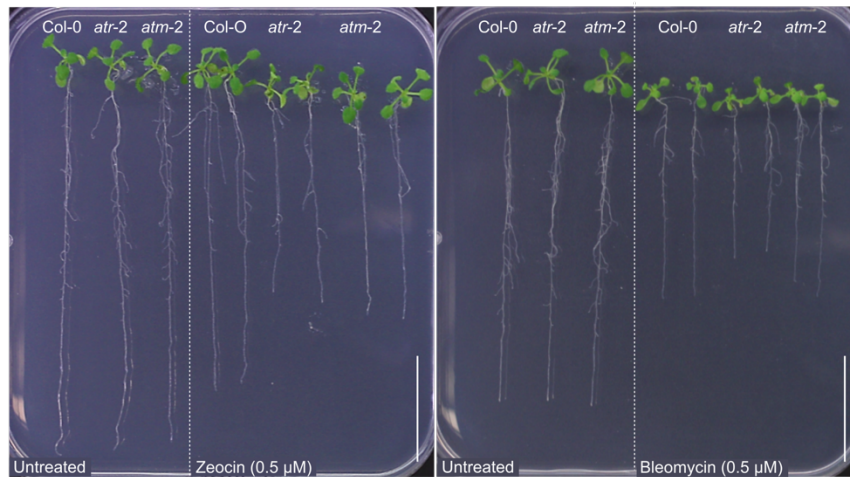
Representation of the loci in *ZmATR* (A) and *ZmATM* (B) targeted by the CRISPR/Cas9 construct. Exons are depicted by green arrows, non-coding regions such as introns and untranslated region are depicted by red arrows, gRNA targets are shown as T1 (target 1) and T2 (target 2) above the genes. Red line with number of base pairs underneath the locus indicates the size of deletion if two cuts happen simultaneously. Mutations detected through Sanger sequencing of a fragment PCR-amplified from gDNA. Letters in blue match the gRNA recognition site, letters in red are the PAM sequence. Dashes indicate deletions, bold black nucleotides indicate insertions.



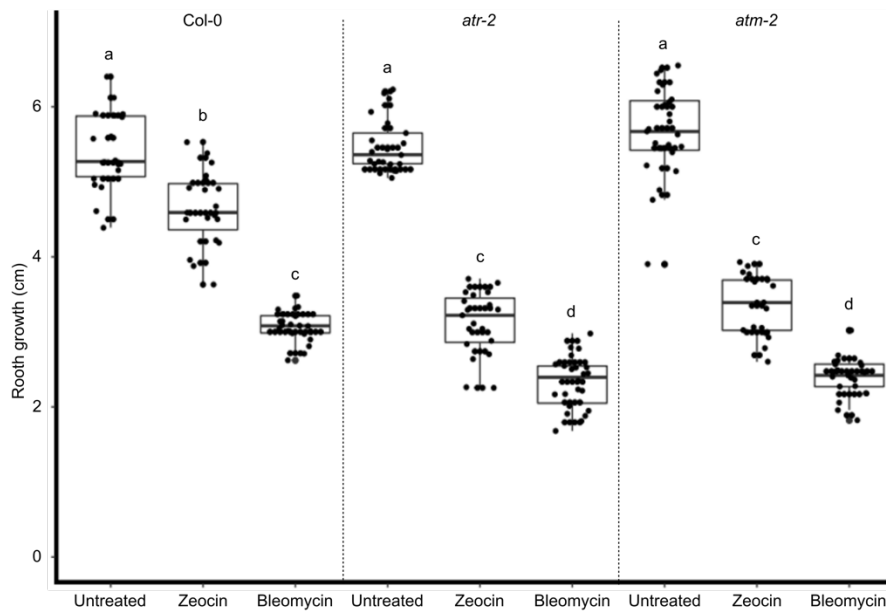
Supplemental Figure 2. Early Transcriptional Response in *Zmatr-b* and *Zmatm-a* Root Tips Treated with HU or Zeocin (Supports Figures 1 and 2).

ATR-B wild type (WT), ATM-A WT, *Zmatr-b*, and *Zmatm-a* seedlings were grown hydroponically for 3 days using the paper roll system. Subsequently, the roots were submerged in 0.5x MS liquid medium (untreated) or supplemented for 90 min with 5 mM HU or 3 h and 6 h with 150 μ M zeocin. RNA was extracted from ten root tips (1-2 mm) and RT-qPCR was performed in cDNA using three biological and three technical repeats. Values are means \pm SE; n = 3 (*P < 0.005, Student's *t*-test).

