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**Running head:** Role of DNA Pol θ in the repair of replication associated DNA damage

## ABSTRACT

Safeguard of genome integrity is a key process in all living organisms. Due to their sessile lifestyle, plants are particularly exposed to all kinds of stress conditions that could induce DNA damage. However, very few genes involved in the maintenance of genome integrity are indispensable to plants' viability. One remarkable exception is the *POLQ* gene that encodes DNA polymerase theta (Pol θ), a non-replicative polymerase involved in Trans-Lesion Synthesis (TLS) during DNA replication and Double-Strand Breaks (DSB) repair. The Arabidopsis *tebichi* (*teb*) mutants, deficient for Pol θ, have been reported to display severe developmental defects, leading to the conclusion that Pol θ is required for normal plant development. However, this essential role of Pol θ in plants is challenged by contradictory reports regarding the phenotypic defects of *teb* mutants, and the recent finding that rice null mutants develop normally. Here we show that the phenotype of *teb* mutants is highly variable. Taking advantage of hypomorphic mutants for the replicative DNA polymerase ε, that display constitutive replicative stress, we show that Pol θ allows maintenance of meristem activity when DNA replication is partially compromised. Furthermore, we found that the phenotype of Pol θ mutants can be aggravated by modifying their growth conditions, suggesting that environmental conditions impact the basal level of replicative stress, and providing evidence for a link between plants' response to adverse conditions, and mechanisms involved in the maintenance of genome integrity.

**Key words:** Genome stability, DNA replication, Pol θ, abiotic stress, plants

**Significance statement:** Pol θ is one of the few proteins involved in DNA repair that appears to be essential for plant development, but there are contradictory reports concerning the phenotype of Pol θ-deficient mutants. Here we show that Pol θ plays a key role in the repair of replication-associated DNA breaks, and that its requirement for plant development depends on growth conditions, providing evidence for a link between abiotic stress responses and the DNA Damage Response.

# INTRODUCTION

Organism's survival depends on the faithful transmission of genetic information. Due to their sessile lifestyle, plants cannot escape stress conditions with the potential to compromise their genome integrity. Indeed, because sunlight is the energy source of plants, they are constantly exposed to UV-radiations, that can cause DNA damage such as pyrimidine dimers. In addition, cellular metabolic activities such as photosynthesis lead to the production of Reactive Oxygen Species (Noctor and Foyer, 2016), that can induce DNA lesions, and whose production can be exacerbated by various biotic and abiotic stress conditions. In plants like in all eukaryotes, DNA lesions are recognized and trigger a signaling cascade called the DNA damage response (DDR) that leads to the activation of cell cycle checkpoints in proliferating cells, and specific DNA repair mechanisms (Ciccia and Elledge, 2010; Yoshiyama *et al.*, 2013; Hu *et al.*, 2016; Nisa *et al.*, 2019). Outcomes of DDR activation may be different depending on the severity of DNA damage and on the efficiency of the repair process: successful repair allows cell survival and resumption of the cell cycle, but if the damage is too severe, it may induce permanent cell proliferation arrest through endoreduplication (Adachi *et al.*, 2011) or even cell death (Fulcher and Sablowski, 2009). The cellular response also depends on the cell type, meristematic cells being more sensitive to DNA damage and more prone to undergo cell death than differentiated cells (Fulcher and Sablowski, 2009).

Because maintenance of genome integrity relies on its faithful duplication in proliferating cells, and its efficient repair in all cell types, DNA polymerases (Pol) play a pivotal role in this process (Burgers, 1998). In eukaryotes, DNA polymerases are distributed between replicative and non-replicative polymerases (Burgers, 1998), and classified into 4 families (A, B, X and Y), based on the primary structure of their catalytic subunit (Makarova and Koonin, 2013). The three replicative polymerases (DNA Pol α, δ and ϵ) belong to the B-family (Jain *et al.*, 2018) whereas non-replicative polymerases can be found in all families, and are involved in different DNA repair pathways. One distinctive feature of replicative polymerases is their tight catalytic sites that confers them a very low error rate (Kunkel, 2004). Consequently, their progression during DNA replication can be blocked by lesions that are too large to be accommodated in their catalytic site, such as bulky adducts or pyrimidine dimers. When replisome progression is prevented by a DNA lesion on the template strand, non-replicative polymerases that have a looser active site can

substitute for canonical replicative ones to perform Trans-Lesion Synthestis (TLS), a process by which they allow the replisome to progress beyond the DNA lesions (Kunkel, 2004; Yang and Gao, 2018). TLS polymerases are thought to associate into a huge complex with stalled replication forks, allowing the choice of the most appropriate one to bypass the lesion, depending on its nature (Powers and Washington, 2018). The high diversity of TLS polymerases likely stems from the fact that they have different so-called cognate lesions, opposite to which they are able to perform error-free DNA synthesis: each TLS polymerase can thus be recruited for efficient error-free bypass of specific lesions (Powers and Washington, 2018). Plant genomes encompass at least 9 non-replicative polymerases, 6 of which have been functionally characterized, and involved in TLS and/or DNA repair: Pol ζ, η, κ, θ, and λ and Reversionless1 (Rev1) (reviewed in (Pedroza-Garcia *et al.*, 2019; Sakamoto, 2019)). Deficiency in non-replicative polymerases usually does not affect overall development, but rather results in hypersensitivity to various DNA-damaging agents (Pedroza-Garcia *et al.*, 2019). One intriguing exception is Pol θ (encoded by the *POLQ* gene) also called TEBICHI in *Arabidopsis thaliana*: *teb* null mutants show severe developmental defects (Inagaki *et al.*, 2006; Inagaki *et al.*, 2009), suggesting that the cellular function of Pol θ is essential for proper development.

The knowledge about the molecular function of Pol θ in plants is scarce in comparison to other eukaryotes. In Human cells, Pol θ can perform error-prone TLS through UV-lesions. Its deficiency results in a dramatic increase of tumorigenesis upon UV exposure, indicating that this error-prone TLS is crucial to avoid collapse of stalled forks (Yoon *et al.*, 2019). Another key function of Pol θ is DSB repair through ALTernative Non-Homologous End Joining (Alt-NHEJ), also called Micro-homology Mediated End Joining (MMEJ) (Beagan and McVey, 2016). Alt-NHEJ is an error-prone pathway for DSB repair in which resection of DNA ends on each side of the break exposes micro-homology of only a few base pairs, that can allow annealing of single stranded DNA (ssDNA) and subsequent end-joining (Chiruvella *et al.*, 2013). Recently, Mateos-Gomez and colleagues demonstrated that through its helicase domain, Pol θ facilitates the displacement of RPA that normally protects resected ends and promotes homologous recombination (HR), thereby favoring Alt-NHEJ over HR (Mateos-Gomez *et al.*, 2017).

This dual role of Pol θ in DSB repair is likely a key factor of the cellular response to replicative (or replication) stress. Replicative stress is a complex phenomenon that arises when fork

progression is stopped or slowed-down. If the obstacle cannot be bypassed (for example through TLS), fork stalling triggers the accumulation of single stranded DNA coated by the replication protein RPA, leading to the activation of the ATR (ATM and Rad3-related) kinase and subsequent DDR signaling (reviewed in (Zeman and Cimprich, 2014)). Pol θ is thus assumed both to prevent replicative stress by avoiding fork stalling at DNA lesions, and to contribute to DNA repair when replicative stress results in fork collapse and subsequent DSB formation. Indeed, in Human cells, *POLQ* deficiency confers hypersensitivity to ATR inhibitors, providing evidence for the role of Pol θ for the repair of DNA replication-induced DNA damage (Wang *et al.*, 2019), and a synthetic lethal genetic screen revealed that various components of the DDR are indispensable to cell survival in the absence of Pol θ. The common feature of all mutations identified in the screen was that they caused accumulation of endogenous DNA damage, indicating that the most prominent role of Pol θ is the repair of replication-associated DSB, regardless of the initial cause of DNA damage (Feng *et al.*, 2019). There is thus accumulating evidence that Pol θ is key for repairing DSBs associated with fork collapse due to replication stress, via Alt-NHEJ (Wang et al., 2019; Kelso et al., 2019). Analysis of plant Pol θ mutants suggests that this dual role is conserved in plants. The *teb* mutants show constitutive activation of the DNA Damage Response (DDR), consistent with role of Pol θ in the maintenance of genome integrity (Inagaki *et al.*, 2006). They are more sensitive to UV, and to the DNA alkylating agent methylmethane sulfonate (MMS) (Inagaki et al., 2006), consistent with a TLS function. Furthermore, Pol θ-dependent Alt-NHEJ was identified as the pathway for T-DNA integration after transformation by agrobacterium (van Kregten *et al.*, 2016), although this finding has lately been questioned by the observation that T-DNA integration remains possible, albeit with a reduced efficiency, in Pol θ null mutants (Nishizawa-Yokoi *et al.*, 2020).

Two important questions still hold regarding the function of plant Pol θ. First, it is not clear whether the TLS or DSB repair function, or both can account for the fact that this protein is required for normal plant development, as most plant mutants deficient for TLS or DNA repair develop normally in the absence of genotoxic stress. Second, there are conflicting reports regarding the developmental defects caused by Pol θ deficiency, and it thus remains unclear to what extend it is indeed required for normal development. As mentioned above, several *teb* alleles (*teb1*, *teb2* and *teb5*) have been described in Arabidopsis and were all reported to display the same phenotypic

alterations including reduced growth, deformed leaves and disorganized root meristems (Inagaki *et al.*, 2006; Inagaki *et al.*, 2009). However, in *Physcomitrella patens*, *polq* mutants were deficient for DSB repair, but did not show any developmental defects (Mara *et al.*, 2019), and authors questioned the requirement of Pol θ for normal development in Arabidopsis, as other groups did not seem to observe severe developmental defects (van Kregten *et al.*, 2016). More recently, *polq* mutants were generated in rice, and reported to develop normally under standard growth conditions, although regeneration from calli was severely impaired (Nishizawa-Yokoi *et al.*, 2020).

To tackle these questions, we carefully re-examined the phenotype of *teb* mutants, finding that it is highly variable. Furthermore, to try and determine the origin of developmental defects caused by Pol θ deficiency, we took advantage of the *pol2a-4* mutant that is partially deficient in the replicative polymerase Pol ε and shows constitutive replicative stress (Pedroza-Garcia *et al.*, 2017). Our results indicate that one key cellular function of Pol θ is to avoid DNA damage accumulation during DNA replication, and that developmental defects observed in *teb* mutants are likely consequences of replicative stress. Finally, we show that the phenotype of *teb* mutants can be aggravated by exposure to abiotic stresses, suggesting that environmental conditions impact the basal level of replicative stress, and providing evidence for a link between plant tolerance to stress, and mechanisms involved in the maintenance of genome integrity.

## RESULTS

Previous work reported the phenotype of *teb* mutants, with stunted growth and deformed leaves (Inagaki *et al.*, 2006). Five alleles of the mutant were initially described, three of which: *teb1*, *teb2* and *teb5* gave rise to the same phenotype and appeared to be full loss of function mutants (Inagaki *et al.*, 2006) (Figure 1A). However, rice mutants did not show developmental defects (Nishizawa-Yokoi *et al.*, 2020), and other groups reported much milder phenotypical defects for *teb2* and *teb5* mutants (van Kregten *et al.*, 2016). To clarify this, we carefully re-examined the phenotype of these mutants. In our growth conditions, most of the *teb2* and *teb5* mutants appeared indistinguishable from the wild-type after 1 month of growth (Figure 1B). We classified *teb* mutants' phenotypes in two categories: wild type like (WTL) plants appeared identical to the wild-type (Col-0) and plants with severe (S) developmental defects showed the previously described

*tebichi* phenotype (Figure 1B). We first checked that both WTL and S plants were homozygous for the *teb* mutation, using primers flanking the T-DNA insertions in *teb2* and *teb5* mutants (Figure 1A, C). We also checked by qPCR that both *teb* alleles we used did not allow the expression of the full length *POLQ* mRNA. To this end we used three primer pairs: one (#1) at the 5' end of the *TEB* gene, upstream both insertions, one flanking the T-DNA insertion site of the *teb5* mutant (#2), and one (#3) in the 3' moiety of the gene (Figure 1A). The first primer pair allowed detection of wild-type levels of mRNA in both mutants, indicating that the 5' extremity of the gene is normally expressed (Figure 1D). However, the *teb2* mutant accumulated no detectable transcripts produced downstream of the insertion. Expression of the 3' moiety of the gene was drastically reduced in *teb5* and no mRNA spanning the insertion site could be detected (Figure 1D). Thus, neither *teb2* nor *teb5* accumulate full length *TEB* mRNA, and are likely knock-out mutants, consistent with previous reports (Inagaki *et al.*, 2006). We next quantified the distribution of *teb* mutants between the two phenotypic categories. In our growth conditions ~ 85-90% of *teb* mutants were in the WTL category and only 10% to 15% in the S category corresponding to the previously described phenotype (Figure 1E).

Since *teb* mutants were shown to display a constitutive upregulation of DNA damage responsive genes (Inagaki et al., 2009), we asked whether the severity of the phenotype may correlate with the levels of expression for DDR genes. We thus determined the expression level of *BRCA1* that is involved in the DNA repair and *SMR7* which is an inhibitor of cell cycle progression, in rosette leaves of *teb* plants. Plants from the two phenotypic classes displayed upregulation both genes as previously reported (Inagaki et al., 2009), but no significant differences were observed between *teb* plants with different phenotype (Figure S1).

