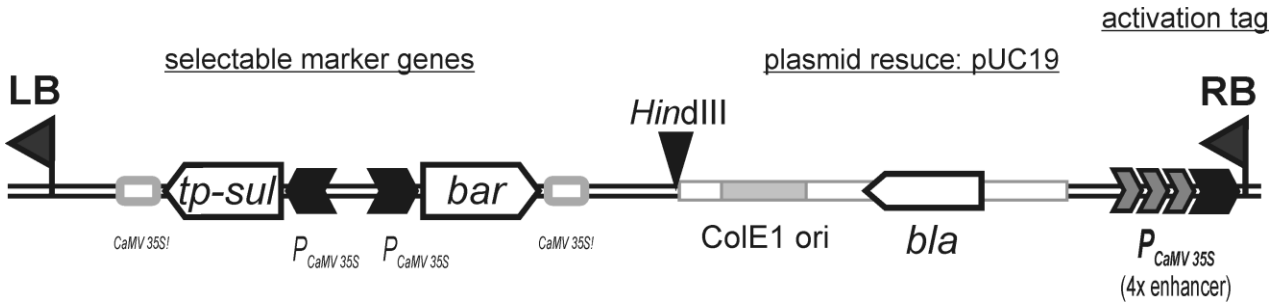
Supplemental Data, Schuermann et al., (2009) Replication Stress Leads to Genome Instabilities in Arabidopsis DNA Polymerase δ Mutants

Supplemental Experiments

Genetic Screen for Increase Frequency of HR

A population of mutant Arabidopsis plants, ecotype *Columbia*-0*,* containing a luciferase-based IR HR substrate was generated by the floral dip method described in Desfeux et al. (2000) using the *Agrobacterium tumefaciens* strain C58CIRifR (Van Larebeke et al., 1974) with the disarmed Ti-plasmid GV3101. The binary plasmid pAC102, a donation of B. Reiss, contained an activation-tag close to the right border of the mutagenizing T-DNA (Supplemental Figure 1). T1 mutant plants were selected for sulfonamide resistance, grown to rosette stage and scored for the frequency of HR. Plants that revealed more than the background level of about 3 HR events were considered as mutants with a hyper-recombination phenotype and subjected to further phenotypic and molecular analysis.