A

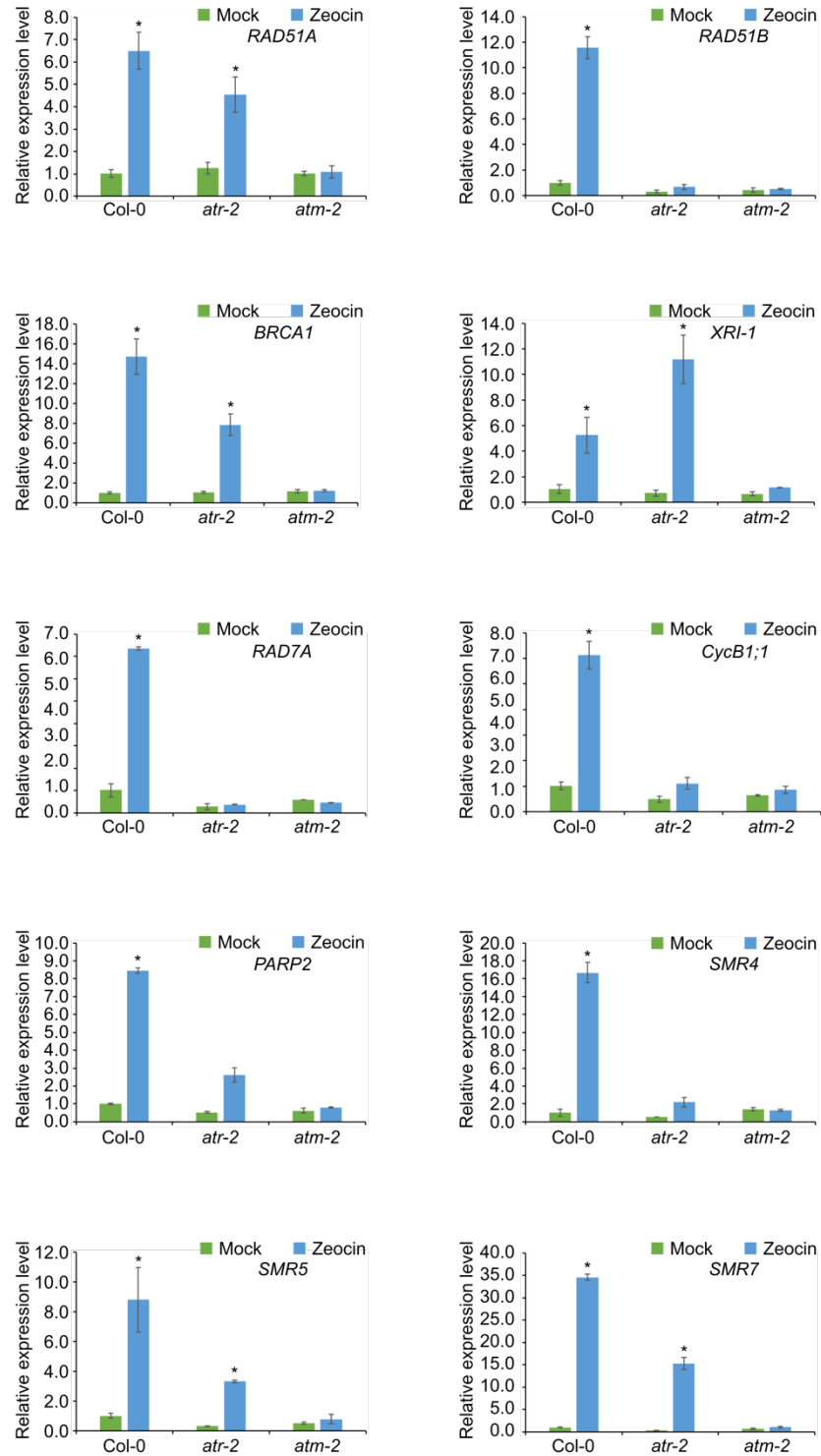


B



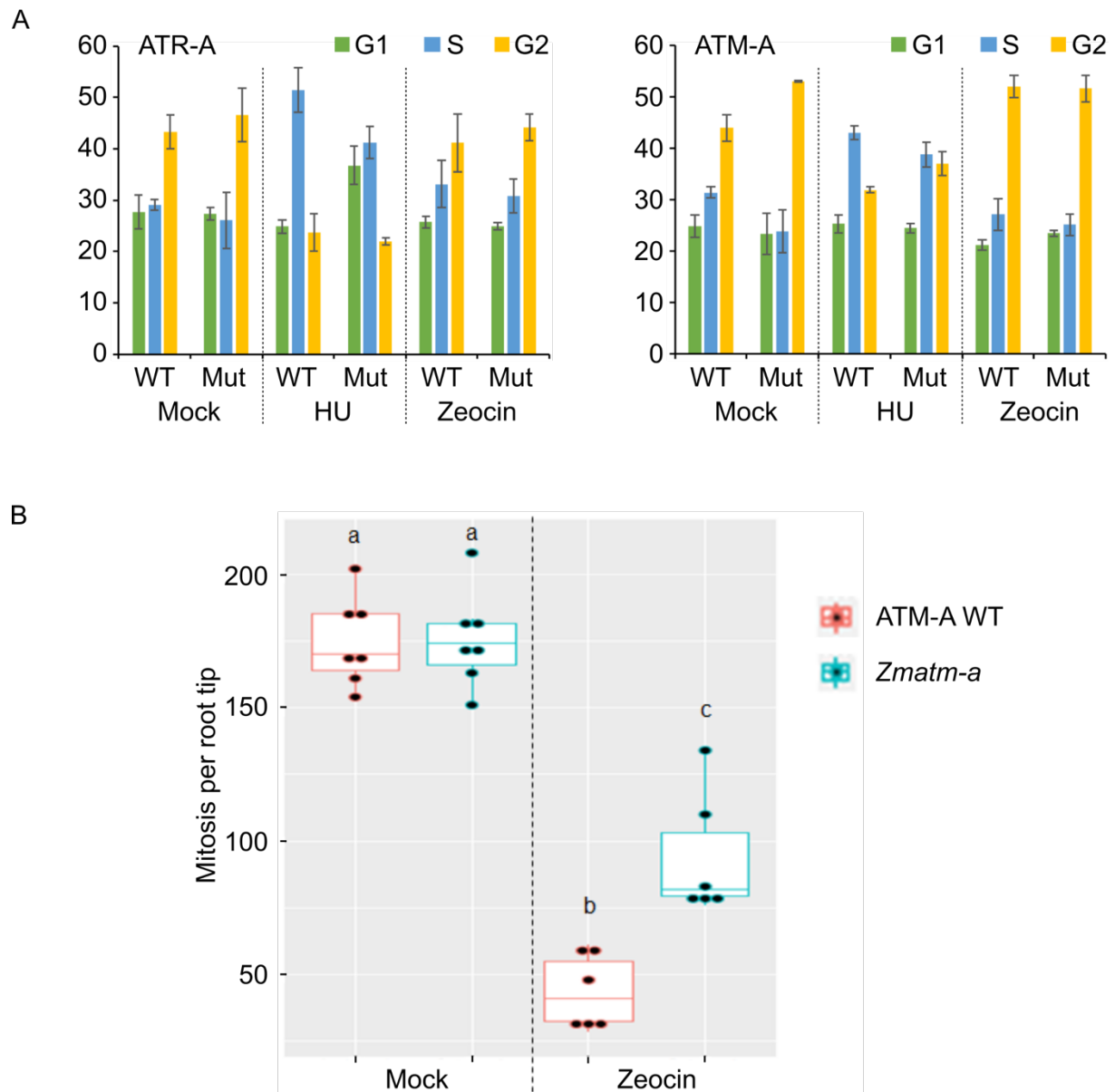
Supplemental Figure 3. *Atatr* and *Atatm* Mutants Display Sensitivity to Zeocin and Bleomycin, Both DSB Inducers (Supports Figure 2).

(A) Representative seedlings of untreated and treated plants. Scale bars = 3.0 cm. (B) Average root length was measured after 10 days ($n \geq 20$ roots for each treatment). Values are average \pm SE. Different letters indicate significantly different values (Student's t -test, $P < 0.05$). Data are representative for two independent experiments.



Supplemental Figure 4. Early Transcriptional Response in *Atatr* and *Atatm* root Tips Treated with Zeocin (Supports Figure 2).