We next asked whether the observed variability in the *teb* mutant phenotype could also be observed earlier during development. Indeed, when analyzing root length of 15-day-old plants, we observed that *teb* mutants displayed a higher proportion of plants with arrested root growth than the wild-type (Figure S2). Likewise, at 10 days after germination, plantlets displayed more variable sizes than the wild-type, with a higher proportion of small plantlets with shorter roots and smaller cotyledons (Figure S3A, B). To determine whether this phenotypic variability related to increased DNA damage accumulation, we performed immuno-labelling of phosphorylated γ-H2AX variant on root tips of wild-type plants and small and big plantlets of *teb* mutants, that forms foci at the

site of DSBs (Charbonnel *et al.*, 2010). As shown on Figure 2, we could observe a significant increase in γ-H2AX labelling in *teb* mutants: the percentage of root tip nuclei showing γ-H2AX foci was around 1% in the wild-type, and around 10 % in both *teb* mutant alleles. However, the percentage of labelled nuclei was not significantly different between big and small plantlets. Consistently, DDR genes activation did not differ significantly between small and big *teb* mutants (Figure S3C, D). Furthermore, plants with arrested root growth did not show severe *teb* mutant plants at later stages: we selected 20 of those plantlets and transferred them to the green house, but none of them developed a severe phenotype after 3 weeks. Collectively, these results indicate that loss of Pol θ results in an increase in DNA damage accumulation in proliferating cells, but that the appearance of the *teb* severe phenotype is stochastic, and does not correlate with significantly higher levels of DNA damage or DDR activation.

One possible explanation for the stochastic appearance of the severe phenotype in *teb* mutants could be the accumulation of mutations as a consequence of defects in DNA repair. Under such a scenario, developmental defects would be expected to be transmitted to the next generation, or to aggravate in the next generation. To test this, the progeny of WTL and S plants was sown, and we evaluated the distribution of plants between the two classes in the next generation. However, the distribution of plants between the 2 classes was the same in the subsequent generation (Figure S4), suggesting that developmental defects are not due to mutations.

**Pol θ is involved in replicative stress tolerance**

Pol θ has been proposed to play a key role in replicating cells (Inagaki *et al.*, 2009), we therefore asked whether replicative stress could increase the proportion of plants showing developmental defects in *teb* mutants. Wild-type and *teb* mutants were germinated on MS supplemented with Hydroxyurea (HU, 0.75mM). At 10 days after germination, the survival rate of *teb* mutants was lower than that of wild-type plants (Figure S5), indicating that *teb* mutants are hypersensitive to replicative stress. After 10 days, surviving plants were transferred to soil, and the proportion of plants with a WTL or S phenotype was assessed after 3 weeks. Wild-type (Col-0) plants subjected to this treatment displayed a growth reduction but did not show other developmental defects such as deformed leaves (Figure S6). By contrast, as shown on Figure 1E, the proportion of plants with severe developmental defects was significantly increased in both *teb2* and *teb5* mutants. The

proportion of S plants increased from less than 15% to almost 30%, indicating that replicative stress may be the cause for developmental defects observed in *teb* mutants.

To further explore the role Pol θ in response to replicative stress, we took advantage of the hypomorphic mutant *pol2a-4*. This mutant (also called *abo4-1*) is partially deficient for the replicative DNA polymerase Pol ε (Yin *et al.*, 2009), and we have shown that it displays constitutive replicative stress (Pedroza-Garcia *et al.*, 2017). The *teb2* and *teb5* mutations were therefore introduced in the *pol2a-4* background by crossing, generating the *pol2a teb2* and *pol2a teb5* double mutants.

Six weeks old plants of all mutant combinations are shown in Figure 3A. Interestingly, *pol2a teb* double mutants displayed severe developmental defects that were fully homogeneous between individuals. To further characterize the developmental defects of *pol2a teb* double mutants, we quantified root length: we observed that *teb* and *pol2a* roots were shorter compared to wild type plants, as previously reported (Inagaki *et al.*, 2006; Pedroza-Garcia *et al.*, 2017). In addition, root length of *pol2a teb* double mutants was significantly reduced compared to single mutants (Figure 3B and 3C). Because *teb* mutants display disorganized meristem and spontaneous cell death in root tips (Inagaki *et al.*, 2006), we evaluated whether these defects were exacerbated in *pol2a teb* double mutants. Root tips of eight-day-old plants from mutant combinations were observed by confocal microscopy after propidium iodide staining. We observed disorganized meristem and cell death in the *teb* mutants, confirming the result of the previous study (Inagaki *et al.*, 2006). Furthermore, meristems were severely compromised in *pol2a teb* double mutants (Figure 4A-F) with disorganized patterning, extensive cell death and differentiation of root hair close to the tip of the root. Finally, meristem length was measured in these mutants, showing that *pol2a* and *teb* mutants have smaller root meristem size compared to the wild type Col-0. Moreover, more drastic reduction of meristem size was observed in the *pol2a teb* double mutants (Figure 4G). Finally, *pol2a teb* double mutants accumulated significantly higher levels of γ-H2AX foci than *teb* single mutants, whereas *pol2a* mutants did not accumulate more DSBs than the wild-type, as previously reported ((Pedroza-Garcia *et al.*, 2017), Figure 2D). Together, these results indicate that cell proliferation is more severely compromised in *pol2a teb* double mutants than in parental lines, likely due to increased accumulation of DNA breaks, consistent with the notion that Pol θ plays a key role in the repair of replication-associated DNA damage.

Our results indicate that loss of Pol θ impairs the repair of replication-associated DNA damage, which could lead to the activation of the DDR response. To test this hypothesis, we next checked the expression of DNA damage responsive genes in all mutant combinations by qRT-PCR (Figure 5). We selected genes representative of different responses triggered by DDR activation such as DNA repair genes (*RAD51* and *BRCA1*) and cell cycle regulation (*SMR5*/7, *WEE1* and *CYCB1*; 1). Expression of all tested genes was induced in the *teb* single mutants and in *pol2a* compared to wild-type Col-0, consistent with previous reports (Inagaki *et al.*, 2006; Pedroza-Garcia *et al.*, 2017), except for the *WEE1* gene in the *teb5* mutant. Furthermore, these genes displayed an even higher up-regulation in *pol2a teb* than in the single mutants (Figure 5), indicating that replicative stress induced by Pol ε deficiency is enhanced by the lack of Pol θ.

**Abiotic stresses aggravate the severity of *teb* mutants' phenotype.**

Taken together, our results indicate that a key cellular function of Pol θ is to allow repair of replication-associated DNA damage. This led us to postulate that the discrepancies between our observations and previous reports regarding the severity of *teb* mutants' phenotype could stem from different intensities of basal replicative stress between laboratories, due to different growth conditions. Under such a scenario, abiotic stresses would be expected to impact the severity of *teb* mutants' phenotypes. To test this hypothesis, we subjected *teb2* and *teb5* mutants to various abiotic stress conditions: high light intensity (HL, 350 µmol x $m^{-2}$ x $s^{-1}$), salt treatment (50 or 100 mM of NaCl). and heat (growth at 32°C). Except for the HL treatment, plants were grown under a low light intensity (LL, 160µmol x $m^{-2}$ x $s^{-1}$). After 3 weeks, we counted the plants in each phenotype category (n>50). These treatments obviously modified the phenotype of wild-type plants but did not induce the appearance of the conspicuous *teb*-like phenotype in Col0 plants (Figure S7). It is worth noting that the HL condition could not be considered as a stress condition for wild-type plants as they grew faster and reached a larger size than under LL conditions (Figure S7). The proportion of S plants increased under HL and high salt stress (100mM) for both *teb2* and *teb5* mutants (Figure 6A, B). By contrast, a lower concentration of salt (50mM) had no impact on the distribution of *teb* mutants between the different phenotypic classes. Likewise, growth at 32°C did not significantly affect the proportion of *teb* mutants with a severe phenotype. We tried increasing the temperature to 37°C, but the proportion of plantlets that did not survive in these growth conditions was over 50% in both wild-type and mutants, which prevented further analysis. To

determine whether abiotic stress conditions affected the level of DDR activation in *teb* mutants, we monitored the expression of DDR marker genes (Figure S8). Results obtained on mature plants were too variable to draw robust conclusions, so experiments were performed on *in vitro* grown plantlets. The two DNA-repair marker genes (*XRI-1* and *BRCA2*) and the cell cycle inhibitor *SMR7* were induced by salt treatment but not by high-light in wild-type plants, while expression of *SMR5* did not change between growth conditions. All these genes were induced in *teb2* and *teb5* mutants, and reached the same levels under control and high-light conditions. By contrast, all tested DDR marker genes were significantly induced in *teb* mutants grown in the presence of salt compared to control conditions, although the relative expression compared to wild-type plants remained in the same range. These results suggest that salt treatment can lead to DDR activation, and that this phenomenon is amplified in *teb* mutants, consistent with the observation that this treatment leads to an increase in the proportion of plants with a S phenotype. The situation for high-light response appears to less clear, but it is worth noting that *in vitro* growth conditions may not be fully comparable to growth on soil that we used for plant phenotyping.

Together, our results suggest that Pol θ is required in proliferating cells for the repair of replication-induced DNA lesions, and that basal levels of replicative stress vary depending on growth conditions, which likely accounts for the variability of *teb* mutants' phenotype.

## DISCUSSION

In mammalian cells, Pol θ mediates both error-prone TLS during DNA replication (Yoon *et al.*, 2019; Yousefzadeh and Wood, 2013) and DSB repair through Alt-NHEJ/MMEJ (Beagan and McVey, 2016). This dual role appears to be conserved in plants. Indeed, previous studies have shown that plant Pol θ is required for plant tolerance to various sources of DNA damage: Arabidopsis *teb* mutants are hypersensitive to damaging agents such as UV, cisplatin, MMC among others (Inagaki *et al.*, 2006), all of which can induce DNA damage in both proliferating and differentiated cells. More recently, Pol θ was involved in the repair of DSB through Alt-NHEJ (van Kregten *et al.*, 2016; Nishizawa-Yokoi *et al.*, 2020), a process that may also occur both in dividing and in differentiated cells. These observations, together with the fact that Pol θ appeared to be required for normal plant development prompted us to ask whether developmental defects observed in *teb* mutants reflect its function in TLS, DNA repair in all cell types, or repair of

replication-associated DNA damage. Here, we were able to show that phenotypic defects triggered by Pol θ deficiency are variable, and that their severity correlates with endogenous replicative stress levels. Indeed, HU treatment increased the proportion of *teb* mutants displaying severe developmental defects. Furthermore, DNA Pol ε deficiency that triggers constitutive replicative stress via ATR activation (Pedroza-Garcia *et al.*, 2017) abolished the variability of phenotypic alterations observed in *teb* mutants: *pol2a teb* double mutants all showed the same developmental defects, including drastically reduced growth, loss of primary root meristem function and extensive cell death in the root meristem. We therefore conclude that Pol θ is required for cellular response to replicative stress, and that this cellular function accounts for the developmental defects triggered by Pol θ deficiency. This hypothesis is consistent with the observation that *POLQ* genetically interacts with *ATR* (Inagaki *et al.*, 2009), whose function is to activate the DDR in response to replicative stress: developmental defects of *teb atr* double mutants are drastically enhanced compared to *teb* mutants, and inactivation of ATR prevents upregulation of the DDR marker gene *CYCB1;1* in *teb*. Taken together, these observations indicate that the activity of plant Pol θ is crucial to avoid accumulation of DNA damage during DNA replication in plants. Consistently, in mammals, the Alt-NHEJ activity is maximal during S-phase (Brambati *et al.*, 2020). Likewise, mutations in Drosophila *MUS308* gene induce hypersensitivity to replication-blocking lesions such as inter-strand cross-links (Harris *et al.*, 1996), and Pol θ was shown to play a key role in replication-associated DSB repair (Alexander *et al.*, 2016). A similar finding was reported in *Caenorhabtidis elegans*, where loss of Pol θ results in dramatic DNA loss around replication barriers such as G-quadruplexes (Koole *et al.*, 2014). Thus, repair of DNA breaks generated by DNA replication appears to be most prominent cellular function of Pol θ both in plants and animals.

Another pending question is how essential this replication-associated DNA repair function is for the normal development of multicellular organisms. The *chaos1* mouse mutants that harbor a point mutation in the *POLQ* gene are viable but show genomic instability, especially in erythrocytes (Shima *et al.*, 2004), but otherwise grow normally. Drosophila mutants also do not show major developmental alterations, except for a thin eggshell phenotype (Alexander *et al.*, 2016). In plants, the situation seems less clear as Arabidopsis *teb* mutants grown under our standard laboratory conditions display very variable phenotypic defects, and rice mutants grow and develop normally

(Nishizawa-Yokoi *et al.*, 2020). Likewise, in the moss *Physcomitrella patens*, loss of Pol θ does not affect development or genetic stability (Mara *et al.*, 2019). The latter observation may relate to the fact that the most prominent DNA repair pathway in moss cells is homologous recombination (HR) rather than Alt-NHEJ: in *P. patens*, mutants deficient for the RAD51 protein, that is required for HR, show developmental defects and hypersensitivity to DNA damaging agents (Markmann-Mulisch *et al.*, 2007). This suggests that in the moss, replication-associated damage is repaired mainly by HR rather than via Alt-NHEJ. The existence of alternative repair mechanisms also likely accounts for the variability observed in Arabidopsis *teb* mutants' phenotypes. HR, or other back-up repair mechanisms such as canonical NHEJ may compensate for Pol θ deficiency when replicative stress levels are relatively low. However, when the intensity of replicative stress increases, Pol θ becomes indispensable to deal with the accumulating DNA damage, and its absence leads to cell death and developmental defects. Our working model for Pol θ cellular function is summarized on Figure 7: in the wild-type, Pol θ avoids for stalling by promoting TLS, and contributes to the repair of DSBs generated by the combination of fork collapse and converging DNA replication coming from a nearby replication origin. Persistent fork stalling or unrepaired DSBs can activate the DDR via ATR, but this event remains very rare. In the absence of Pol θ, both TLS and DSB repair via Atl-NHEJ are compromised, leading to persistent DNA damage that activates ATR signaling and the DDR. Interestingly, contrasting requirements for Pol θ activity may exist between cell types in higher plants such as Arabidopsis. Indeed, Pol θ appears to be strictly required for T-DNA integration when plants are transformed by floral-dip, but not when transformation is done on somatic cells (Nishizawa-Yokoi *et al.*, 2020). The stochastic appearance of severe developmental phenotypes in teb mutants, and the fact that they are not heritable through sexual reproduction may be due to the appearance of mutations in somatic cells. Such a hypothesis would imply that meristematic cells that will give rise to the germline rely on other DNA repair pathways than Alt-NHEJ, or are more readily eliminated by programmed cell death than the neighboring initials. A similar situation may exist in animals since in mice, POLQ deficiency affects genomic stability mainly in erythrocytes (Shima *et al.*, 2004).