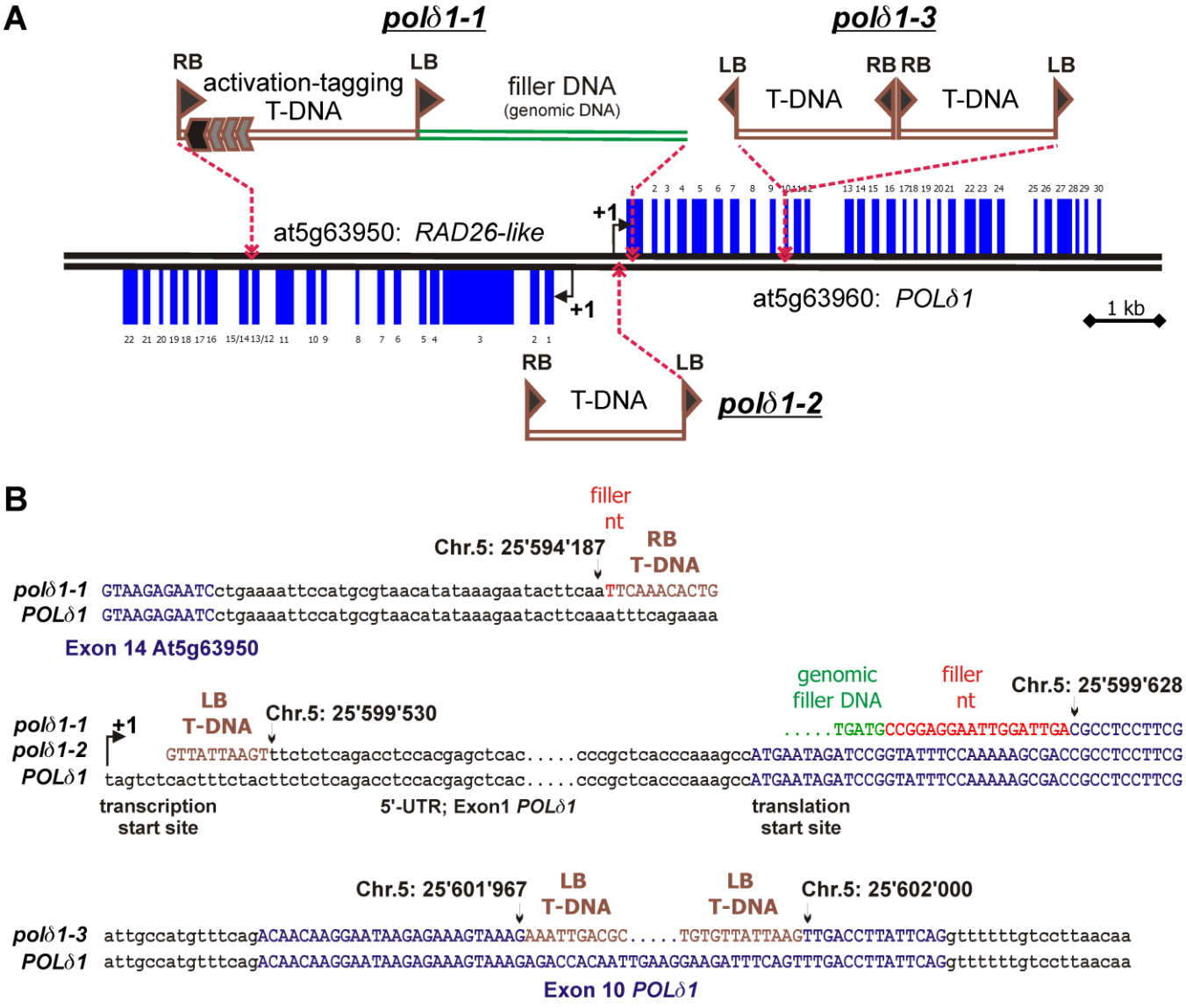


Supplemental Figure 1. Schematic Representation of the Mutagenizing T-DNA

The T-DNA of binary vector pAC102 contains a strong viral CaMV 35S promoter with repeated enhancer elements for activation-tagging of genes near the integration site of the right border (RB). The unique restriction site *Hin*dIII and the pUC19 backbone sequence, containing the bacterial ColE1 origin of replication and the *ampicilin resistance* gene (*bla*), enables the identification of the T-DNA integration site by plasmid rescue. Mutagenized T1 plants can be selected for the resistance to the herbicides sulfonamide or phosphinotricin, conferred by the *tp-sul* or *bar* expression cassettes, respectively.

Molecular Analysis of Insertional *polδ1* Mutant Plants

Plasmid rescue (Kiessling et al., 1984) was applied to identify the site of T-DNA integration in the hyper-recombination mutant *polδ1-1*. The right border of the T-DNA containing the activation-tag was found to be inserted into intron 13 of the *RAD26*-like gene At5g63950 (Supplemental Figure 2). The integration site of the left border turned out to be complex. Genomic Arabidopsis DNA and some filler nucleotides replace the genomic sequence up to 32 bp after the translation start site of the *POLδ1* gene (Supplemental Figure 2A), interrupting the ORF.



Supplemental Figure 2. T-DNA Mutant Alleles of the *POLδ1* Gene.