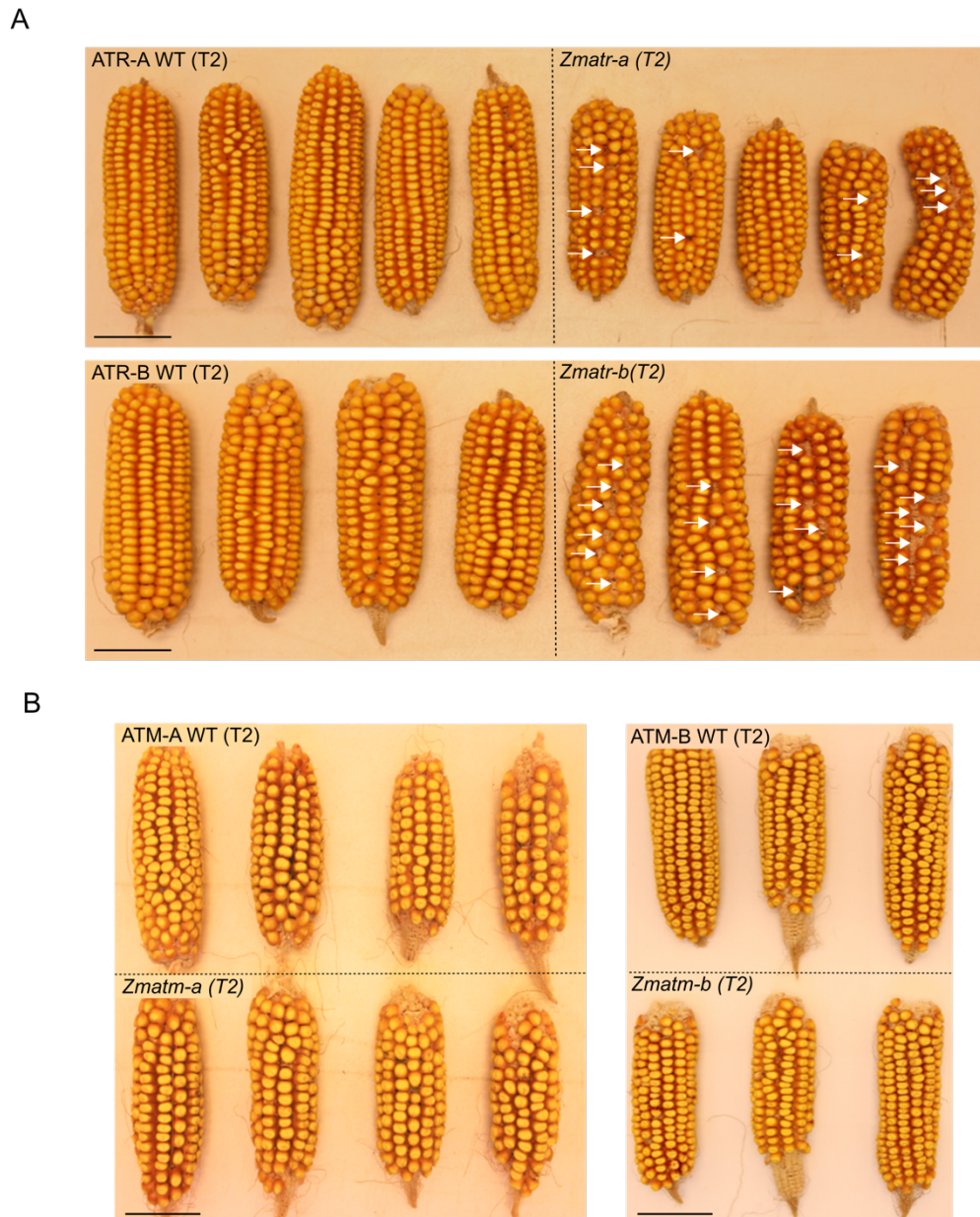
Wild type (*Col-0*), *Atatr-2*, and *Atatm-2* seedlings were grown for 5 days on 0.5x MS and transferred to genotoxin-supplemented medium (20 μ M zeocin) for 6 h. RNA was extracted from ~200 root tips (1-2 mm) for RT-qPCR analysis using three biological and three technical repeats. Values are means \pm SE; n = 3 (*P<0.005, Student's *t*-test).



Supplemental Figure 5. *Zmatr* and *Zmatm* Root Tips Display Impaired Checkpoint Activation in Response to DNA Stress (Supports Figure 4).

(A) Flow cytometry analysis of untreated (mock) and treated root tips of ATR-A wild type (WT), *Zmatr-a* (Mut), ATM-A WT and *Zmatm-a* seedlings. Seedlings were grown hydroponically for 3 days using the paper roll system, after which the roots were treated with 5 mM HU or 150 μ M zeocin for 24 h. The proportion of nuclei in G1, S, and G2 is indicated in the graphs. Data are representative of ten independent observations. Values are means \pm SE; $n = 3$.

(B) Number of cells in mitosis per root tip in untreated and treated (150 μ M zeocin for 24 h) ATM-A WT and *Zmatm-a* seedlings. Values are means \pm SE; $n=6-7$ (* $P < 0.001$, Student's t -test).

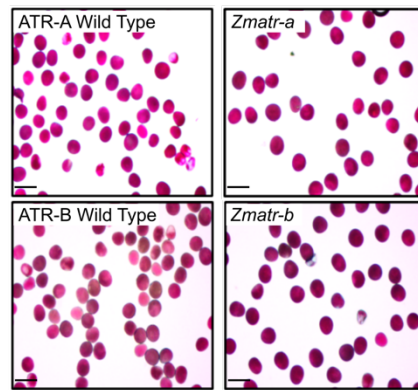


Supplemental Figure 6. Cob Phenotype of Wild-Type and *ZmATR* and *ZmATM* Mutant Plants (Supports Figure 5).

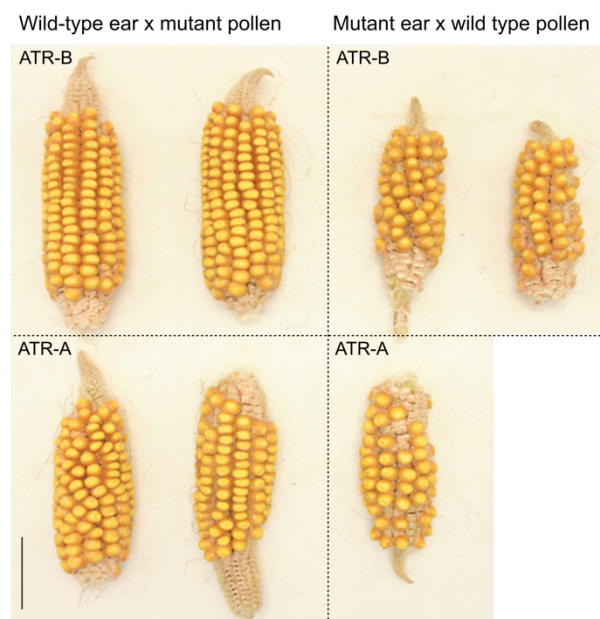
(A) Representative F₂ cobs of ATR-A and ATR-B wild-type plants, and *Zmatr-a* and *Zmatr-b* mutants. Arrows indicate gaps left by early death of kernels. Scale bars = 4 cm.