Requirement for Pol θ-dependent DNA repair may differ not only between cell types, but also between genomic regions. In Drosophila follicle cells, replication associated damage is repaired preferentially by HR or Pol θ-dependent Alt-NHEJ depending on the loci (Alexander *et al.*, 2016),

suggesting that Pol θ-mediated DNA repair is the prevalent DNA repair mechanism after fork collapse only at a subset of genomic regions. Likewise, in Arabidopsis, the *teb* mutations specifically affects the expression of genes with a nearby Helitron as well as that of tandem and dispersed duplicated genes (Inagaki *et al.*, 2009). Authors postulated that the *teb* mutation affects the chromatin state at these loci due to failed HR. One more likely hypothesis would be that Pol θ is preferentially involved in DNA repair at genomic regions that could otherwise engage in illegitimate HR with duplicated loci to avoid loss of genetic information. In the absence of Pol θ, DNA repair at these genomic regions would be compromised or delayed, which could indeed impair the proper re-establishment of chromatin states after DNA replication. This hypothesis could account for the fact that developmental defects triggered by Pol θ vary in severity and are not transmitted to the next generation through sexual reproduction. Indeed, if developmental defects associated with Pol θ deficiency were due to mutations caused by altered DNA repair, they would be expected to be stochastic in terms of plants' aspect because mutations could occur anywhere in the genome, and heritable. On the contrary, the *teb* mutation gives rise to remarkably similar phenotypical defects that are not heritable, and appear with a variable frequency. Defects in cell cycle progression very likely contribute to these developmental defects. However, it is tempting to speculate that they could also partly be due to changes in gene expression triggered by defects in the restoration of chromatin states after DNA replication. If Pol θ is preferentially involved in DNA repair at specific genomic contexts, this model would also explain why plants with severe developmental defects all look identical.

Finally, our results not only provide evidence for the role of Pol θ during DNA replication, but also reveal that abiotic stresses can enhance the requirement for Pol θ, indicating that levels of replicative stress in dividing cells may differ depending on growth conditions. How abiotic stresses affect genome integrity in plants remains to be fully elucidated (Nisa *et al.*, 2019). The effect of UV light or heavy metals on DNA is well documented (Chen *et al.*, 2019), but the consequences of other stresses such as temperature changes, drought, salinity or light intensity have been less explored, although there is accumulating evidence that DDR signaling may be a relevant element in plants' response to these stimuli. Indeed, the ANAC044 and ANAC085 transcription factors, that are activated by DNA damage also contribute to the induction of a G2-arrest in response to heat stress (Takahashi *et al.*, 2019). Whether their role reflects the accumulation of DNA damage

in response to heat, or the recruitment of this DDR branch to respond to heat stress remains to be clarified, but these results suggest that DDR signaling may play a more prominent role than previously anticipated in plants' response to environmental stresses. In line with this hypothesis, root meristem maintenance under chilling conditions requires DDR signaling components (Hong *et al.*, 2017). Our results indicate that at least high light and salt may induce replicative stress in plants, as evidenced by the aggravation of *teb* mutants' phenotypes. By contrast, we could not detect any effect of heat stress. This may be due to the fact that we exposed plants to a slightly less severe heat stress than Takahashi and colleagues (Takahashi *et al.*, 2019) because prolonged growth at 37°C resulted in a high mortality rate in both wild-type and mutant plants. However, this could also mean that all types of abiotic stresses do not affect DNA replication in the same way. Future work should help elucidate how much mechanisms involved in the maintenance of genome integrity contribute to plants developmental plasticity in response to stress, and whether different stress conditions affect genome integrity in different ways.

## MATERIAL AND METHODS

### Plant material and growth conditions

All *Arabidopsis thaliana* mutants used in this study are in the wild type Columbia-0 (Col-0) background. *teb2* (SALK_035610) and *teb5* (SALK_018851) mutants were a kind gift from M. van Kregten (Leiden University).

Seeds were surface sterilized and treated with bayrochlore$^{TM}$ for 20 min, then washed with sterile water and kept at 4◦C for 2 days. They were next sown on commercially available 0.5× Murashige and Skoog (MS, Duchefa) medium solidified with 0.8% agar (Phyto-Agar HP696, Kalys). Plates were then transferred to a long day (16 h light, 8 h night, 21◦C) *in vitro* growth chamber. After 2 weeks plants were transferred to soil under short day conditions (8 h light 20◦C, 16 h night at 18◦C) for one week and after that transferred to a long day growth chamber (16 h light, 8 h night, 21◦C) for phenotypic analysis.

Genotyping of the *teb2* and *teb5* mutants was performed using the Lba1/RP primer combination for the mutant allele and the LP/RP primer combination for the wild-type allele. Sequence of primers used can be found in Table S1.

**Genotoxic test**

Wild type Col-0 and *teb* mutants *teb2* and *teb5*) were germinated on MS medium (Control condition) and some were germinated on MS supplemented hydroxyurea (HU) concentration was 0.75 mM. After 2 weeks, these mutant plants were transferred to soil. Then after 10 days, the survival rate of these plants was measured.

**RNA extraction and quantitative RT-PCR**

Total RNA was extracted from flower buds using NucleoSpin® RNA protocol (MACHEREY-NAGEL). First strand cDNA was synthesized from 2µg of total RNAs by using ImProm-II ™ Reverse Transcription System (Promega) according to the manufacturer's instructions. 1/50$^{th}$ of the synthesized cDNA was mixed with 100nM of each primer and LightCycler 480 Sybr Green I mastermix (Roche Applied Science) for quantitative PCR analysis. Products were amplified and fluorescent signals acquired with a LightCycler 480 detection system. The specificity of amplification products was determined by melting curves. Data were from triplicates and are representative of at least two biological replicates. The sequence of primers used in this study is provided in Supplementary Table 1. DDR-related genes expressions were normalized by using housekeeping gene *ACTIN*. Similar results were observed in 3 independent experiments.

**Immuno-fluorescence**

Immuno-labelling of γ-H2AX foci was performed as described previously (Charbonnel *et al.*, 2010). Slides were imaged with an epifluorescence microscope (AxioImager Z.2; Carl Zeiss) fitted with a metal halide lamp and the appropriate shifted free filter sets for imaging DAPI and Alexa 488 dyes. Images were acquired with a cooled CCD camera (AxioCam 506 monochrome; Carl Zeiss) operated using Zen Blue software (Carl Zeiss).

**Confocal microscopy imaging**

Root tips of 8-day-old plantlets were stained with propidium iodide (PI, 10µM) and then root meristems were observed using 20X water immersion lens on a Zeiss LSM 880 laser scanning confocal microscope using a 561nm laser for excitation. Fluorescence was acquired between 565 nm and 700 nm. Representative images were collected from 10 to 15 roots with three biological replicates.

**Abiotic stress**

In this study three different abiotic stress were applied on *teb* mutants. These mutant seeds were grown on ½ MS medium and germinated *in vitro* and after 10 days transferred to pots (soil). Control plants were kept at 20°C under low light intensity (LL, 160µmol x $m^{-2}$ x $s^{-1}$), and watered with water. Plants were either subjected to high light intensity (HL, 350 µmol x $m^{-2}$ x $s^{-1}$) at 20°C, or transferred in a growth cabinet at 32°C (16h day, 8h night, 28°C at night) under LL or kept at 20°C under LL but watered with two NaCl solutions (50mM or 100mM). For the higher salt concentration, plants were first watered with NaCl for 3 days, and the concentration was then increased to 100mM. Distribution of plants between the three phenotypic classes was documented after three weeks of these stresses. Chi-squared tests was used to compare the distributions between phenotypic classes. Experiments were performed twice giving similar results.

**Accession numbers**

Accession numbers of the genes mentioned in this study are as follows: *TEBICHI* (AT4G32700), *POL2A* (AT1G08260), *CYCB1;1* (AT4G37490), *RAD51* (AT5G20850), *WEE1* (AT1G02970), *BRCA2* (AT1G80210), *SMR5* (AT1G07500), *SMR7* (AT3G27630).

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**Conflict of interest**

Authors declare no conflict of interest.

## REFERENCES


**Adachi, S., Minamisawa, K., Okushima, Y., et al.** (2011) Programmed induction of endoreduplication by DNA double-strand breaks in Arabidopsis. *Proc Natl Acad Sci U S A*, **108**, 10004–10009. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21613568.

**Alexander, J.L., Beagan, K., Orr-Weaver, T.L. and McVey, M.** (2016) Multiple mechanisms contribute to double-strand break repair at rereplication forks in Drosophila follicle cells. *Proc. Natl. Acad. Sci. U. S. A.*, **113**, 13809–13814. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27849606 [Accessed June 17, 2020].

**Beagan, K. and McVey, M.** (2016) Linking DNA polymerase theta structure and function in health and disease. *Cell. Mol. Life Sci.*, **73**, 603–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26514729 [Accessed June 16, 2020].

**Brambati, A., Barry, R.M. and Sfeir, A.** (2020) DNA polymerase theta (Polu)-an error-prone polymerase necessary for genome stability. *Curr Opin Genet Dev*, **60**, 119–126. Available at: https://doi.org/10.1016/j.gde.2020.02.017.

**Burgers, P.M.J.** (1998) Eukaryotic DNA polymerases in DNA replication and DNA repair. *Chromosoma*.

**Charbonnel, C., Gallego, M.E. and White, C.I.** (2010) Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants. *Plant J.*, **64**, 280–290. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21070408 [Accessed October 14, 2015].

**Chen, P., Sjogren, C.A., Larsen, P.B. and Schnittger, A.** (2019) A multi-level level response to DNA damage induced by Aluminium. *Plant J.*, **98**, 479-491.

**Chiruvella, K.K., Liang, Z. and Wilson, T.E.** (2013) Repair of double-strand breaks by end joining. *Cold Spring Harb. Perspect. Biol.*, **5**, a012757. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23637284 [Accessed June 16, 2020].

**Ciccia, A. and Elledge, S.J.** (2010) The DNA Damage Response: Making It Safe to Play with Knives. *Mol. Cell*.

**Feng, W., Simpson, D.A., Carvajal-Garcia, J., et al.** (2019) Genetic determinants of cellular addiction to DNA polymerase theta. *Nat. Commun.*, **10**, 1–13. Available at: https://doi.org/10.1038/s41467-019-12234-1 [Accessed October 26, 2020].

**Fulcher, N. and Sablowski, R.** (2009) Hypersensitivity to DNA damage in plant stem cell niches. *Proc Natl Acad Sci U S A*, **106**, 20984–20988. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19933334.

**Harris, P. V, Mazina, O.M., Leonhardt, E.A., Case, R.B., Boyd, J.B. and Burtis, K.C.** (1996) Molecular cloning of Drosophila mus308, a gene involved in DNA cross-link repair with homology to prokaryotic DNA polymerase I genes. *Mol. Cell. Biol.*

**Hong, J.H., Savina, M., Du, J., Devendran, A., Kannivadi Ramakanth, K., Tian, X., Sim, W.S., Mironova, V. V. and Xu, J.** (2017) A Sacrifice-for-Survival Mechanism Protects Root Stem Cell Niche from Chilling Stress. *Cell*, **170**, 102-113.e14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28648662 [Accessed November 18, 2018].

**Hu, Z., Cools, T. and Veylder, L. De** (2016) Mechanisms Used by Plants to Cope with DNA Damage. *Annu. Rev. Plant Biol.*, **67**, 439–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26653616 [Accessed July 14, 2016].

**Inagaki, S., Nakamura, K. and Morikami, A.** (2009) A link among DNA replication, recombination, and gene expression revealed by genetic and genomic analysis of TEBICHI gene of Arabidopsis thaliana P. S. Schnable, ed. *PLoS Genet*, **5**, e1000613. Available at: https://dx.plos.org/10.1371/journal.pgen.1000613 [Accessed July 30, 2019].

**Inagaki, S., Suzuki, T., Ohto, M., Urawa, H., Horiuchi, T., Nakamura, K. and Morikami, A.** (2006) Arabidopsis TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. *Plant Cell*, **18**, 879–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16517762 [Accessed July 30, 2019].

**Jain, R., Aggarwal, A.K. and Rechkoblit, O.** (2018) Eukaryotic DNA polymerases. *Curr. Opin. Struct. Biol.*, **53**, 77–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30005324 [Accessed February 12, 2019].

**Koole, W., Schendel, R. Van, Karambelas, A.E., Heteren, J.T. Van, Okihara, K.L. and Tijsterman, M.** (2014) A polymerase theta-dependent repair pathway suppresses extensive genomic instability at endogenous G4 DNA sites. *Nat. Commun.*, **5**. Available at: https://pubmed.ncbi.nlm.nih.gov/24496117/ [Accessed October 31, 2020].