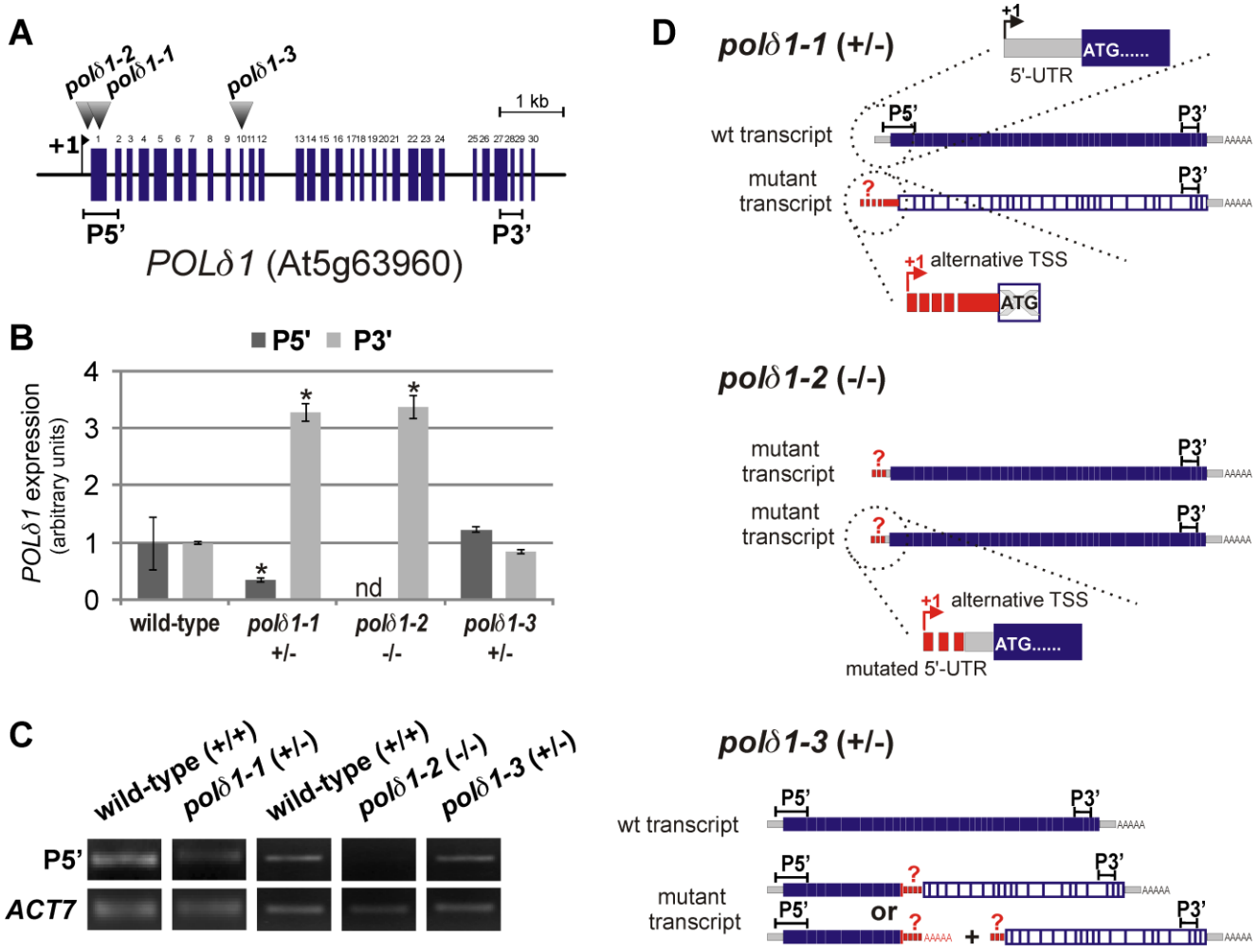
(**A**) Schematic representation of the *POLδ1* locus and the sites of T-DNA integration (red arrows), giving rise to the three insertional mutant alleles. (**B**) Sequence details of the T-DNA integration sites. Blue, protein-coding exon; brown, T-DNA; red, filler nucleotides; +1, transcription start site.

Apart from the *polδ1-1* allele isolated in a forward genetic screen in our lab, we also obtained two additional Arabidopsis lines with T-DNA insertional mutations in the *POLδ1* gene from the public collection of the SALK institute. In the *polδ1-1* and *-3* alleles, the T-DNA was found to interrupt the open reading frame whereas the 5' untranslated region, close to the transcription start site, was altered in the *polδ1-2* allele (Supplemental Figure 2). In agreement with the previously reported essential function of the orthologous proteins in budding yeast (Sitney et al., 1989) or mouse (Uchimura et al., 2009), no homozygous plants for *polδ1-1* and *-3* alleles could be isolated. The latter allele was previously described as *emb 2780* (www.seedgenes.org) and is, like *polδ1-1*, preventing the expression of the full length and functional gene product. In contrast, homozygous *polδ1-2* plants could be isolated.

Expression Analysis of Plants with Insertional *POLδ1* Alleles

Expression data of the insertional mutant lines did not allow a firm conclusion on the cause of the dominant HR phenotype: a reduced endogenous transcript level encoding the full length POLδ1 protein was observed in all lines but also aberrant transcripts were detected (Supplemental Figure 3). These aberrant transcripts could potentially lead to the production of a protein with a dominant-negative influence on the correct function of POLδ1.