(B) Representative F₂ cobs of ATM-A and ATM-B wild-type plants, and *Zmatm-a* and *Zmatm-b* mutants. Scale bars = 4 cm.

A



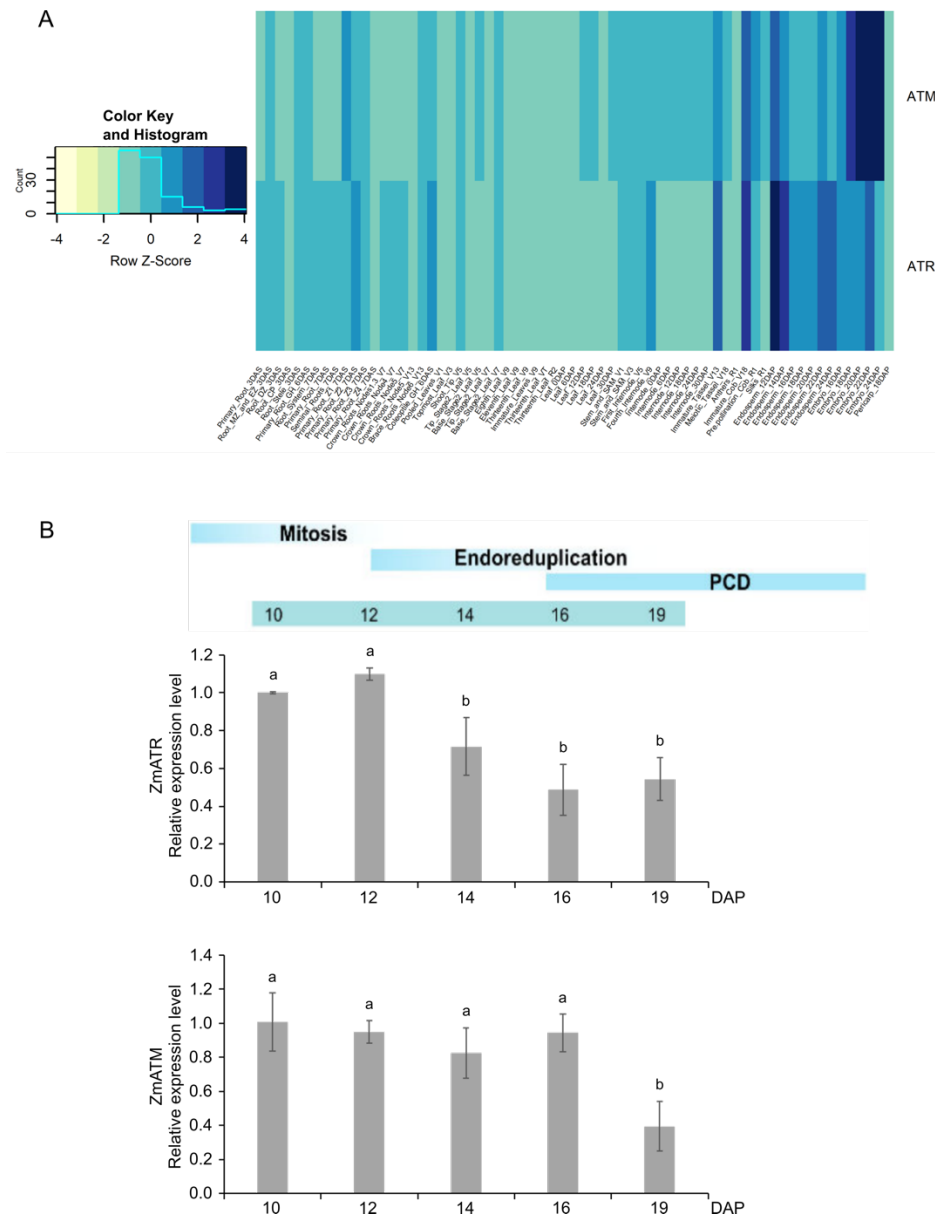
B



Supplemental Figure 7. Pollen Viability of *Zmatr* Is Not Affected, but Reciprocal Crosses Indicate a Maternal Defect (Supports Figure 5).

(A) Alexander staining on ATR wild-type and *Zmatr* pollen. Scale bars = 50 μ m.

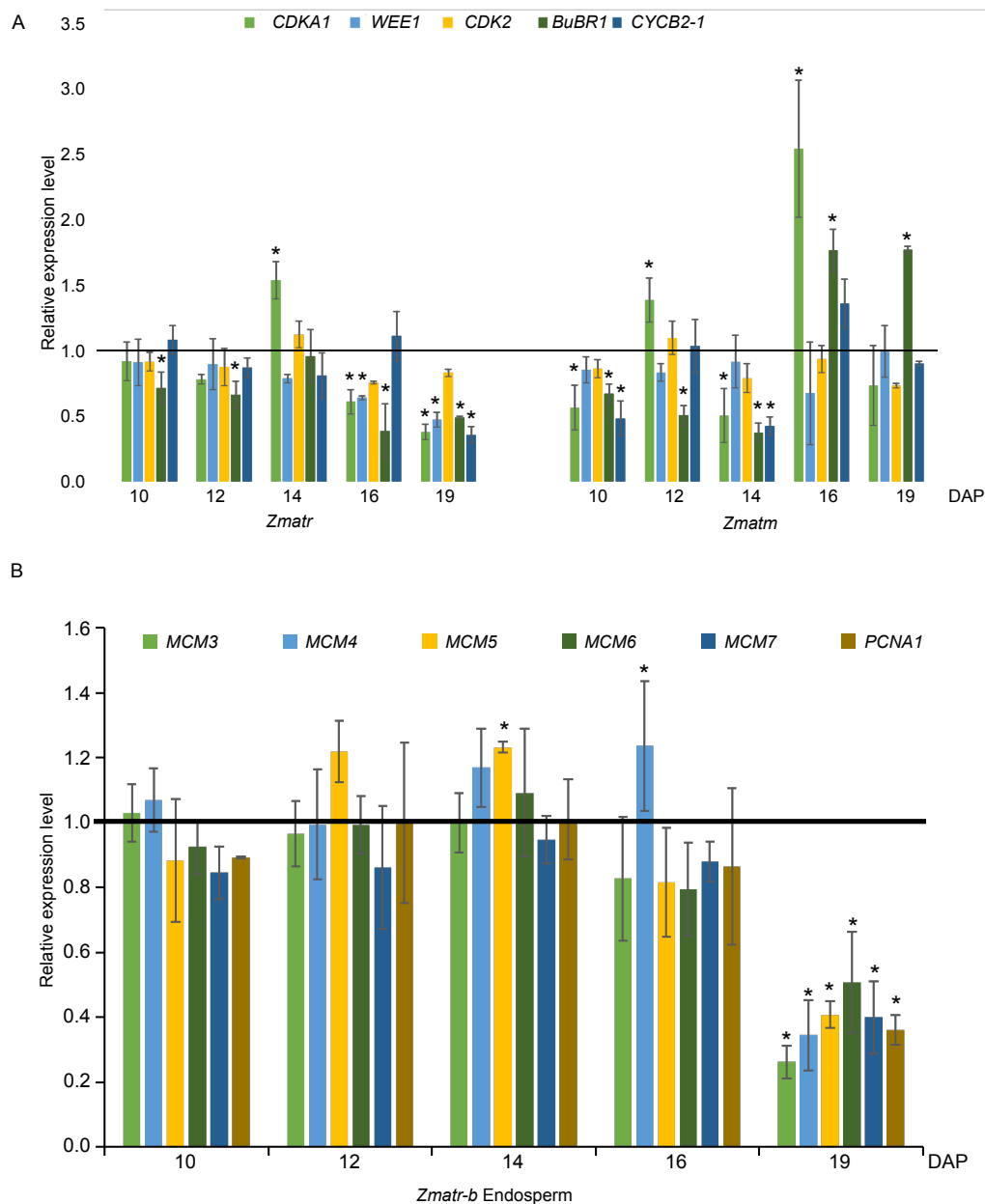
(B) Mature ears of reciprocal crosses. Scale bar = 4 cm.