**Kregten, M. van, Pater, S. de, Romeijn, R., Schendel, R. van, Hooykaas, P.J.J. and Tijsterman, M.** (2016) T-DNA integration in plants results from polymerase-θ-mediated DNA repair. *Nat. Plants*, **2**, 16164. Available at: http://www.nature.com/articles/nplants2016164 [Accessed July 30, 2019].

**Kunkel, T.A.** (2004) DNA replication fidelity. *J. Biol. Chem.*, **279**, 16895–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14988392 [Accessed February 12, 2019].

**Makarova, K.S. and Koonin, E. V** (2013) Archaeology of eukaryotic DNA replication. *Cold Spring Harb. Perspect. Biol.*, **5**, a012963. Available at: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a012963 [Accessed September 9, 2019].

**Mara, K., Charlot, F., Guyon-Debast, A., Schaefer, D.G., Collonnier, C., Grelon, M. and Nogué, F.** (2019) POLQ plays a key role in the repair of CRISPR/Cas9-induced double-stranded breaks in the moss Physcomitrella patens. *New Phytol.*

**Markmann-Mulisch, U., Wendeler, E., Zobell, O., Schween, G., Steinbiss, H.H. and Reiss, B.** (2007) Differential requirements for RAD51 in Physcomitrella patens and Arabidopsis thaliana development and DNA damage repair. *Plant Cell*, **19**, 3080–3089. Available at: https://pubmed.ncbi.nlm.nih.gov/17921313/ [Accessed October 31, 2020].

**Mateos-Gomez, P.A., Kent, T., Deng, S.K., McDevitt, S., Kashkina, E., Hoang, T.M., Pomerantz, R.T. and Sfeir, A.** (2017) The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. *Nat. Struct. Mol. Biol.*, **24**, 1116–1123. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29058711 [Accessed June 16, 2020].

**Nisa, M.-U., Huang, Y., Benhamed, M. and Raynaud, C.** (2019) The Plant DNA Damage Response: Signaling Pathways Leading to Growth Inhibition and Putative Role in Response to Stress Conditions. *Front. Plant Sci.*, **10**, 653. Available at: http://www.ncbi.nlm.nih.gov/pubmed/31164899 [Accessed July 28, 2019].

**Nishizawa-Yokoi, A., Saika, H., Hara, N., Lee, L., Toki, S. and Gelvin, S.B.** (2020) *Agrobacterium* T-DNA integration in somatic cells does not require the activity of DNA polymerase theta. *New Phytol.*, nph.17032. Available at: https://onlinelibrary.wiley.com/doi/10.1111/nph.17032 [Accessed October 31, 2020].

**Noctor, G. and Foyer, C.H.H.** (2016) Intracellular Redox Compartmentation and ROS-Related Communication in Regulation and Signaling. *Plant Physiol.*, **171**, 1581–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27208308 [Accessed March 6, 2019].

**Pedroza-Garcia, J.-A., Veylder, L. De and Raynaud, C.** (2019) Plant DNA Polymerases. *Int. J. Mol. Sci.*, **20**. Available at: http://www.ncbi.nlm.nih.gov/pubmed/31569730 [Accessed June 16, 2020].

**Pedroza-Garcia, J.A., Mazubert, C., Olmo, I. Del, et al.** (2017) Function of the plant DNA Polymerase epsilon in replicative stress sensing, a genetic analysis. *Plant Physiol.*, **173**, 1735-1749.

**Powers, K.T. and Washington, M.T.** (2018) Eukaryotic translesion synthesis: Choosing the right tool for the job. *DNA Repair (Amst).*, **71**, 127–134. Available at: https://www.sciencedirect.com/science/article/pii/S1568786418301812?via%3Dihub [Accessed February 12, 2019].

**Sakamoto, A.N.** (2019) Translesion Synthesis in Plants: Ultraviolet Resistance and Beyond. *Front. Plant Sci.*, **10**, 1208. Available at: www.frontiersin.org [Accessed November 18, 2020].

**Shima, N., Munroe, R.J. and Schimenti, J.C.** (2004) The mouse genomic instability mutation chaos1 is an allele of Polq that exhibits genetic interaction with Atm. *Mol. Cell. Biol.*, **24**, 10381–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15542845 [Accessed June 17, 2020].

**Takahashi, N., Ogita, N., Takahashi, T., Taniguchi, S., Tanaka, M., Seki, M. and Umeda, M.** (2019) A regulatory module controlling stress-induced cell cycle arrest in Arabidopsis. *Elife*, **8**. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30944065 [Accessed April 24, 2019].

**Wang, Z., Song, Y., Li, S., Kurian, S., Xiang, R., Chiba, T. and Wu, X.** (2019) DNA polymerase (POLQ) is important for repair of DNA double-strand breaks caused by fork collapse. *J. Biol. Chem.*, **294**, 3909–3919. Available at: /pmc/articles/PMC6422074/?report=abstract [Accessed October 31, 2020].

**Yang, W. and Gao, Y.** (2018) Translesion and Repair DNA Polymerases: Diverse Structure and Mechanism. *Annu. Rev. Biochem.*, **87**, 239–261. Available at: https://www.annualreviews.org/doi/10.1146/annurev-biochem-062917-012405 [Accessed September 9, 2019].

**Yin, H., Zhang, X., Liu, J., Wang, Y., He, J., Yang, T., Hong, X., Yang, Q. and Gong, Z.** (2009) Epigenetic regulation, somatic homologous recombination, and abscisic acid signaling are influenced by DNA polymerase epsilon mutation in Arabidopsis. *Plant Cell*, **21**, 386–402. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19244142.

**Yoon, J.-H., McArthur, M.J., Park, J., Basu, D., Wakamiya, M., Prakash, L. and Prakash, S.** (2019) Error-Prone Replication through UV Lesions by DNA Polymerase θ Protects against Skin Cancers. *Cell*, **176**, 1295-1309.e15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30773314 [Accessed June 17, 2020].

**Yoshiyama, K.O., Kobayashi, J., Ogita, N., Ueda, M., Kimura, S., Maki, H. and Umeda, M.** (2013) ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in Arabidopsis. *EMBO Rep*, **14**, 817–822. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23907539.

**Yousefzadeh, M.J. and Wood, R.D.** (2013) DNA polymerase POLQ and cellular defense against DNA damage. *DNA Repair (Amst).*, **12**, 1–9. Available at: /pmc/articles/PMC3534860/?report=abstract [Accessed November 18, 2020].

**Zeman, M.K. and Cimprich, K.A.** (2014) Causes and consequences of replication stress. *Nat.*

*Cell Biol.*, **16**, 2–9. Available at: /pmc/articles/PMC4354890/ [Accessed March 10, 2021].

## Figure Legends

**Figure 1: The *tebichi* mutation results in variable developmental defects that can be enhanced by replicative stress**

A: Representative phenotypes observed in *teb-2* and *teb-5* homozygous mutants after 1 month of growth on soil. Plants were classified in 3 categories: wild-type like (WT), intermediate (I) with only mild growth reduction and a few deformed or twisted leaves (arrowhead), or severe (S) with clear growth reduction and abnormal leaf shape. Bar = 1cm.

B: Structure of the *POLQ* gene and position of the T-DNA insertions in the *teb2* and *teb5* alleles. Exons are indicated by grey boxes and introns by a grey line. Primers used for genotyping are indicated by arrows.

C: Result of genotyping for *teb* mutants with wild-type-like (WTL) or severe (S) phenotype. Both types of plants were found to be homozygous for the *teb* mutation.

D: qPCR quantification of *POLQ* expression in *teb* mutants. Actin was used for normalization. The position of primer pairs is indicated by corresponding numbers.

E: Distribution of *teb* mutants between the three phenotypic classes with and without HU treatment. Plants were germinated on control (MS) or hydroxyurea supplemented medium (HU) to a final concentration of 0.75mM. They were transferred to soil after 12 days, and phenotypes were observed after one month. Asterisks denote significant differences between distributions (Chi-squared test, $p< 0.01$). Blind scoring was performed on wild-type and *teb* mutants, the proportion of severe phenotypes observed in wild-type plants was below 2% in all conditions.

**Figure 2: *teb* mutants show DSB accumulation in root meristems**

A-C: representative images of *teb2* root tip nuclei after g-H2AX immuno-staining (A: DAPI fluorescence, B: Alexa 488 fluorescence, C: merged image). Bar = 10µm, arrows indicate nuclei with g-H2AX foci. D: quantification of γ-H2AX foci in the indicated genotypes (n>1500 nuclei imaged from 10 root tips for all genotypes). Different letters indicate statistically different values,

ANOVA followed by a post-hoc Tukey test $p<0.01$). Data are representative of 2 biological replicates.

**Figure 3: Constitutive replicative stress aggravates the phenotype of *teb* mutants**

A: Phenotype of the wild-type (Col0), *teb-2*, *teb-5*, *pol2a-4*, *pol2a teb2* and *pol2a teb5* mutants after 40 days of growth under standard conditions (160 µmol photon x $m^{-2}xs^{-1}$, 16h light, 20°C). Bar = 1cm.

B: Root length of the wild-type (Col0), *teb-2*, *teb-5*, *pol2a-4*, *pol2a teb2* and *pol2a teb5*. Plants were grown vertically *in vitro* for one week.

C: Quantification of the root length in the different genotypes. Data are from at least 20 measurements for each line and are representative of 2 independent experiments. Different letters indicate statistically significant differences (ANOVA and Tukey test $p<0.01$).

**Figure 4: The root meristem of *teb pol2a* double mutants is severely compromised**

A-F: Confocal images of root tips of 8-day-old plants stained with propidium iodide. A: WT (Col0), B: *teb2*, C: *teb5*, D: *pol2a-4*, E: *pol2a teb2* F: *pol2a teb5*. The meristem of *teb* mutants showed abnormal organization and cell death. This defect was exacerbated in *pol2a teb* double mutants with root hair differentiating close to the root tip and meristem organization being dramatically altered. Red arrow indicates the limit of the root apical meristem. Bar = 50µm for all panels.

G: Meristem length was measured in all mutant combinations. Values are from at least 10 roots and are representative of two independent experiments. Different letters indicate statistically significant differences (ANOVA and Tukey test $p<0.01$).

**Figure 5: DDR genes are hyper-induced in *teb pol2a* double mutants**

Total RNA was extracted from twelve-day-old plantlets. Expression of selected genes was assessed by real-time qPCR and normalized to actin. We monitored the expressions of genes involved in cell-cycle arrest (*SMR5*, *SMR7* and *WEE1*), DNA repair (*RAD51* and *BRCA2*) and both (*CYCB1;1*). Values are Fold change compared to the wild-type Col-0. Graphs represent average of 3 technical replicates +/- standard deviation and are representative of 3 independent biological

replicates. Different letters above bars denote statistically relevant differences (ANOVA followed by Tukey test, performed on raw data before normalization, $p<0.01$).

**Figure 6: Some abiotic stresses aggravate the developmental defects of *teb* mutants.**

A: Distribution of *teb* mutants between the different classes in plants grown in low light (LL, 160µmol x $m^{-2}$ x $s^{-1}$) or high light (HL, 350 µmol x $m^{-2}$ x $s^{-1}$).

B: Distribution of *teb* mutants between the different classes in plants grown at standard temperature (20°C) or under heat stress (32°C). Plants were germinated *in vitro* and transferred to soil after 10 days. After 3 days of growth under control conditions at 160µmol x $m^{-2}$ x $s^{-1}$ plants were kept under the same conditions or transferred to 32°C under the same light intensity.

C: Distribution of *teb* mutants between the different classes in plants watered with or without salt to the indicated concentration. Plants were germinated *in vitro* and transferred to soil after 10 days. After 3 days of growth under control conditions (160µmol x $m^{-2}$ x $s^{-1}$, 20°C, salt-treated plants were watered with a solution containing NaCl (50mM), for the 100mM treatment, salt concentration was increased to 100mM after 2days.

For all panels, n.s. indicates non-significant differences and asterisks denote significant differences between distributions (Chi-squared test, $p< 0.01$). Blind scoring was performed on wild-type and *teb* mutants in all growth conditions, the proportion of severe phenotypes observed in wild-type plants was below 2%.

**Figure 7: Model for the role of Pol θ during replicative stress response**

A: In the wild-type, replication blocking lesions induce fork stalling. Pol θ can allow TLS through some lesions such as pyrimidine dimers. If efficient lesion bypass cannot be achieved, replisome disassembly and persistent fork stalling activates the DDR through ATR signalling, and DNA synthesis from a converging fork can lead to the formation of a double-ended DSB. Pol θ contributes to the repair of these lesions through Alt-NHEJ but other pathways such as HR or NHEJ likely contribute to DSB repair. B: In the absence of Pol θ, TLS through some lesions is compromised, leading to an increased frequency of fork collapse and persistent stalling. Furthermore, Alt-NHEJ is also compromised; leading to an increased frequency of failed repair, constitutive activation of the DDR through ATR signalling, cell death and stochastic

developmental defects. Abiotic stress and replicative stress can modify this equilibrium by enhancing the accumulation of more replication-blocking lesions, leading to an increased frequency of developmental defects in Pol θ deficient lines.