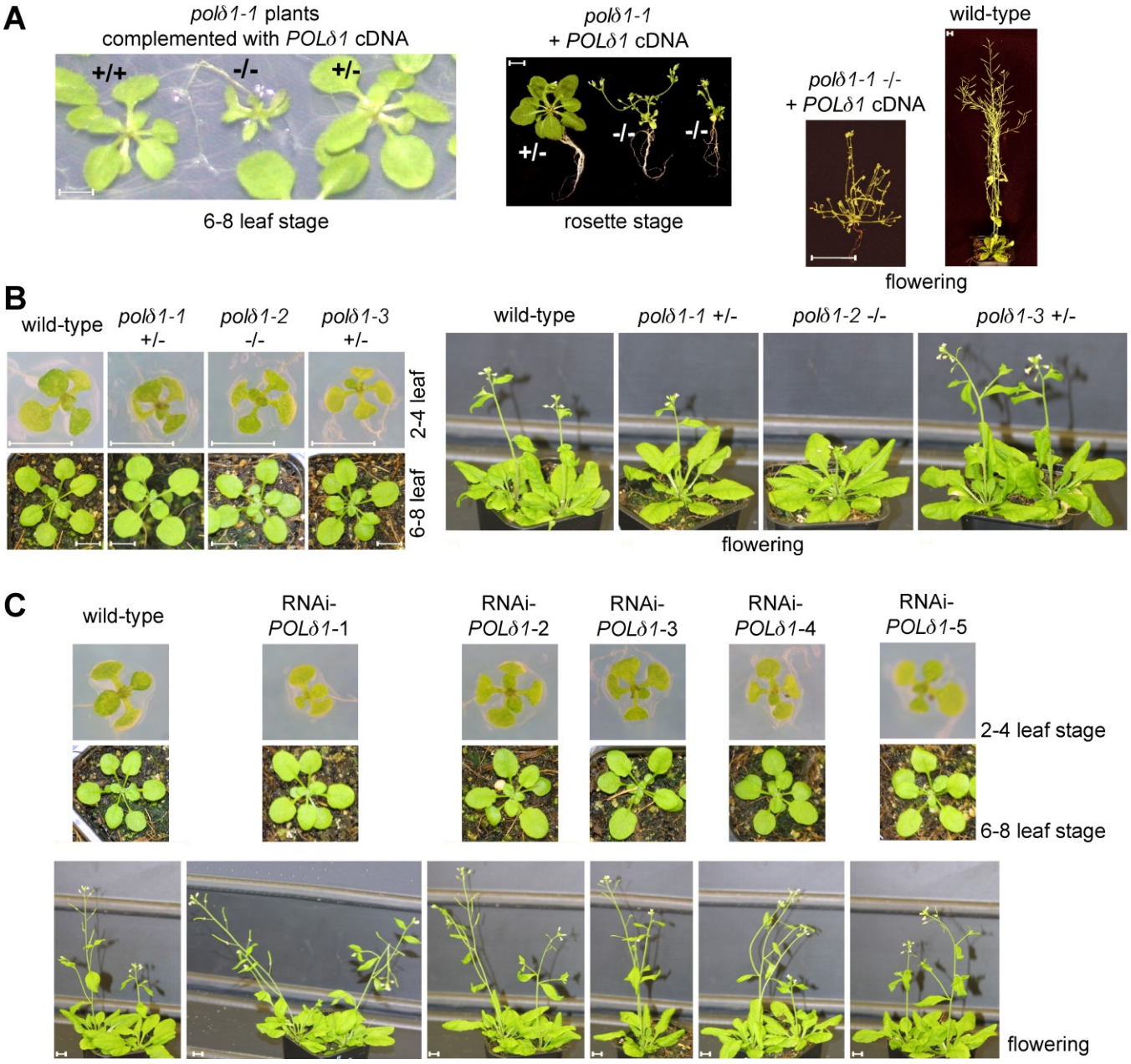
Compared to wild-type plants, hemizygous *polδ1-1* plants express about half as much of the wild-type transcript and about 3 times more mutant transcript that lacks the translation start codon and is likely to be non-coding (Supplemental Figure 3B and 3D). In homozygous *polδ1-2* plants, an about 3-fold enhanced transcription of an abnormal mRNA was found, which is nevertheless expected to code for a complete POLδ1 protein since only the promoter sequence and the very 5'-part of the UTR but not the translation initiation sequence is altered. This data suggests that the HR increase of this line originated from a tissue- or cell cycle-specific misregulation rather than from an allelic depletion of POLδ1 expression. We could not exactly determine the mutant transcript in hemizygous *polδ1-3* plants. However, due to the insertion of the T-DNA in the protein-coding sequence, the anticipated mutant transcript contains either rather extended ectopic sequences or is stopped prematurely and an alternative promoter sequence in the mutagenizing T-DNA drives the expression of non-coding aberrant mRNA corresponding to the 3'-region of the *POLδ1* gene.



Supplemental Figure 3. *POLδ1* Expression Analysis of T-DNA Mutant Plants.

(**A**) Schematic representation of the *POLδ1* gene and the three T-DNA insertional mutant lines. 1+, indicates the transcription start site; blue boxes, protein-coding sequences; triangles, the position of the insertional mutation. P5' and P3' depict the 5' and 3' regions of the *POLδ1* transcript that are amplified in the qRT-PCR analysis. (**B**) qRT-PCR analysis using primers that discriminate between wild-type and mutant *POLδ1* transcripts. Asterisks mark significant differences of the expression level compared to wild-type control plants ($n \geq 3$ experimental replica). (**C**) RT-PCR detecting the 5'-extremity of the wild-type *POLδ1* transcript in all three insertional mutants. Samples were taken in the exponential phase of above qRT-PCR reactions and analyzed by gel electrophoresis. The ubiquitously expressed Arabidopsis *ACTIN7* gene (*ACT7*) was used as internal control. (**D**) Putative transcripts expressed in insertional *polδ1-1* mutant plants, deviated from the analysis of expression and of the insertion site (Supplemental Figure 2). Blue and white boxes represent exons with in frame and out of frame protein coding sequences, respectively. Red color indicates aberrant and not completely defined sequences. TSS, transcription start sites.