Supplemental Figure 8. Expression Levels of *ZmATR* and *ZmATM* in Different Tissues and During the Endosperm Development in Wild-Type Plants (Supports Figures 5 and 6).

(A) Heatmap of expression levels for the *ZmATR* and *ZmATM* genes in different tissues in wild-type plants. Expression values were extracted from Stelpflug et al. (2016) and were normalized per row to obtain Z-scores. Heat map was generated by scaling Z-scores per sample.

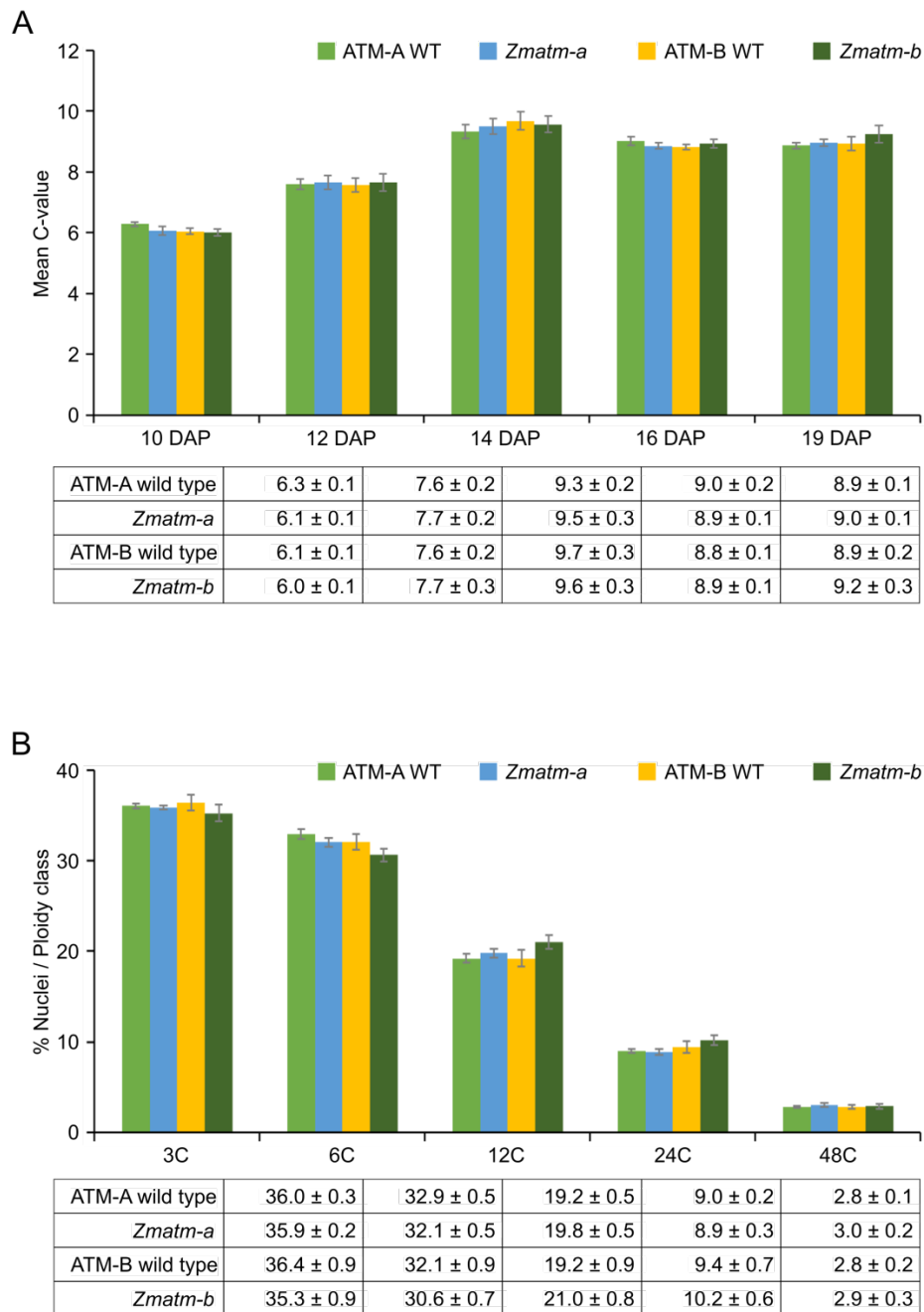
(B) Expression levels of *ZmATR* and *ZmATM* in wild-type endosperm. RNA was extracted at the indicated days after pollination (DAP) of an endosperm pool extracted of at least three kernels of three cobs, and mRNAs were measured by RT-qPCR. Each bar shows the mean \pm SD of three biological replicates. Different letters indicate significantly different values. ($p < 0.001$, mixed model analysis, Tukey correction for multiple testing).



Supplemental Figure 9. Expression Levels of Genes Associated with Cell Cycle Regulation and Replication Initiation During the Development of *Zmatr* and/or *Zmatm* Endosperms (Supports Figure 5 and 6).

(A) Expression levels of cell cycle regulation-related genes in *Zmatr-b* and *Zmatm-a* endosperm.