## Supplemental Figures

**Figure S1: Levels of DDR genes induction do not correlate with the severity of *teb* mutants' phenotype.**

**Figure S2: Root growth defects show some heterogeneity in *teb* mutants**

**Figure S3: Heterogeneous phenotypes of *teb* mutant plantlets do not correlate with different levels of DDR genes activation**

**Figure S4: The *teb* phenotype does not aggravate over generations**

**Figure S5: *teb* mutants are hypersensitive to replicative stress**

**Figure S6: Representation phenotypes of wild-type (Col0) and *teb* mutants exposed to HU before transfer to the green house**

**Figure S7: Representation phenotypes of wild-type (Col0) and *teb* mutants grown under different conditions.**

**Figure S8: Salt treatment, but not increasing light intensity activates DDR gene expression in both wild-type and *teb* mutants.**

## Supplemental Tables

**Table S1: Primer sequences**

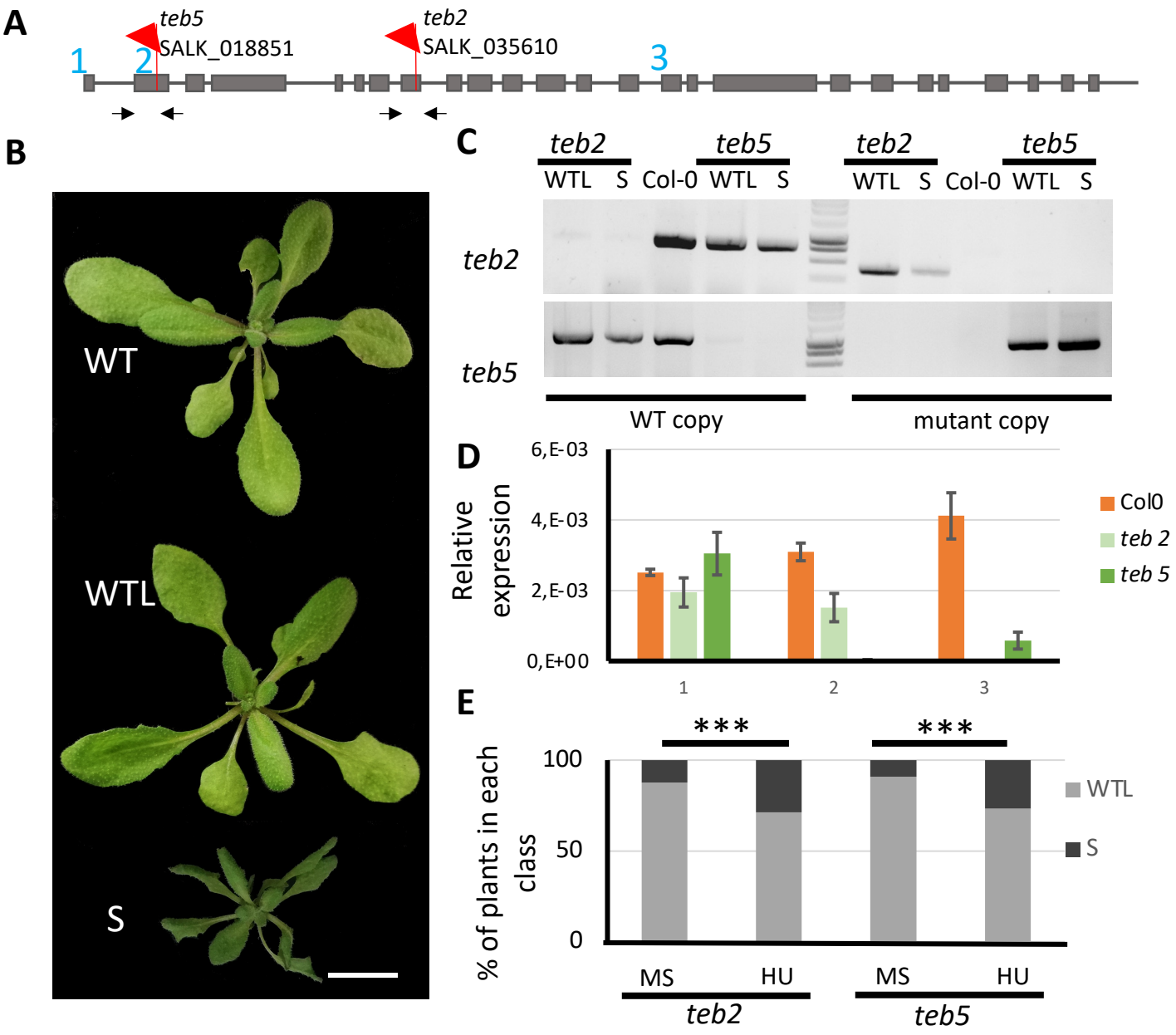



**Figure 1. The *tebichi* mutation results in variable developmental defects that can be enhanced by replicative stress**

A: Structure of the *POLQ* gene and position of the T-DNA insertions in the *teb2* and *teb5* alleles. Exons are indicated by grey boxes and introns by a grey line. Primers used for genotyping are indicated by arrows. B: Representative phenotypes observed in *teb-2* and *teb-5* homozygous mutants after 1 month of growth on soil. Plants were classified in 2 categories: wild-type like (WT) and severe (S) with clear growth reduction and abnormal leaf shape. Bar = 1cm. C: Result of genotyping for *teb* mutants with wild-type-like (WTL) or severe (S) phenotype. Both types of plants were found to be homozygous for the *teb* mutation. D: qPCR quantification of *POLQ* expression in *teb* mutants. Actin was used for normalization. The position of primer pairs is indicated by corresponding numbers in (A). Data are average +/- S.D. of three technical replicates and representative of three independent experiments. E: Distribution of *teb* mutants between the two phenotypic classes with and without HU treatment. Plants were germinated on control (MS) or hydroxyurea supplemented medium (HU) to a final concentration of 0.75 mM. They were transferred to soil after 12 days, and phenotypes were observed after one month (n>50). Asterisks denote statistically relevant differences between distributions ($\chi^2$-test, $p < 0.01$). Blind scoring was performed on wild-type and *teb* mutants, the proportion of severe phenotypes observed in wild-type plants was below 2% in all conditions.

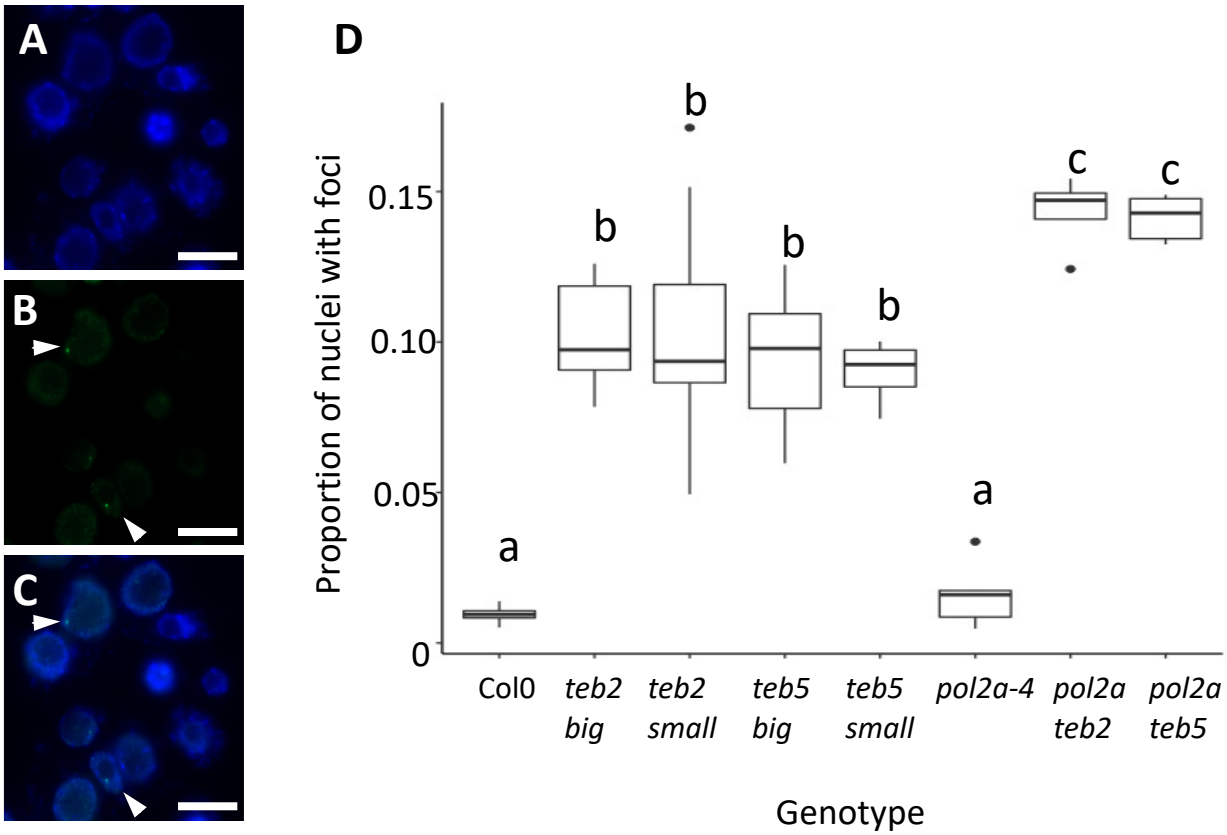
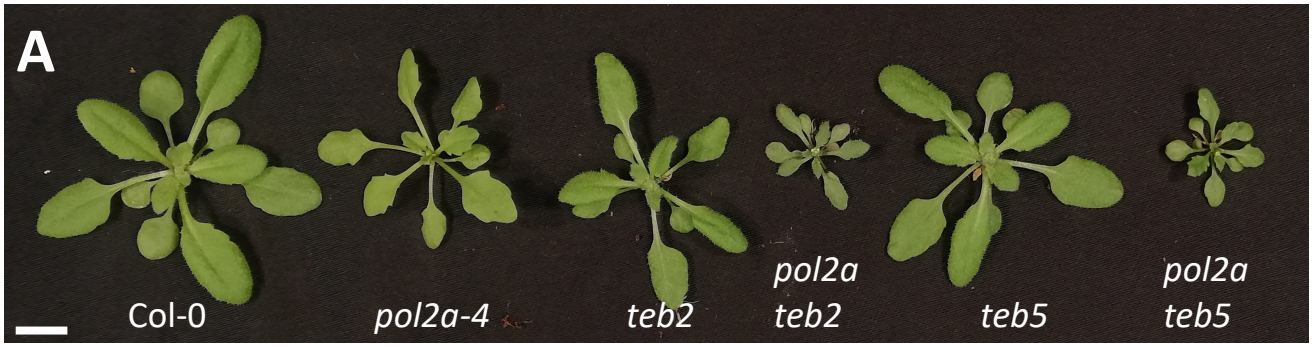



**Figure 2. *teb* mutants show DSB accumulation in root meristems**

A-C: representative images of *teb2* root tip nuclei after γ-H2AX immuno-staining (A: DAPI fluorescence, B: Alexa 488 fluorescence, C: merged image). Bar = 10 µm, arrows indicate nuclei with γ-H2AX foci. D: quantification of γ-H2AX foci in the indicated genotypes (n>1500 nuclei imaged from 10 root tips for all genotypes). Different letters indicate statistically different values, ANOVA followed by a post-hoc Tukey test $p<0.01$). Data are representative of 2 biological replicates.





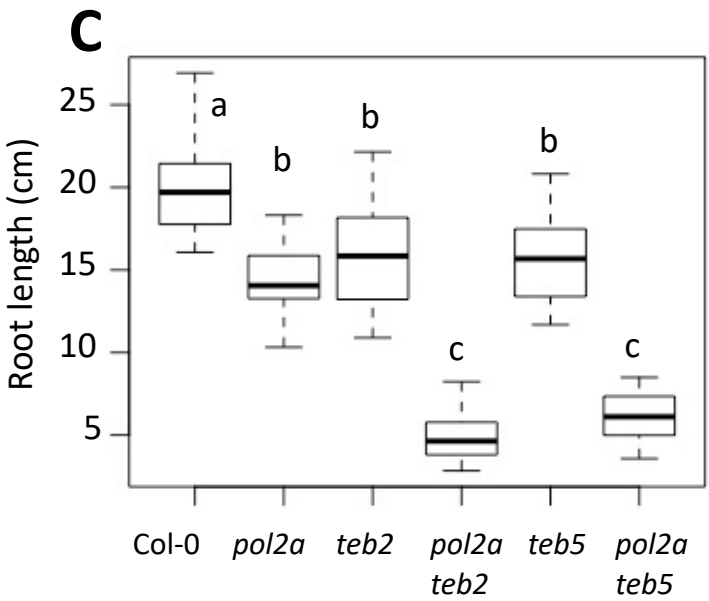



**Figure 3. Constitutive replicative stress aggravates the phenotype of *teb* mutants**

A: Phenotype of the wild-type (Col0), *teb-2*, *teb-5*, *pol2a-4*, *pol2a teb2* and *pol2a teb5* mutants after 40 days of growth under standard conditions (160 µmol photon x $m^{-2}xs^{-1}$, 16h light, 20°C). Bar = 1 cm.

B: Root length of the wild-type (Col0), *teb-2*, *teb-5*, *pol2a-4*, *pol2a teb2* and *pol2a teb5*. Plants were grown vertically *in vitro* for one week.

C: Quantification of the root length in the different genotypes. Data are from at least 20 measurements for each line and are representative of 2 independent experiments. Different letters indicate statistically relevant differences (ANOVA and Tukey test $p<0.01$).