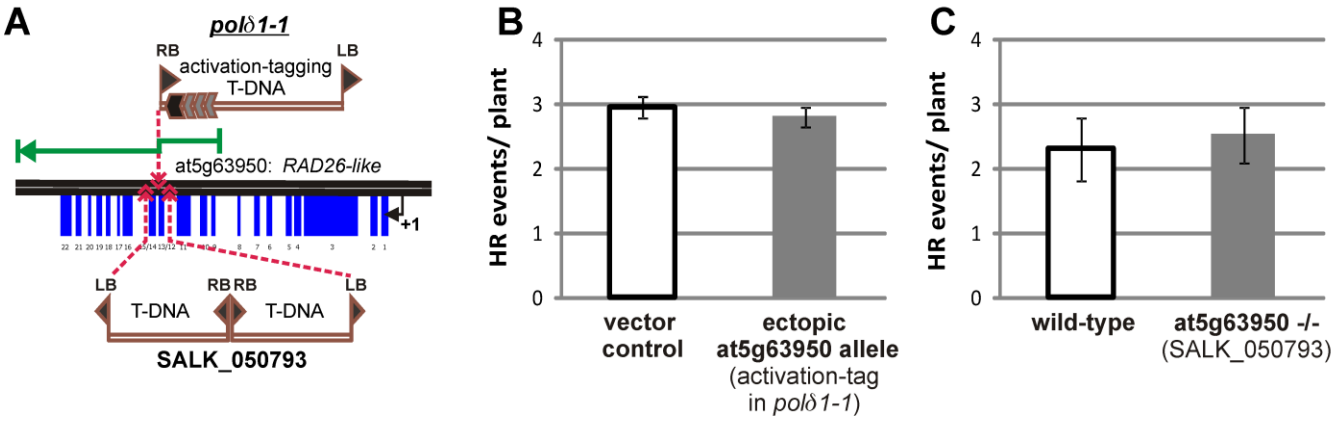
Plants with *polδ1* Mutations Appear Morphological Normal



Supplemental Figure 4. Phenotypic Comparison of *polδ1* Mutant and Wild-Type Plants.

(**A**) The segregating offspring of a hemizygous *polδ1-1* plant that ectopically expressed the cDNA of POLδ1 was analyzed for the zygosity of the *polδ1-1* allele by PCR. Complemented homozygous *polδ1-1* mutant plants revealed pleiotropic growth phenotypes such as reduced size, altered morphology, fasciated stems and reduced fertility. (**B**) Insertional *polδ1* mutant plants were phenotypically compared with wild-type plants at various developmental stages and did not show any alteration. (**C**) Comparison between wild-type plants and epigenetic *POLδ1* mutants. No significant phenotypic alterations were observed throughout all developmental stages for most of the mutant lines. Only germinating RNAi-*POLδ1*-1 plants exhibited faint growth retardation in early development. Size bars; 10 mm.