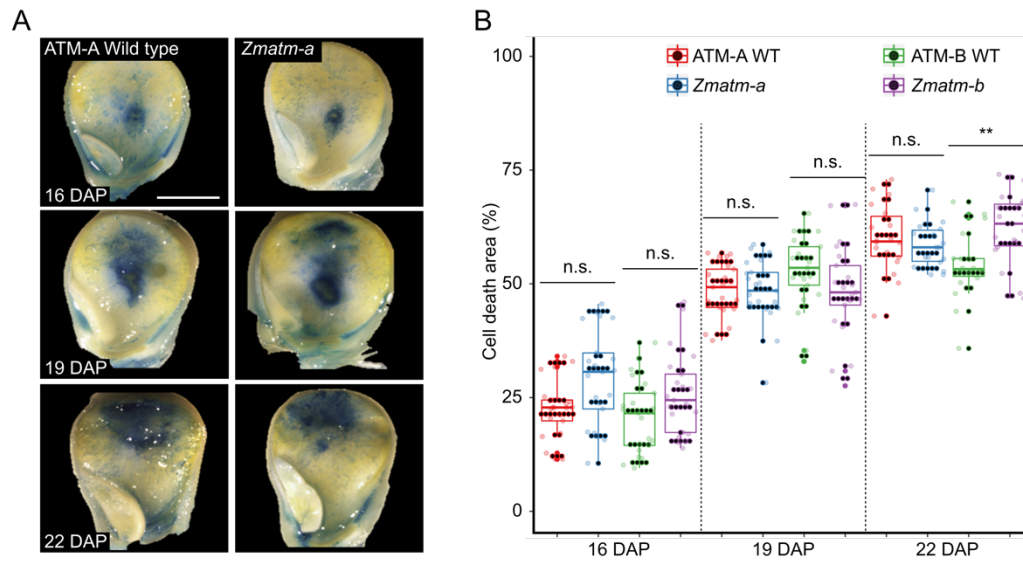
(B) Expression levels of replication initiation-related genes in *Zmatr-b* endosperm. RNA was extracted from an endosperm pool extracted from at least three kernels of three cobs for each genotype, and analyzed by RT-qPCR. The horizontal black line indicates the reference expression level of one unit in control samples (respective to wild-type sample of same genotype and time). Each bar shows the mean \pm SD of three biological replicates (*, $P < 0.001$, Student's *t*-test).



Supplemental Figure 10. Endoreduplication Is Not Affected in *Zmatm* Endosperm (Supports Figure 7).

(A) The mean C-value in wild-type (WT) ATM and mutant *Zmatm* endosperm at five developmental stages between 10 and 19 days after pollination (DAP).

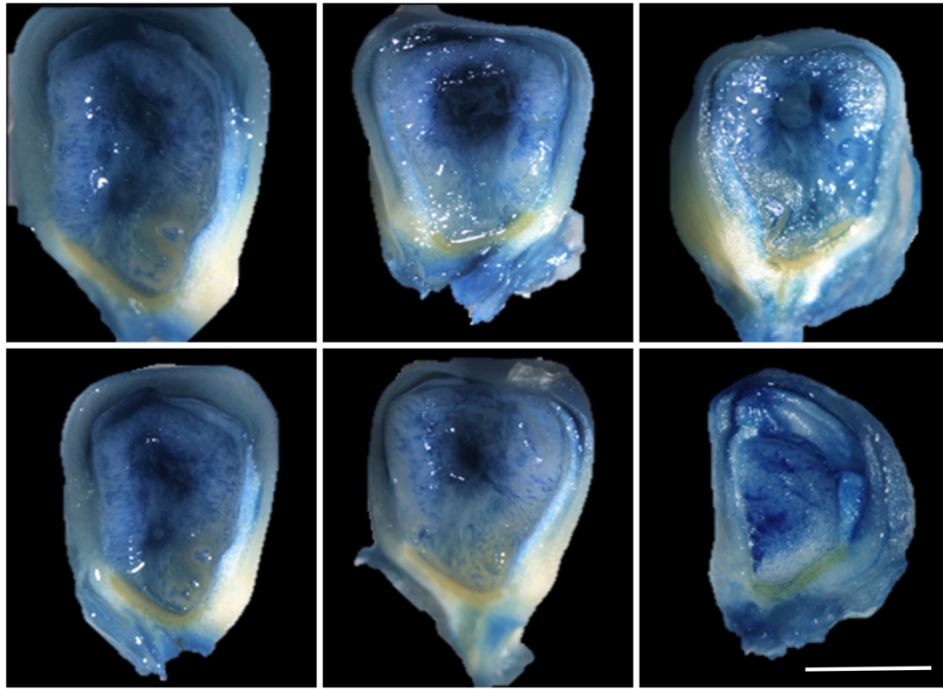
(B) Distribution of endosperm nuclei (expressed as a percentage of the total number of nuclei) among different ploidy classes in 19-DAP ATM-WT and *Zmatm* endosperms. Values are means ± SE; n=9-11, although no significant differences were found ($P < 0.001$, Student's *t*-test). All analyses were carried out from at least three kernels of three independent cobs for each genotype.



Supplemental Figure 11. *Zmatm* Endosperm Does Not Display Differences in Cell Death Timing or Abundance (Supports Figure 8).