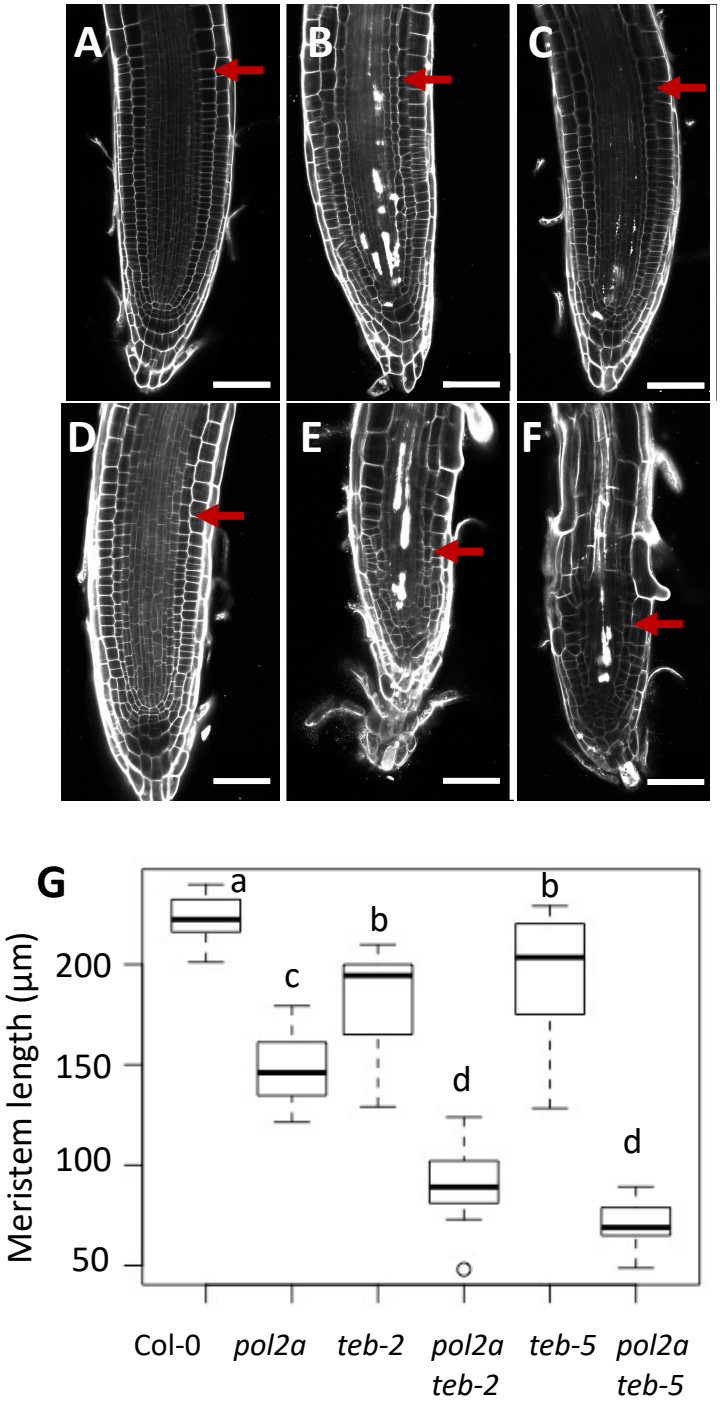



**Figure 4. The root meristem of *teb pol2a* double mutants is severely compromised**

A-F: Confocal images of root tips of 8-day-old plants stained with propidium iodide. A: WT (Col0), B: *teb2*, C: *teb5*, D: *pol2a-4*, E: *pol2a teb2* F: *pol2a teb5*. The meristem of *teb* mutants showed abnormal organization and cell death. This defect was exacerbated in *pol2a teb* double mutants with root hair differentiating close to the root tip and meristem organization being dramatically altered. Red arrow indicates the limit of the root apical meristem. Bar = 50 µm for all panels.

G: Meristem length was measured in all mutant combinations. Values are from at least 10 roots and are representative of two independent experiments. Different letters indicate statistically relevant differences (ANOVA and Tukey test $p<0.01$).

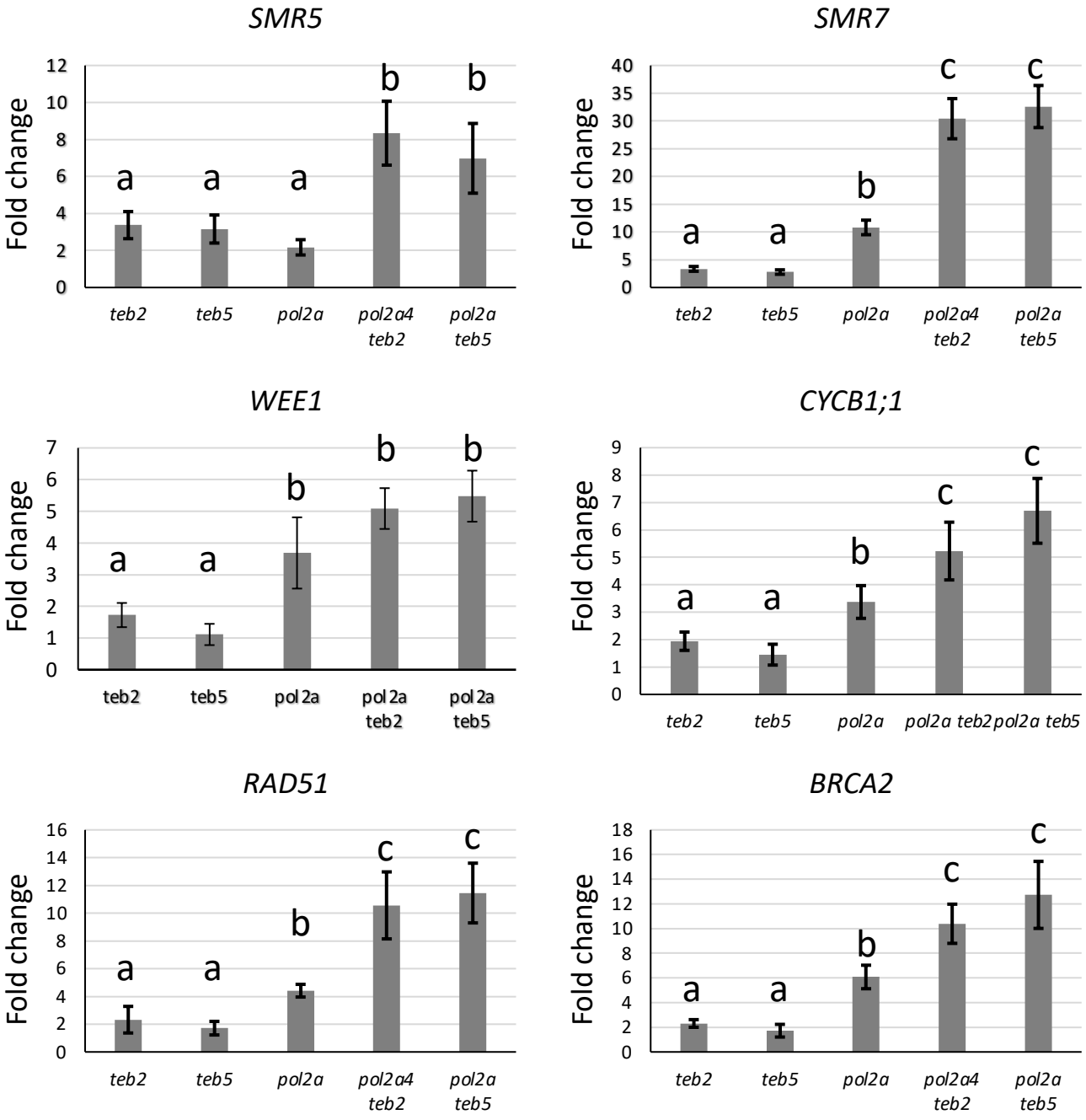



**Figure 5. DDR genes are hyper-induced in *teb pol2a* double mutants**

Total RNA was extracted from twelve-day-old plantlets. Expression of selected genes was assessed by real-time qPCR and normalized to actin. We monitored the expressions of genes involved in cell-cycle arrest (*SMR5*, *SMR7* and *WEE1*), DNA repair (*RAD51* and *BRCA2*) and both (*CYCB1;1*). Values are Fold change compared to the wild-type Col-0. Graphs represent average of 3 technical replicates +/- standard deviation and are representative of 3 independent biological replicates. Different letters above bars denote statistically relevant differences (ANOVA followed by Tukey test, performed on raw data before normalization, $p<0.01$).

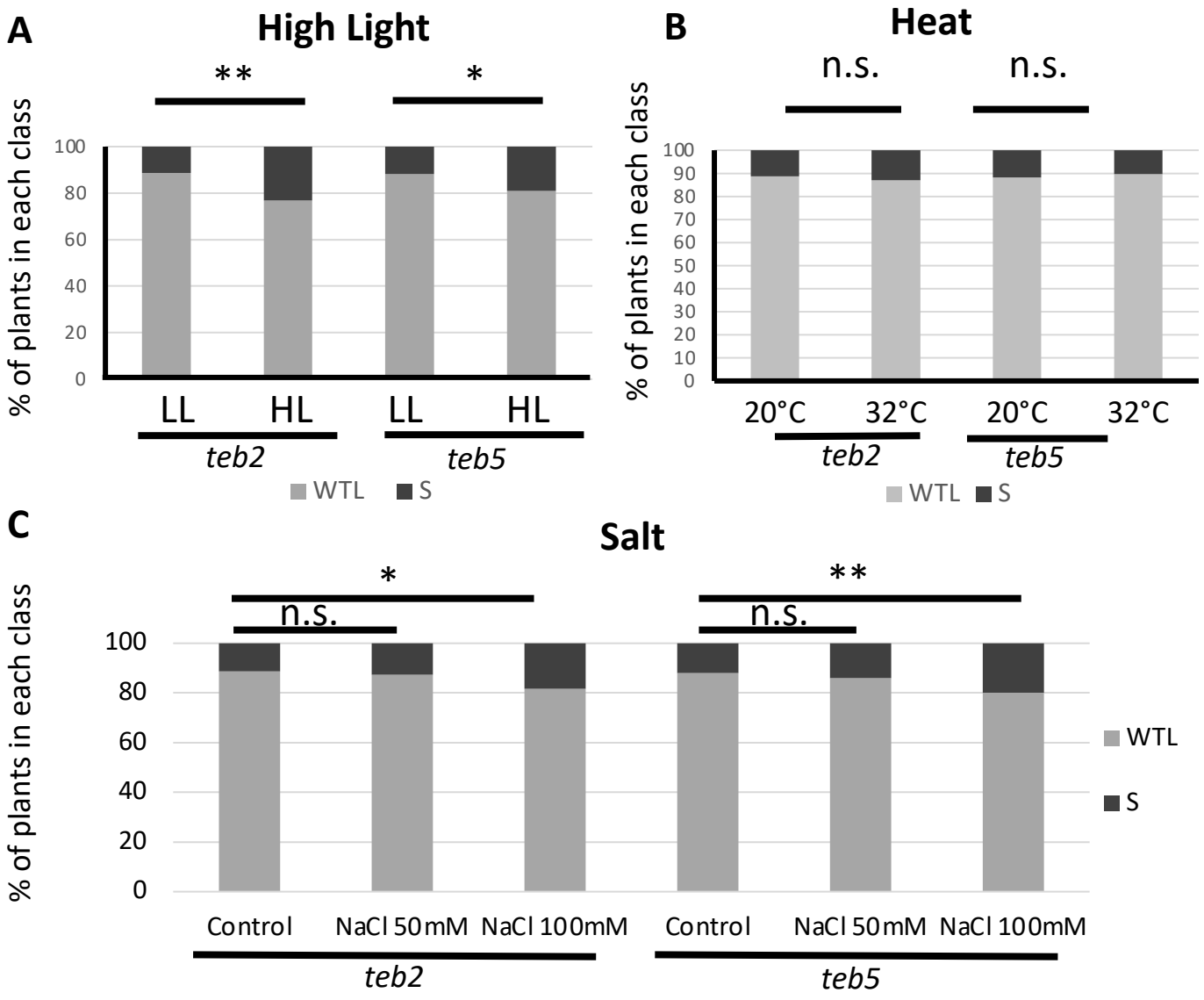



**Figure 6: Some abiotic stresses aggravate the developmental defects of *teb* mutants.**

A: Distribution of *teb* mutants between the different classes in plants grown in low light (LL, 160µmol x $m^{-2}$ x $s^{-1}$) or high light (HL, 350 µmol x $m^{-2}$ x $s^{-1}$).

B: Distribution of *teb* mutants between the different classes in plants grown at standard temperature (20°C) or under heat stress (32°C). Plants were germinated *in vitro* and transferred to soil after 10 days. After 3 days of growth under control conditions at 160µmol x $m^{-2}$ x $s^{-1}$ plants were kept under the same conditions or transferred to 32°C under the same light intensity.

C: Distribution of *teb* mutants between the different classes in plants watered with or without salt to the indicated concentration. Plants were germinated *in vitro* and transferred to soil after 10 days. After 3 days of growth under control conditions (160 µmol x $m^{-2}$ x $s^{-1}$, 20°C, salt-treated plants were watered with a solution containing NaCl (50 mM), for the 100 mM treatment, salt concentration was increased to 100 mM after 2days.

For all panels, n.s. indicates non-significant differences and asterisks denote statistically relevant differences between distributions ($\chi^2$-test, $p < 0.01$). Blind scoring was performed on wild-type and *teb* mutants in all growth conditions, the proportion of severe phenotypes observed in wild-type plants was below 2%.