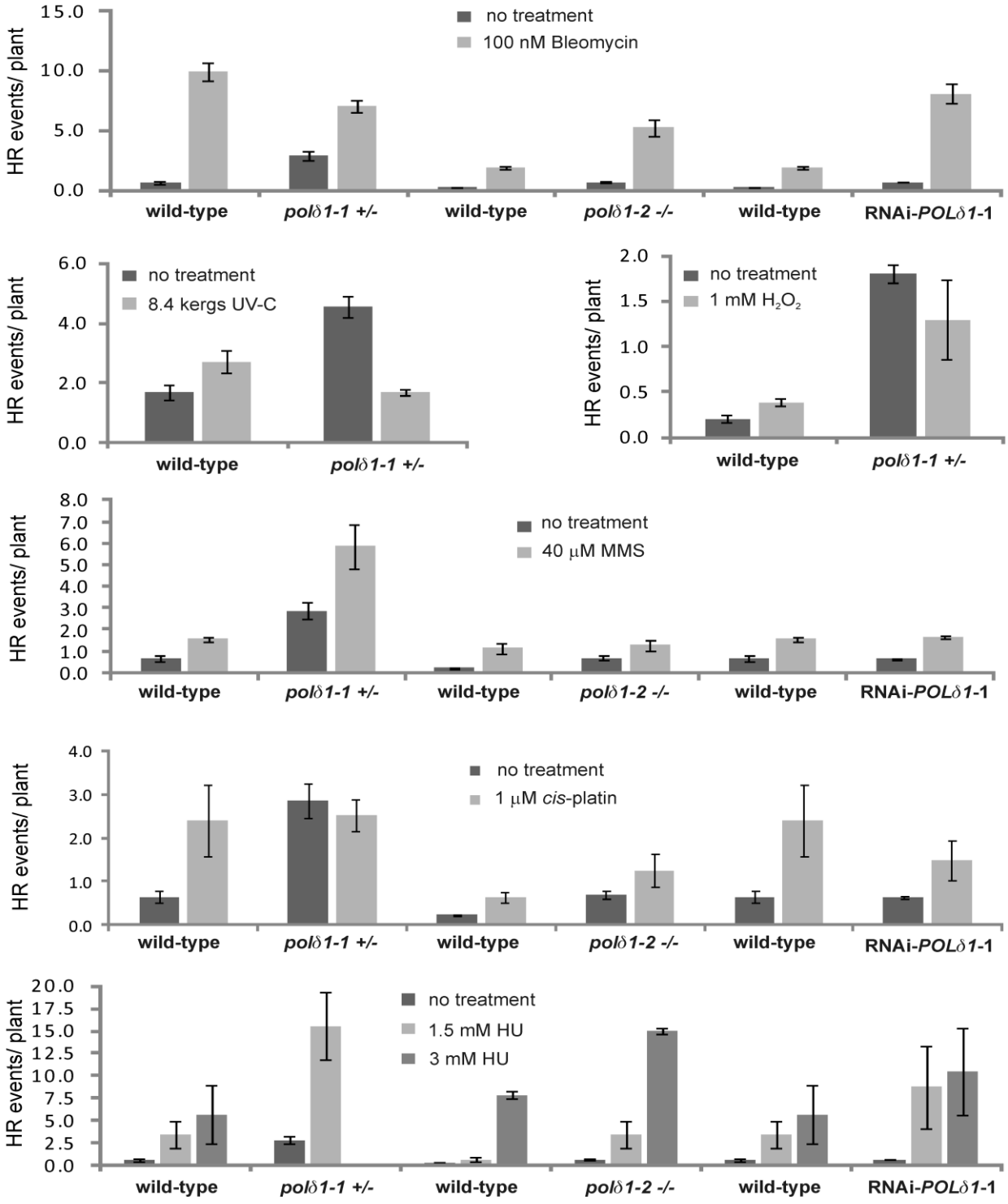
The *Rad26*-like Gene At5g63950 Adjacent to *POLδ1* Is Unlikely to Increase the HR Frequency



Supplemental Figure 5. The *RAD26*-like Gene At5g63950 Does Not Alter the HR Frequency.

(**A**) Scheme of at5g63950 gene and sites of T-DNA integration in *polδ1-1* mutant plants and the line SALK_050793. The green arrow indicates the genomic region amplified from DNA of hemizygous *polδ1-1* plants to generate the ectopic at5g63950 allele containing the activation-tag of the mutagenizing T-DNA and the 3' region of at5g63950. Red arrows, T-DNA integration site; blue, protein-coding exon; brown, mutagenizing T-DNA; +1, transcription start site. (**B**) The *luciferase*-based substrate line 50B was transformed either with a vector control or with the ectopic at5g63950 allele, which was present in mutant line *polδ1-1* and could have had an influence on HR in a dominant-negative manner. The CaMV 35S promoter of the activation-tag drives the expression of at5g63950 downstream of the T-DNA integration site. The HR frequencies of the T2 control and ectopic at5g63950 allele population were assessed and statistically analyzed by the Student's *t*-test (*p*-value 0.51). (**C**) The HR frequency of mutant plants homozygous for the at5g63950 T-DNA mutation was compared with out-segregating wild-type plants in HR substrate line 50B. Statistical analysis by the Student's *t*-test did not reveal a significant difference wild-type and mutant plants (*p*-value 0.74). Error bars, SEM ($n \geq 3$).