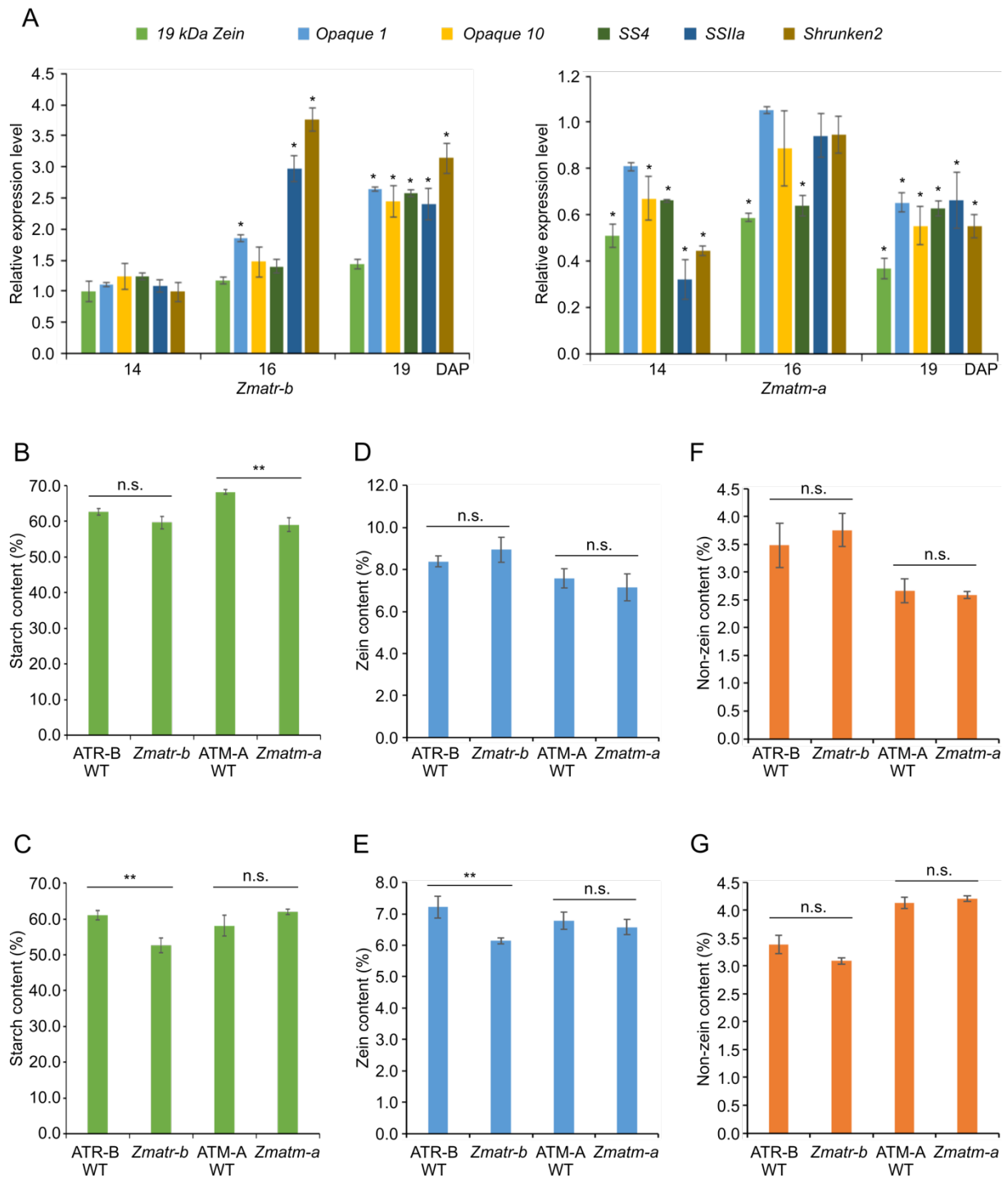
(A) Progression of endosperm cell death in ATM-A wild-type (WT) and *Zmatm-a* kernels as indicated by Evans Blue stain at indicated days after pollination (DAP). Scale bar = 3 mm.

(B) Quantification of the percentage of cell death within the endosperm area in ATM-A and ATM-B WT, and *Zmatm-a* and *Zmatm-b* mutants. All analyses were carried out from at least seven kernels of three independent cobs for each genotype. Values are means \pm SE; $n=21$ (**, $P<0.005$, Student's *t*-test; n.s = no significant).



Supplemental Figure 12. Cell Death in Endosperm of *Zmatr* Aborted Kernels (Supports Figure 8).

Endosperm cell death in *Zmatr* abnormal kernels at 16 days after pollination, as indicated by Evans Blue stain. Scale bar = 3 mm.



Supplemental Figure 13. Starch and Protein Content in *Zmatr* and *Zmatm* Endosperm (Supports Figures 5, 7, and 8).

(A) Expression levels of genes associated with the zein and starch biosynthesis in endosperms of *Zmatr-b* and *Zmatm-a* at indicated days after pollination (DAP). RNA was extracted from an endosperm pool extracted from at least three kernels of three cobs for each genotype for RT-qPCR analysis. The horizontal black line indicates the reference expression level of one unit in control samples (respective

to wild-type sample of same genotype and time). Each bar shows the mean \pm SD (* $P < 0.05$, Student's t -test) of three biological replicates.

(B and C) Starch contents in *Zmatr* and *Zmatm* endosperm at 19 DAP (B) and in mature endosperm (C). Percentage represents mg per 100 mg of endosperm flour for each genotype. Values are means \pm SE; $n = 3$ (**, $P < 0.005$, Student's t -test; n.s = no significant).

(D-G) Zein (D, E) and non-zein (F, G) protein content (%) in *Zmatr* and *Zmatm* endosperm at 20 DAP (D and F) and in mature endosperm (E and G). Values are means \pm SE; $n = 3$ (**, $P < 0.005$, Student's t -test; n.s = not significant).

Supplemental Table 1. Percentage of Abnormal Kernels in *Zmatr* Cobs.

Line	Plant	Total kernels	# abnormal kernels	% abnormal kernels
<i>Zmatr-a</i>	1	254	15	5.9
	2	230	22	9.6
	3	185	12	6.5
	4	244	13	5.3
	5	210	36	17.1
<i>Zmatr-b</i>	1	165	14	8.5
	2	180	9	5.0
	3	194	12	6.2
	4	171	28	16.4
ATR-A WT	1	386	1	0.3
	2	335	0	0.0
	3	376	0	0.0
	4	281	2	0.7
	5	300	0	0.0
ATR-B WT	1	372	1	0.3
	2	352	2	0.6
	3	328	0	0.0
	4	270	0	0.0

Supplemental Table 2. Primers Used in This Study

CRISPR/Cas9 construct		qRT primers (sequence 5' to 3')	
Gene ID	Primer name	Forward	Reverse
	BAR-qRT	TGTCTCGATGTAGTGGTTGACG	ACACGCTGAAATCACCAGTC
	zCAS9-qRT	TAATGCTTGTGCTGCTCGAC	TGCCGTCCAAGTATGTGAAC

Genotyping of maize mutants		Primers (sequence 5' to 3')	
Gene ID	Gene name	Forward	Reverse
Zm00001d040166	ZmATM – target 1	GTAAGCTTCATGTGGAATGGGTC	AATGACCAGCAAAACAGGGAA
Zm00001d040166	ZmATM – target 2	ACATGTTCTATCCTCCAAGTACTG	ACACGAGATGGACTGGCCT
Zm00001d014813	ZmATR – target 1	CTGGAAGAGTGCAGAGGATATCC	GATTGCTCTGCACATCCTTTTAG
Zm00001d014813	ZmATR – target 2	CCTAAAGTTACTCTAGCAAGGGC	GGCGATCATGCCTTGAAGGT