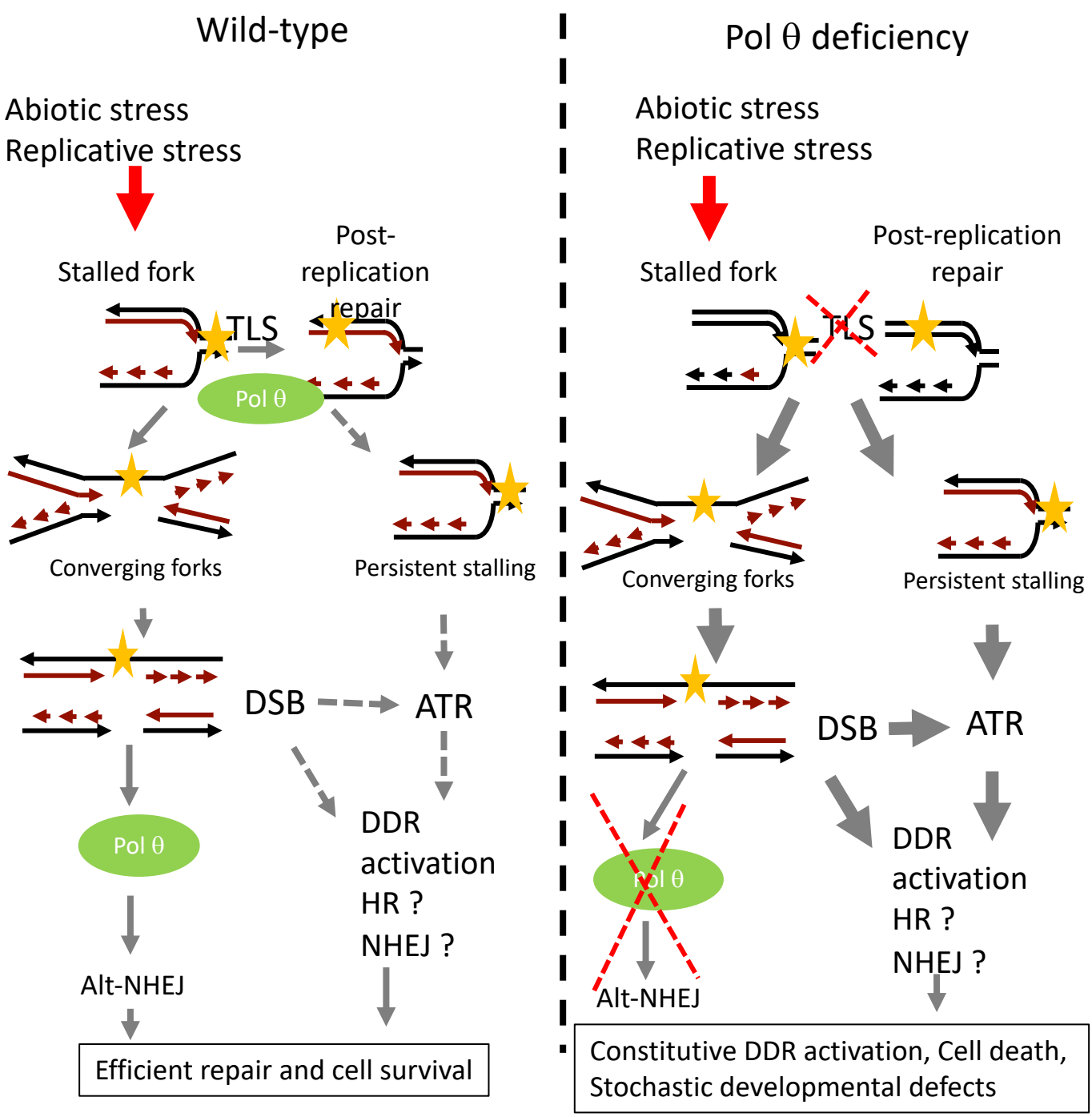



**Figure 7: Model for the role of Pol θ during replicative stress response**

A: In the wild-type, replication blocking lesions induce fork stalling. Pol θ can allow TLS through some lesions such as pyrimidine dimers. If efficient lesion bypass cannot be achieved, replisome disassembly and persistent fork stalling activates the DDR through ATR signalling, and DNA synthesis from a converging fork can lead to the formation of a double-ended DSB. Pol θ contributes to the repair of these lesions through Alt-NHEJ but other pathways such as HR or NHEJ likely contribute to DSB repair. B: In the absence of Pol θ, TLS through some lesions is compromised, leading to an increased frequency of fork collapse and persistent stalling. Furthermore, Alt-NHEJ is also compromised, leading to an increased frequency of failed repair, constitutive activation of the DDR through ATR signalling, cell death and stochastic developmental defects. Abiotic stress and replicative stress can modify this equilibrium by enhancing the accumulation of more replication-blocking lesions, leading to an increased frequency of developmental defects in Pol θ deficient lines.

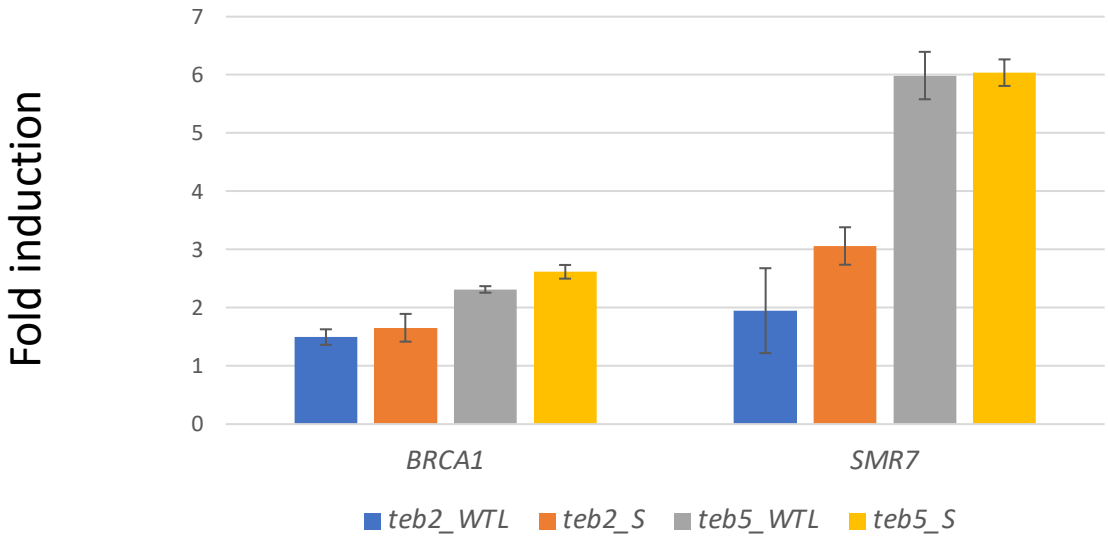



**Figure S1. Levels of DDR genes induction do not correlate with the severity of *teb* mutants' phenotype.**
Expression of the DDR marker genes *BRCA1* and *SMR7* was monitored by qRT-PCR in rosette leaves of WTL and S *tebichi* mutants. Expression was normalized to that of *ACTIN*. Data are average +/- standard deviation of 3 technical replicates and representative of two independent experiments.


**Figure S2. Root growth defects show some heterogeneity in *teb* mutants**
Plantlets of the wild-type (Col-0) and *teb2* and *teb5* mutants were grown in vitro for 5 days, and aligned on half strength MS plates to be grown vertically. After 2 weeks, root growth of some plants appeared completely arrested in all genotypes (red arrowheads). The proportion of plants with arrested root growth was significantly higher in *teb* mutants (16% in *teb2* and 17% in *teb5*) than in the wild-type (7%), n = 75 for all genotypes, $\chi^2$ p-value< 0.001. Bar = 2cm for all pictures.

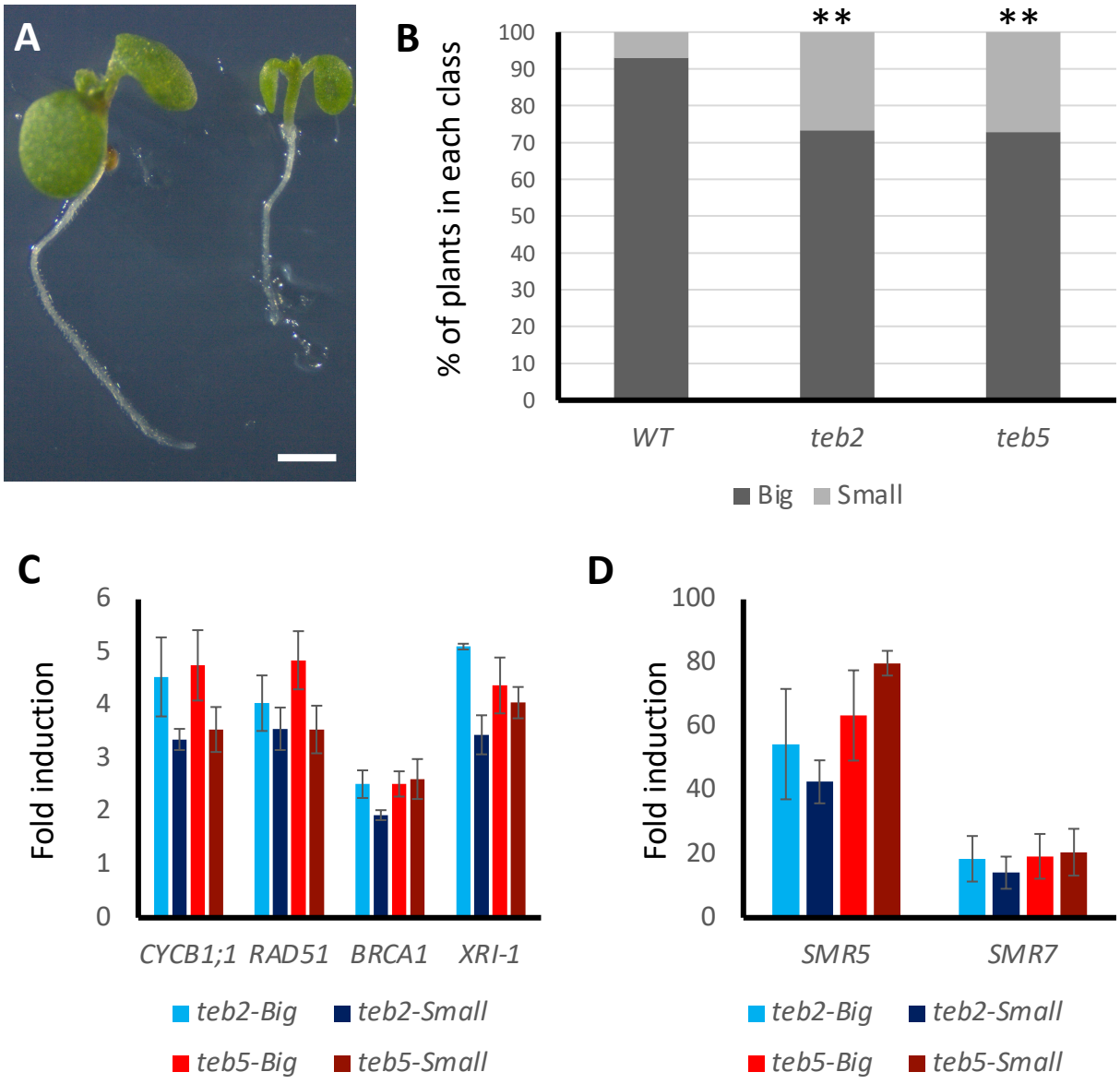



**Figure S3. Heterogeneous phenotypes of *teb* mutant plantlets do not correlate with different levels of DDR genes activation**

A: Representative picture of "big" and "small" plantlets observed among the *teb* mutants 10 days after germination, Bar = 500µm. B: Percentage of "big" and "small" plants among wild-type (WT) and *teb2* and *teb5* mutants (n>150 for all genotypes). ** denote statistically relevant differences $\chi 2$ test p-value < 0.01. Data are representative for 3 independent experiments. C-D: qPCR analysis of DDR maker genes expression in small and big *teb* mutant plantlets. Expression levels were normalized using ACTIN as a reference gene, and results are expressed as fold-changed compared to the wild-type (Col-0). Data are average +/- standard deviation obtained from 3 technical replicates and are representative of 2 independent experiments.

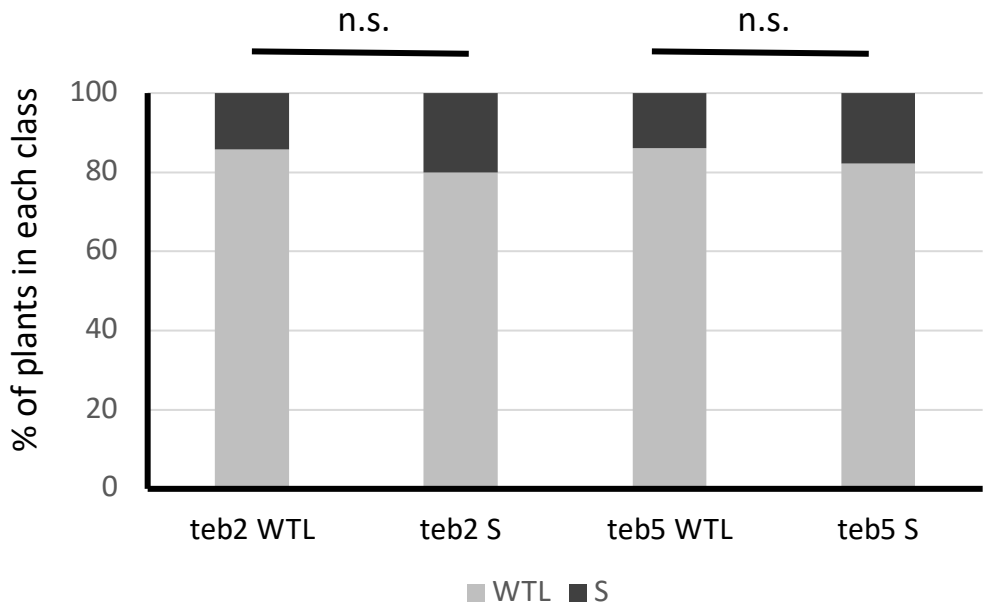



**Figure S4. The *teb* phenotype does not aggravate over generations**

Seeds from *teb* mutants with WTL and S phenotype were harvested and the distribution of individuals in each phenotypic category was estimated at the next generation. All three phenotypic classes were found in the progeny of each type of mutant, and no difference was observed in the distribution among the different classes between the three types of plants ($\chi^2$-test $p>0.05$).