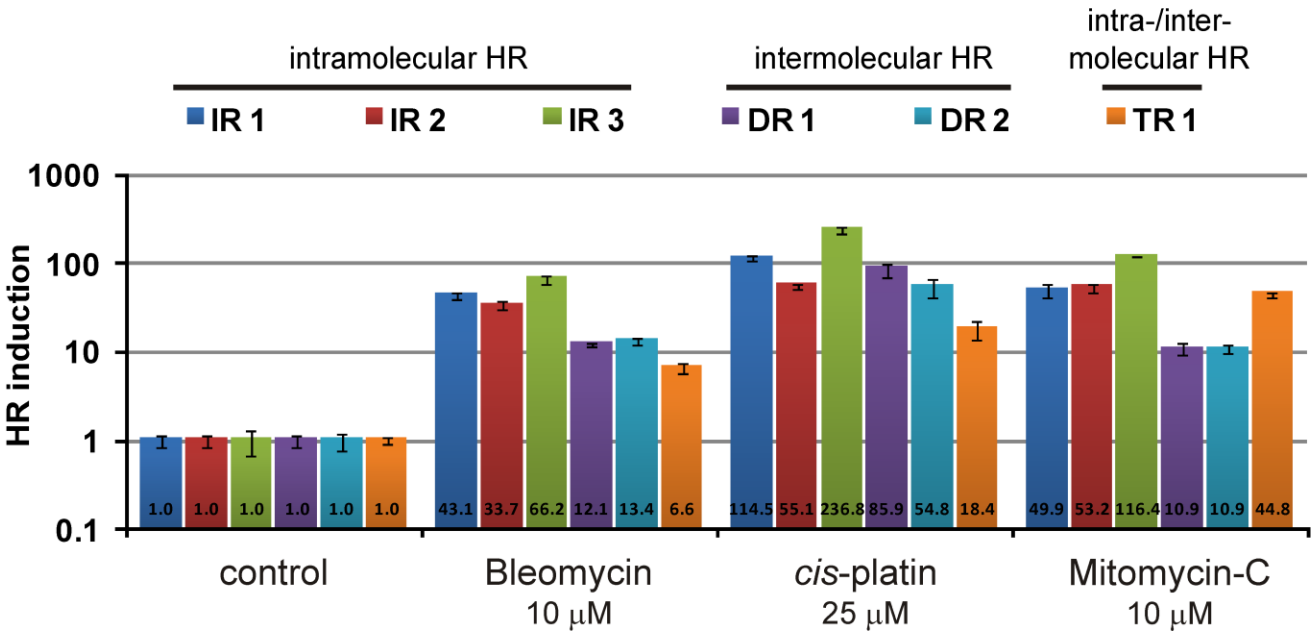
The HR Frequency of *polδ1* Mutants Is Responding to Genotoxic Treatments Less than That of Wild-Type Plants



Supplemental Figure 6. Induction of HR in *polδ1* Mutant and Wild-Type Plants.

Two-week-old wild-type and SHR class *polδ1* mutant plantlets were challenged with various genotoxic or replication stalling agents. Intramolecular HR events (substrate line IR1) with and without treatment were assessed in the different genetic backgrounds. Error bars, SEM ($n \geq 4$).

Assessment of HR Induction by Various DNA-Damaging Agents



Supplemental Figure 7. The Induction of HR by DNA-Damage in Substrate Lines with Different Spatial Arrangement of Repeats

Two-week-old Arabidopsis seedlings carrying recombination substrates with inverted, direct or tandem repeats (IR, DR, TR) were challenged with the DSB-inducing agents Bleomycin, Mitomycin-C or the DNA cross-linker *cis*-platin. HR induction relative to the spontaneous rate was calculated. Error bars, SEM of independent experiments ($n \geq 4$).

Supplemental Table 1: List of Oligo-Nucleotides

Purpose	Forward primer	Reverse primer
Cloning of *POLδ1* cDNA	GCGCAAGCTTATCCATGGGAAATAGATCCGGTATTTCCAA	GCTCCCTAGGTACCGTTGTTGCCTGGCTACAG
Cloning of hairpin-spliced RNAi constructs (sense)	GCGAAGCTTTGATAGAGATGTTCCAGGGG	CGTGTACACTCGAGAAGAAGTGGCTTGCTAATCTG
Cloning of hairpin-spliced RNAi constructs (antisense)	CGTGTACACTCGAGAAGAAGTGGCTTGCTAATCTG	GCGCCTAGGTGATAGAGATGTTCCAGGGG
Cloning of $P_{POLδ1}$*::GUS* fusion	CCGCCATGGCTACACCGTTTTCTGGAG	CGCGCCATGGCTTTGGGTGAGCGGGAAA
First strand cDNA synthesis		GACCACGCGTATCGATGTCGAC$(T)_{16}$(A/C/G)
Genotyping *polδ1-1* KO	AACACATGACACCTCTGGATAC	ATCTTCGTCGCCGTACAAGAG
Genotyping *polδ1-1* wt	TGCCTAATTGTCTTGGTCATG	ATCTTCGTCGCCGTACAAGAG
Genotyping *polδ1-2* KO	TGGTTCACGTAGTGGGCCATCG	CAATGCAACTGTAATCTTCAG
Genotyping *polδ1-2* wt	ATGGCTACACCGTTTTCTGGAG	ATCTTCGTCGCCGTACAAGAG
Genotyping *polδ1-3* KO	GCGTGGACCGCTTGCTGCAACT	AAACAGCAAGACGCCTCCTGG
Genotyping *polδ1-3* wt	CTGCTGATGTATGAGATGTATTGG	AAACAGCAAGACGCCTCCTGG
qRT-PCR, *ACT7* control	TGGACAAGTCATAACCATCGGAGC	TGTGAACAATCGATGGACCTGAC
qRT-PCR, 5' of *POLδ1* transcript (P5')	TTCTCTCAGACCTCCACGAGC	CATCATCTAGGGTTCCGATGG
qRT-PCR, 3' of *POLδ1* transcript (P3')	CAGCGGTATAATGAAGTTTGC	AGCTCAGCCACTTGAGACACG
qRT-PCR of epigenetic *POLδ1* mutants	TTTGATTCGTGATGTTGATCC	GAGCTGAGACAGAATTCAGCG
qRT-PCR for *ATR*	GACCCAGAACATGATTGATGG	ACTCCACAAGAGGATCATGG
qRT-PCR for *BRCA1*	ACCATTGATTGGATTAAGGC	TTCAAAGCTCTTTGTCTTCC
qRT-PCR for *PARP2*	TCTCAAGGTCTGCGAATAGC	TTCATTCATGTCTCCCAAAGC
qRT-PCR for *RAD17*	CATGTTCATCCAGATGGCAC	TATATCTGCCACTCTTGACCC
qRT-PCR for *RNR2A*	AGAGATCATCTCTTTCGTGC	GAAGAAGATACCTTCGACGC
qRT-PCR for *RAD51*	TTAGCAGATGAGTTTGGTGTG	GGCATGAGCCATGATATTCC