qPCR for maize		qRT primers (sequence 5' to 3')	
Gene ID	Primer name	Forward	Reverse
Zm00001d036904	ZmEF-a-qRT	AGTCCGTTGAGATGCACCATG	CACATACCCACGCTTCAGATCC
AF16884.1	Zm18s rRNA-qRT	ACCTTACCAGCCCTTGACATATG	GACTTGACCAACATCTCAGCAC
Zm00001d014813	ZmATR-qRT	TCAAGCCTACAGGATCAGCT	ACTCCATGCATGCACCAAG
Zm00001d040166	ZmATM-qRT	GATGTACAGGAGTTTCATGCTTC	CGCAGATAGGTCATTCTACG
Zm00001d021898	ZmRAD51A-qRT	ACGCAGAGGGTACATTCAGACC	CAGCACCATTTCAGTCCAAACCTG
Zm00001d041757	ZmRAD51B-qRT	GTGCTAGCCAACTTCATGCA	GAGTGTGGCACAACCTGAGTC
Zm00001d018151	ZmRAD54-qRT	TGTTCTGCTGGCGAAGAGGTTTC	TGAGCTGCTGATCGCACCATAG
Zm00001d038667	ZmBRCA1-qRT	CTCGCAAAAATAGCTGGTGTGC	AGAAACTTCAGTGTGCGCTTGC
Zm00001d042357	ZmXRI1-qRT	GCAGACGCATGTTGCAGT	GTGAGCAATCGACTAGCCA
Zm00001d050104	ZmRAD7A-qRT	GAAATGCTGCACCAACACG	TGCTTGCAATTGGCAGGATC
Zm00001d045192	ZmRNR1-qRT	ACTCATGCTTCACCGACG	GACACCAATTCTCCAGCAG
Zm00001d003164	ZmTSO2-qRT	AGAAGGCGTCCGTAATGTCCAG	AACTCAGAAGTCCTCGTCGATGC
Zm00001d027373	ZmCDKA1-qRT	CTTCGAGTACCTGGATCTGGA	CGCAACACCGTGGAGTATC
Zm00001d053998	ZmWEE1-qRT	GACTTCCTGAGCCACAATCTATG	GTCGTCGTCGAACTCGTTG
Zm00001d002662	ZmCyclinB2_1-qRT	AAGCTGCAGATGCAGATAAACAGC	GCTGGCAACGATTGATAGCACAC
Zm00001d015863	ZmBUBR1-qRT	TCAAAGTGTGGCTGGAATACGC	ACTCCAGCAACGATGCGTAAGAC

Zm00001d048497	ZmCDK2-qRT	CACGGCAACATCGTCAGGTAC	TCTTAGCAAACCTCCGGGCAAGAG
Zm00001d032501	ZmBFN1-qRT	ACAGGAGATACAACCTGACTGA	CGAGAGCCGTCTGTATGATCT
Zm00001d009594	ZmPASPA3-qRT	GCACTGGAATTCGTAGCGTA	GTAGGTCAGGATGAGCTCCT
Zm00001d009392	ZmMCM3-qRT	GTGCCATGGTTCTTGCTGATCGTGGTGT	GGAAAGCAGTGAATCCGGAAGCCCGATA
Zm00001d009374	ZmMCM4-qRT	GAGCTCGAGGTCTTGACTTGCAGACAC	TGCTTCGCTGAGACGGATCAAACCTCTCT
Zm00001d018384	ZmMCM5-qRT	AAAATGAGGCAGCAAGCTCACGAGACAG	ATCTTGCAGCATCAACGGTGGAGACATT
Zm00001d010406	ZmMCM6-qRT	ATGACCAAGCAGCGGATAATGG	ATGCCAGCTAAACCATCTCCATC
Zm00001d030614	ZmMCM7-qRT	GCAGAGCACGTACCAAAGGGCCACATTC	GCAACTAATCCTGCGCGCATCGCTCTAA
Zm00001d018415	ZmPCNA-qRT	CCATCGTCCGCATGCCTTCTTCTGAGTT	CAAAGGTCAGGGAAACCGGCTCTTGCAT
Zm00001d048851	Zm19kDa_zein-qRT	TTAGGTGGGACCCTATAGCCTTGC	ATGTGTCACACGTTTCCATTGGC
Zm00001d052110	ZmOpaque1-qRT	CAAAGTCAACACGTGGGCAAT	GCTGGGTGGGCACCAA
Zm00001d033654	ZmOpaque10-qRT	ATACCCTTGCTTGTGATG	GCTGAAGTCTGATGGCTC
Zm00001d010821	ZmSS4-qRT	ACCGGCGGTTTGAATGACAGTG	AGGTGAAGCCATTTTCGCACCTC
Zm00001d037234	ZmSSIIa-qRT	TGCTGTGCCACTCACTGTTTACC	TCAGCCCTAACGAGCAAAGGAC
Zm00001d044129	ZmShrunken2-qRT	TGGGAAGGTCCCAGTTGGAATAGG	TCAGCCTCTTGGATGCCCTTAC

qPCR for *Arabidopsis thaliana*

		qRT primers (sequence 5' to 3')	
Gene ID	Primer name	Forward	Reverse
AT1G02780	EMB2386_qRT	CTCTCGTTCCAGAGCTCGCAAAA	AAGAACACGCATCCTACGCATCC
AT3G22110	PAC1_qRT	TCTCTTTGCAGGATGGGACAAGC	AGACTGAGCCGCCTGATTGTTTG
AT3G56340	RPS26E_qRT	GACTTTCAAGCGCAGGAATGGTG	CCTTGTCTTGGGGCAACACTTT
AT5G20850	RAD51A-qRT	GACTCTTACAGATAGCTGACAGGT	GCGGTAGCACTATCGACAATC
AT2G28560	RAD51B-qRT	GAGCTCAGAGACAACCTCAGT	ATACTGACTAGTTTCATCGCGGT
AT4G21070	BRCA1-qRT	AGGTGAACCTGTCTCTGCGGATTT	TTCTCCGGCTTCTTGTCAACTCCA
AT5G48720	XRI1-qRT	GCTACCTGATGACTTAACTTTGGTTC	CATTTGGAGAAGATCGAGTCACAG
AT2G06040	RAD7A-qRT	CGATTCTTCGATCTCGCGAAAT	GTAGTGTGAGTGGGTTTCATCAAC
AT4G37490	CYCB1-1-qRT	GGAAGCAACAAGAAGAAGGGAG	AGGGATCAAAGCCACAGCG
AT2G31320	PARP2-qRT	AGCCTGAAGGCCCGGGTAACA	GCTGTCTCAGTTTTGGCTGCCG
AT5G02220	SMR4-qRT	GAAGGATGTACGACGCCGA	TCTTCGAGGCTGTGCGTAG
AT1G07500	SMR5-qRT	AAACTACGACGACGGAGATACG	GCTACCACCGAGAAGAACAAGT
AT3G27630	SMR7-qRT	GCCAAAACATCGATTCGGGCTTC	TCGCCGTGGGAGTGATACAAAT

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