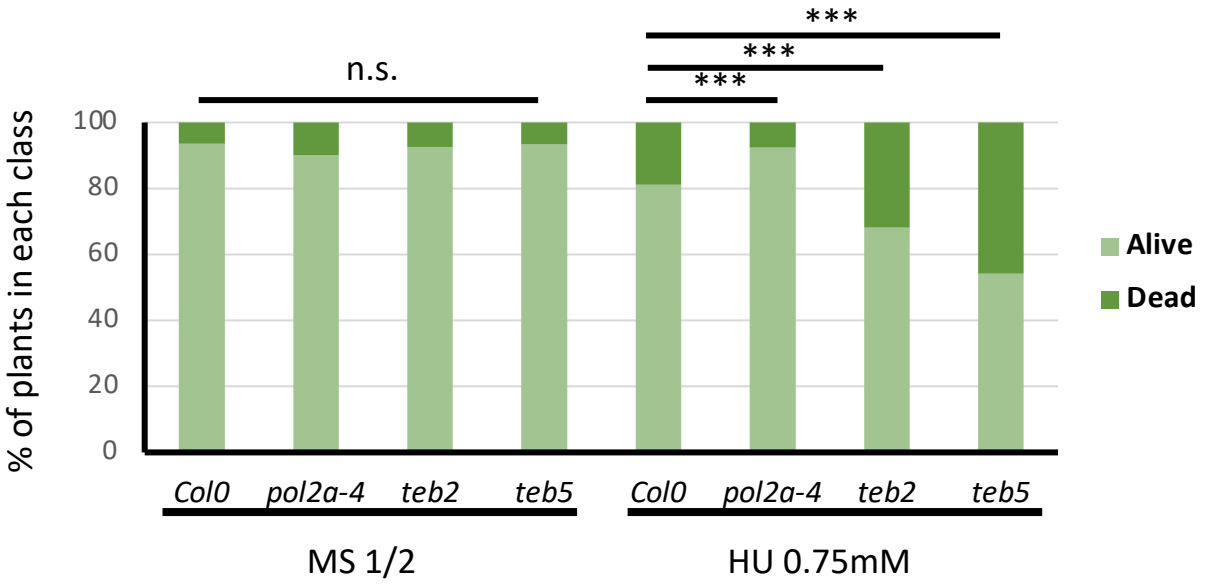



**Figure S5. *teb* mutants are hypersensitive to replicative stress**

Wild-type (Col0) and *teb* mutants (*teb2* and *teb5*) were germinated on MS supplemented or not with HU to a final concentration of 0.75mM. After 10 days, the survival rate was measured (n>100). While the survival rate on control medium was similar for all genotypes, *teb* mutants showed a higher proportion of dead plantlets on HU supplemented medium ($\chi^2$-test, p<0.001). The *pol2a-4* that was shown to be tolerant to HU (Pedroza-Garcia et al, 2017) was used as a control.

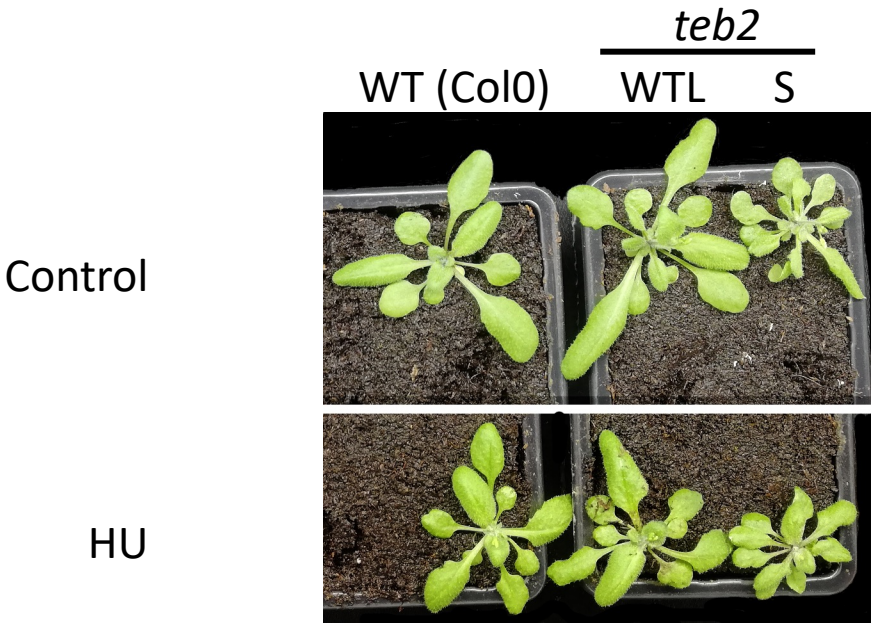



**Figure S6. Representative phenotypes of wild-type (Col0) and *teb* mutants exposed to HU before transfer to the green house**

Plants were grown for 10 days on half strength MS with or without HU (0.75mM). Surviving plants were transferred to the green house and grown for 3 weeks. Wild-type plants pre-treated with HU were slightly smaller than plants grown on MS alone. However, the characteristic *teb*-like phenotype was observed only amongst *teb* mutants.

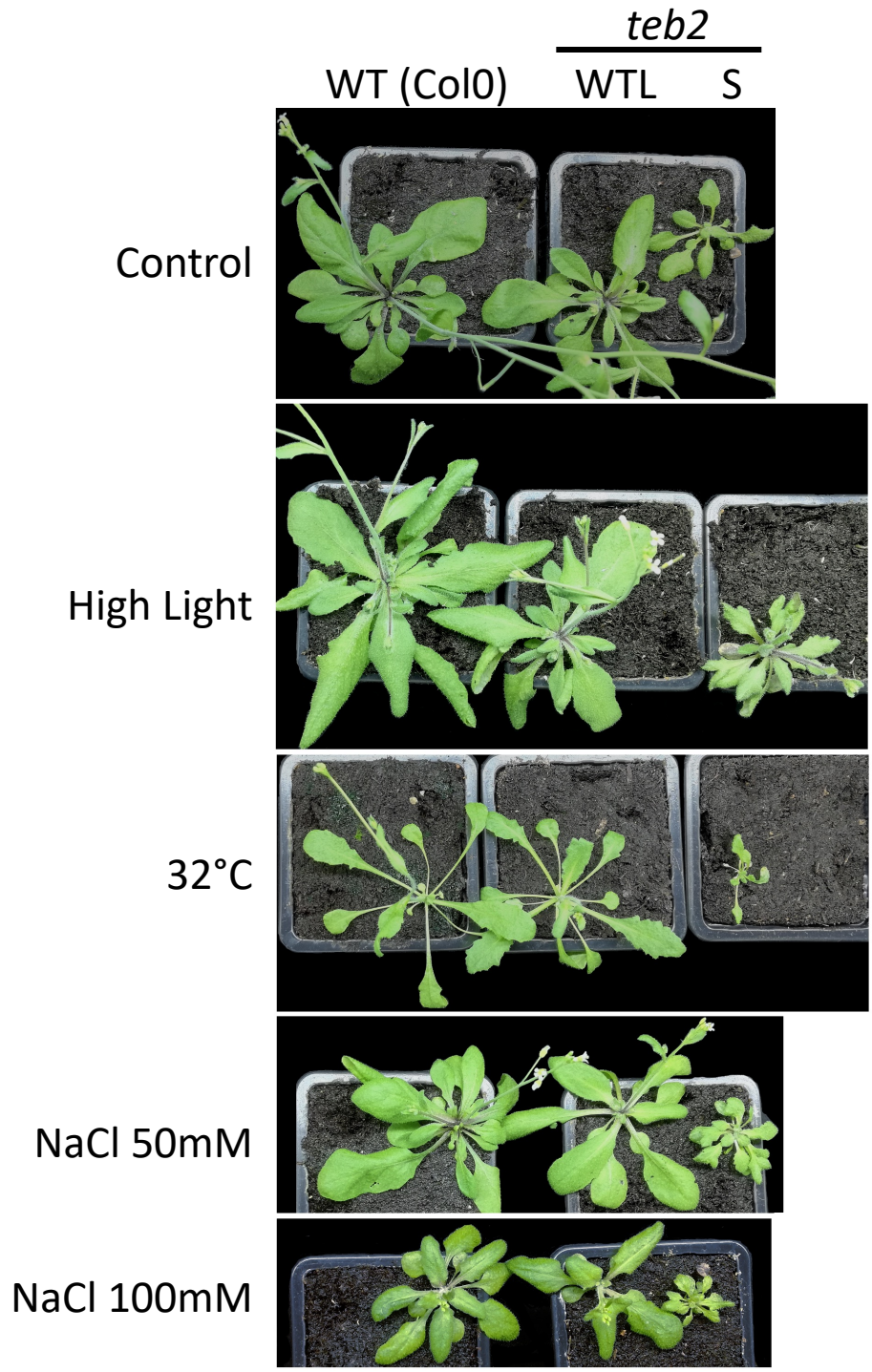



**Figure S7. Representative phenotypes of wild-type (Col0) and *teb* mutants grown under different conditions.**

Plants were grown for 1 month under the indicated conditions (see methods for details). Some of these growth conditions significantly altered the phenotype of wild-type plants: plants grown under high light were slightly larger with rolled leaves, plants grown at 32°C showed typical phenotype of plants acclimated to heat including elongated petiole and small leaf blade, while plants grown in the presence of salt showed reduced growth. The same modifications were observed in *teb* WTL plants. Moreover, none of these conditions induced the typical *teb*-like phenotype in wild-type plants.

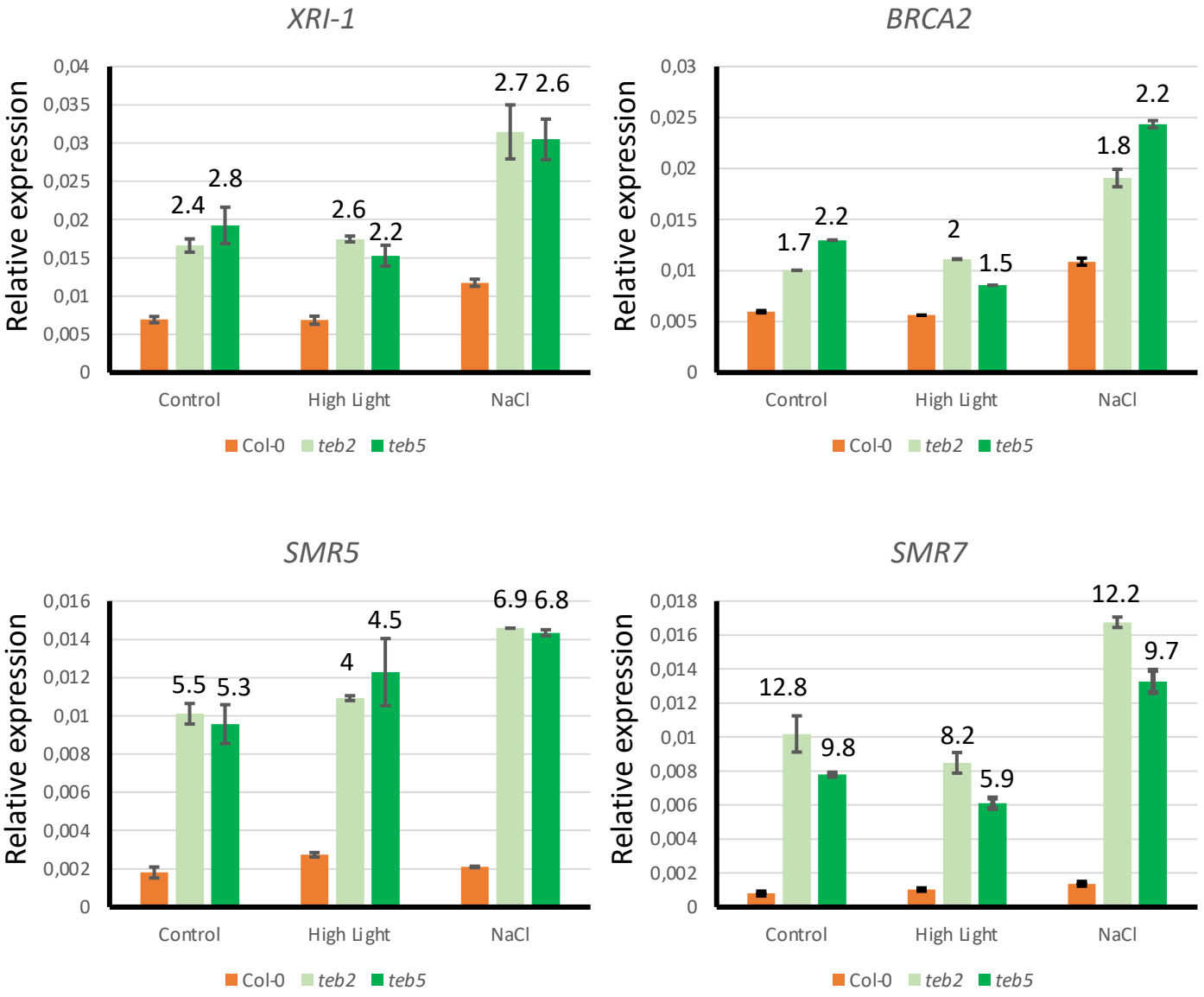



**Figure S8. Salt treatment, but not increasing light intensity activates DDR gene expression in both wild-type and *teb* mutants.**

Expression of DDR marker genes associated with DNA repair (*XRI-1* and *BRCA2*) or cell cycle arrest (*SMR5* and *SMR7*) was monitored by RT-qPCR in wild-type and *teb* mutants grown under standard conditions, germinated on NaCl supplemented medium (100mM) or exposed to high light. Data are average +/- S.D. obtained on 3 technical replicates and are representative of two independent experiments. They show relative expression of the selected genes compared to actin. Figures above bars represent the fold-induction compared to wild-type plants grown under the same conditions.