Supplemental Methods

Cloning of the *POLδ1* cDNA and the complementation vector

An Arabidopsis cDNA library obtained from a callus culture was used to amplify the coding region of *POLδ1* by "Expand™ high fidelity PCR system" (Roche Diagnostics, Rotkreuz, Switzerland). Transcription initiation and polyadenylation sites were mapped by circularization RT-PCR as described (Couttet et al., 1997).

The cDNA was re-amplified using primers with suitable restriction sites (5'-GCGCAAGCTT ATCCATGGGA AATAGATCCG GTATTTCCAA) and (5'-GCTCCCTAGG TACCGTTGTT GCCTGGCTAC AG) and subsequently introduced into the binary vector pC23C, a derivative of pCAMBIA2300 containing the CmYLCV promoter sequence (Stavolone et al., 2003). Primary transformants were genotyped and selected for the complementing construct and the *polδ1-1* mutation. In the following generation, the effect of the ectopic *POLδ1* expression on the HRF of hemizygous *polδ1-1* mutant plants was evaluated.

Construction of RNAi Vectors

For the hairpin-spliced RNAi constructs *POLδ1*-specific sequences of the 3' region of the cDNA (gi: DQ160246, position 2,550 – 2,872) were amplified (Supplemental Table 1) and cloned into the plant expression vectors poEXhp (Fritsch et al., 2004) and pRM derived from the alcohol-inducible system (Roslan et al., 2001), giving rise to RNAi-*POLδ1* lines 1 and 2-4, respectively. In addition, the line RNAi-*POLδ1*-5 was generated using the Arabidopsis U6 snRNA promoter to drive a 320 nt long *POLδ1* antisense RNA, corresponding the nucleotides 2,550 - 2,872 of gi: DQ160246.

qPCR Analysis

Quantitative PCR (qPCR) was performed with the SYBRGreen-based QuantiTect from Qiagen (Hombrechtikon, Switzerland) using the Rotorgene RG-3000 real-time PCR system (Corbett Research, Sydney, Australia) according to the providers' recommendations. 3 µg of total RNA was reverse transcribed with the "Ready-To-Go™ You-Prime First-Strand Beads" (GE Healthcare Life Sciences, Uppsala, Sweden) according to the supplier's indications. The cDNA was diluted 10 times with 10 mM Tris-HCl pH8. qPCR reactions of 10 µL final volume containing 0.5 µM of each primer and 1 µL of the diluted cDNA preparation were run with the following program: 95°C for 15 min, 45 cycles of 95°C for 10 s, 56°C for 20 s, 65°C for 30 s.

For each primer combination corresponding to a given target (Supplemental Table 1), the specificity and optimal annealing temperature were evaluated prior to qPCR analysis. For every target, a standard curve was established by the software package of the Rotorgene RG-3000 using a serial dilution of RT reaction derived from wild-type plants. Applying the relevant standard curve, a Ct value was calculated for every qPCR sample of a given target and subtracted from the Ct value of the internal standard *ACT7* according to the formula ΔCt = Ct(ACT7) - Ct(target). Finally, the expression level relative to wild-type controls was calculated with the formula $2^{\Delta Ct(mutant)-\Delta Ct(wild\text{-}type)}$.

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